*Biomedical Research 2017; 28 (16): 7157-7166* *ISSN 0970-938X*
*www.biomedres.info*

# Methods for detection of the misuse of "anti-oestrogens and aromatase inhibitors" in sport.

**Stanislava Ivanova[1*], Kalin Ivanov[1], Elina Petkova[2], Stanislav Gueorgiev[2], Denitsa Kiradzhiyska[3]**

[1]Faculty of Pharmacy, Department of Pharmacognosy and Pharmaceutical Chemistry, Medical University of Plovdiv, Plovdiv, Vasil Aprilov Blvd. 15-A, Bulgaria

[2]Faculty of Pharmacy, Department of Pharmaceutical Sciences, Medical University of Plovdiv, Plovdiv, Vasil Aprilov Blvd. 15-A, Bulgaria

[3]Faculty of Pharmacy, Department of Chemical Sciences, Medical University of Plovdiv, Plovdiv, Vasil Aprilov Blvd. 15-A, Bulgaria

## Abstract

**Oestrogen blockers and aromatase inhibitors which are used mainly for treatment of breast cancer are considered as essential medicines in contemporary clinical practice. A current social and health problem is that many athletes abuse with these two categories of medicines to counter the side effects of anabolic steroids as part of their steroid cycle, to increase testosterone concentration by stimulation of testosterone biosynthesis and to reduce and prevent symptoms of excess oestrogen. This paper reviews the most appropriate methods for detection of these substances and their metabolites in biological samples. It also summarises the historical development of these drugs and their pharmacological aspects. We have described the difficulties and strengths of the analytical procedures that could be used for doping control of these substances. Literature search was done through Medline/PubMed, Scopus Database, Web of Knowledge search as well as an internet-based search with predefined keywords. We have also analysed many WADA's protocols. The present review is based on a total of 69 publications.**

**Keywords:** Anti-oestrogens, Aromatase inhibitors, Tamoxifene, Anastrozole, Letrozole, Doping.

*Accepted on July 24, 2017*

## Introduction

The term anti-oestrogens unite some oestrogen blockers and selective oestrogen receptor modulators. Although Aromatase Inhibitors (AIs) could be considered anti-oestrogens by some definitions, they are distinct class medicines [1]. Aromatase inhibitors (letrozole, anastrozole, exemestane) inhibit the production of oestrogens by inhibition of aromatase, whereas oestrogen blockers (tamoxifen, toremifene and clomiphene) block the oestrogen receptors and thus prevent the interaction of oestrogen with the receptors [2-23]. Both classes of drugs are used mainly for treatment of breast cancer. World Health Organisation has included many of these drugs in the List of Essential Medicines. Unfortunately these two categories of medicines are not only used for treating pathological conditions but are also used by athletes to counter the side effects of anabolic steroids as part of their steroid cycle to increase testosterone concentration by stimulation of testosterone biosynthesis and to reduce and prevent symptoms of oestrogen excess-gynecomastia and water retention. Since 1st September 2001 the use of aromatase inhibitors is prohibited by WADA. It is considered that if an athlete use aromatase inhibitors or anti-oestrogen, he abuse also with anabolic steroids. Blockade of oestrogen action is also considered as form of indirect androgen doping that can stimulate sustained, albeit modest, increases in endogenous Luteinizing Hormone (LH) secretion in physiological pulsatile patterns sufficient to maintain a modest increase in blood testosterone concentrations [24-26]. It has been established that these medicines consistently increase blood testosterone concentrations in men by up to 50% [24]. Anti-oestrogen causes reflex increases in pituitary gonadotrophin secretion and circulating testosterone levels [24,27-37]. Similar increases in blood testosterone concentrations are reported with aromatase inhibitors such as exemestane and anastrozole [24,38,39].

## Materials and Methods

Literature search was done through Medline/PubMed, Scopus Database, Web of Knowledge search as well as an internet-based search with keywords "doping", "sport", "aromatase inhibitors", "anti-estrogens", "anti-oestrogens", "estrogen blockers", "tamoxifen", "clomiphene", "exemestane", "letrozole", "anastrozole" and "formestane". The present mini review is based on a total of 69 publications satisfying the

search criteria. Our study was limited to the most used by athlete's antiestrogens agents and aromatase inhibitors.

## Results and Discussion

World Anti-Doping Agency (WADA) has united the science and medicine to develop adequate and effective methods for detection of doping substances. The aim of WADA is to create a clean sport culture, because the doping abuse leads to many negative consequences in different aspects: professional, social and health. The abuse with antiestrogens and aromatase inhibitors is a current and deep problem for professional sport. WADA have funded many project to identify and detect these doping substances in biological samples, exploring new models for enhanced detection. Many independent researchers also work in this area but the methods for detection for antiestrogens and aromatase inhibitors still remains a difficult issue. According to WADA Prohibited List antiestrogens and aromatase inhibitors are classified as hormone and metabolic modulators. Hormone and metabolic modulators are divided into 5 categories (Table 1).

*Table 1. Hormone and metabolic modulators prohibited by WADA [4].*

| Aromatase inhibitors including, but not limited to | Selective estrogen receptor modulators (SERMs) including, but not limited to | Other anti-estrogenic substances including, but not limited to | Agents modifying myostatin function(s) including, but not limited to | Metabolic modulators |
|---|---|---|---|---|
| Anastrozole | Raloxifene | Clomiphene | Myostatin inhibitors | Activators of the AMP-activated protein kinase |
| Androsta-1,4,6-triene-3,17-dione (androstatrienedione) | Tamoxifen | Cyclofenil | | Insulin's and insulin-mimetics |
| Exemestane | Toremifene | Fulvestrant | | Meldonium |
| Formestane | | | | Trimetazidine |
| Letrozole | | | | |
| Testolactone | | | | |

The first three categories (Aromatase inhibitors, SERMs and other anti-estrogenic substances) are used in sport by athletes who abused with anabolic-androgenic steroids (AAS) to prevent gynecomastya and to improve testosterone levels (indirect doping). Androgens could be easily converted to oestrogen and the athlete would develop gynecomastia. Aromatase is the enzyme responsible for conversion of androgens to oestrogen and is the key enzyme in oestrogen biosynthesis [6].

Gynecomastia is defined as the presence of palpable breast tissue in males [5] and could be caused by many different factors including drugs (Table 2).

*Table 2. Factors causing gynecomastia [5].*

| Cause | Etiology |
|---|---|
| Estrogen excess | 1. Exogenous estrogens: Exposure to aromatizable androgens (abuse with AAS). |
| | 2. Endogenous estrogens: Increased secretion from testis, increased secretion from adrenals, aromatase excess syndrome, hyperthyroidism, alcoholic cirrhosis and others. |
| Androgen deficiency | Hypogonadism. |
| Altered serum androgen/estrogen ratio | 1. Puberty. |
| | 2. Refeeding gynecomastia. |
| | 3. Hepatic cirrhosis. |
| | 4. Renal failure and dialysis. |
| | 5. Hyperthyroidism. |
| | 6. Drugs. |
| Decreased androgen action | 1. Use of androgen receptor antagonists like bicalutamide and other. |
| | 2. Absent or defective androgen receptors. |
| | 3. Kennedy disease. |

We have found that in the last few years most popular AIs among the athletes are: letrozole and anastrozole and among the oestrogen blockers clomiphene and tamoxifen. We have found that both female and male athletes abuse with these medicines. In 2013, because of positive testing for clomiphene Camarena-Williams has received a 6 month suspension from the US Anti-Doping Agency. In 2014 Gabrielle Lemos Garcia, a Brazilian Jiu-Jitsu female athlete tested positive for clomiphene. In the same year the bowling player Shannon O'Keefe also tested positive for clomiphene. In 2015 Tara Harbert-a baseball player failed doping tests. She gave positive samples for tamoxifen metabolites. July 2016 UFC athlete Jon Jones tested positive for two banned substances, clomiphene and letrozole. In 2016 another UFC athlete Brock Lesnar gave positive samples for clomiphene and its metabolite 4-hydroxyclomiphene. In October 2016 the UFC athlete Adam Hunter has received a two-year punishment after testing positive for multiple prohibited substances. His samples were positive for tamoxifen metabolite, boldenone metabolites, methandienone metabolites, drostanolone metabolite, and clenbuterol. Not all athletes who use these doping substances give positive doping samples. Some athletes could refuse testing and receive a penalty and some athletes could be found "clean" if all metabolites of prohibited substances have been eliminated in the time when they are tested. Usually athletes use clomiphene as post anabolic steroid cycle therapy, because it stimulates the pituitary to release more Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH), which will in turn stimulate enhanced natural testosterone production. This gives many benefits for athletes who abused with AAS, because after AAS cycle the natural testosterone levels are very low due to suppression caused by anabolic steroid use. Tamoxifen Citrate is used both during and post anabolic steroid cycle and very often is combined with AI drug. Letrozole and Anastrozole are used by athletes mainly during AAS cycles.

*Table 3. Half-life of some aromatase inhibitors.*

| AI | Generation | Therapeutic form | Half life | Other information |
|---|---|---|---|---|
| Formestane | II$^{nd}$ | Ampule (depot) | 30 h | Nowadays it is not used in clinical practice. However the substance is available on black market as topical gel forms. These forms are mainly used by bodybuilders. |

## *Aromatase inhibitors*

The implementation of aromatase inhibitors for treatment of early and metastatic breast cancer has been one of the major improvements in endocrine therapy of breast cancer [17]. AIs have been developed as a non-surgical therapy to reduce estrogen production in patients with hormone-responsive tumours [7]. It is important to note that aromatase inhibition is dose dependent. Aromatase inhibitors could be classified as first, second and third generation (Table 3). AI could be also divided by chemical structure in two other categories: steroidal and non-steroidal. Steroidal inhibitors such as formestane and exemestane inhibit aromatase activity by mimicking the substrate androstenedione. Letrozole and anastrozole which are non-steroidal enzyme inhibitors inhibit enzyme activity by binding with the heme iron of the enzyme.

First-generation aromatase inhibitors such as aminoglutethimide were found relatively weak and nonspecific because they can also block other steroidogenic enzymes [7-9]. As result of the efforts to generate a second generation AI was synthesised formestane (4-hydroxyandrost-4-ene-3, 17-dione). It was biotransformed by Rhizopus oryzae to yield the known 4 beta, 5 alpha-dihydroxyandrostane-3, 17-dione as the major product and bioconverted by Beauveria bassiana to afford the known reduced 4, 17 beta-dihydroxyandrost-4-en-3-one and 3 alpha, 17 beta-dihydroxy-5 beta-androstan-4-one and the new 4, 11 alpha,17 beta-trihydroxyandrost-4-en-3-one [13]. The second-generation AIs: 4-hydroxyandrostenedione (formestane) and fadrozole (CGS 16,949A), were found to be significantly more potent and better tolerated than aminoglutethimide but have not shown any significant benefit over tamoxifen [7,10]. In the late 1980's and early 1990's has begun the development of third generation AI. The scientists have already identified the two different mechanisms of aromatase inhibition which led to the development of the third-generation AIs. III$^{rd}$ generation AI can be divided in two categories: type I and type II. Type I aromatase inhibitors are androgen analogues, they are considered as aromatase in-activators. Type I interfere with the substrate-binding site of the enzyme and block the enzymatic complex by producing an unbreakable covalent bond between the inhibitor and the enzyme protein [7]. Type II are non-steroidal: anastrozole which was patented in 1987 and approved for medical use in 1995 [15] and letrozole, which block the electron transfer chain by the cytochrome P450 prosthetic group of the aromatase complex, acting as competitive inhibitors reversibly bound to the active enzymatic site [7].

## *Anti- oestrogens*

Anti-oestrogens are substances that are capable of antagonising many of the actions of oestrogens.

They have long half-lives in serum, and during their action *in vivo* undergo biotransformation to more polar forms that have a higher affinity for the oestrogen receptors [19]. The original class of anti-oestrogens are drugs that bind competitively to oestrogen receptor α and/or β to block oestrogen action. The original anti-oestrogens are the non-steroidal drugs:

cholorotrianisene, etamoxytriphetol, clomiphene and tamoxifen [24,28]. Subsequently, newer oestrogen receptor blockers have been developed as a class of partial or mixed estrogen analogues that have tissue-specific oestrogen agonist or antagonist effects: raloxifene, toremifene, droloxifene, lasoxifene, idoxifene, arzoxifene and bazedoxefine [24]. Fulvestrant is a new drug also considered anti-oestrogen. It is a steroidal oestrogen analogue which is the first in its class. It is a selective oestrogen receptor degrader. It doesn't block the estrogen receptor but destabilises and destroys it [29].

Almost sixty years ago, Lerner et al. discovered the first non-steroidal anti-oestrogen [2]. In 1960 Jensen et al. have identified the target for drug action of the anti-oestrogens the oestrogen receptors. Tamoxifen which was synthesised in 1967 is an anti-oestrogen that is used in the adjuvant endocrine therapy of early breast cancer and malignant breast disorders [21,22]. In males it increases the endogenous production of androgens. Because of its properties, tamoxifen is often misused in some sports. Nowadays, the biochemical mechanism of action of tamoxifen is widely understood and involves two active metabolites [40]. Tamoxifen is a prodrug which is metabolised in the liver by the cytochrome P450 isoform CYP2D6 and CYP3A4 into active metabolites such as 4-hydroxytamoxifen (4-OHT) and N-desmethyl-4-hydroxytamoxifen which have 30-100 times more affinity with the oestrogen receptors than tamoxifen itself [26,27,41]. Tamoxifen is excreted mainly in faeces. Biphasic excretion has been reported, and the terminal half-time may be longer than 7 d [22].

In 1956, Palopoli et al. have synthesised clomiphene. In 1959 clomiphene was patented. Eight years later it was approved for medical use in the United States. The introduction of clomiphene citrate into clinical medicine in 1967 made a revolution in the treatment of infertility and of polycystic ovarian syndrome [30]. Later it was approved for other therapeutic indications. Clomiphene is considered a very important medicine and is included in World Health Organization's List of Essential Medicines. By its chemical structure clomiphene is a triphenylethylene derivative. It is a mixture of two geometric isomers: enclomiphene and zuclomiphene, which contribute to oestrogenic and antiestrogenic properties of clomiphene. Because of the presence of the diethyl-amino group, clomiphene is sometimes called a catechol-oestrogen [30]. It acts as a selective oestrogen receptor modulator (SERM), similar to tamoxifen and raloxifene [42]. Clomiphene citrate is excreted principally through the intestines. Five days after oral administration, 51% has been excreted. However, some clomiphene continues to be excreted for at least 6 weeks [30,43]. So an athlete could give a positive doping for clomiphene even 6 weeks after last intake.

### *Side effects of anti-oestrogens and aromatase inhibitors*

AI and EB are prescription drugs with many benefits for some pathological conditions but also could cause many side effects. Unfortunately these medicines could be easily bought *via* different illegal internet sites by people of all ages. We have found that these online stores offer many original products, generics and also products of dubious origin and quality. It is important to note that the online sale of prescription drugs is illegal and could lead to many negative health consequences. We have found that although Clinical Practice Guidelines in Oncology recommend that anti-estrogen therapy should alternates with AI therapy, many athletes use both groups of drugs simultaneously which leads to more serious side effects.

Although anti-oestrogens and aromatase inhibitors are very attractive for many athletes, we have found that the proportion benefit-risk shows that any kind of abuse with these drugs is extremely dangerous.

The major side effects of anti-oestrogens abuse are: blurred vision, pain, constipation, cough, diarrhoea, hot flashes, loss of appetite, nausea, sore throat, stomach pain or upset, sweating, tingling or burning sensation, trouble sleeping, vomiting, weakness, weight gain, vomiting. About 25% of athletes abused with anti-oestrogens announced for hot flashes, nausea, and vomiting.

The side effects of anti-oestrogens use could be systematised in 12 different categories:

1. Musculoskeletal (back, bone, breast, joint, or pelvic pain).
2. Metabolic (hypercalcemia).
3. Hepatic side effects (jaundice, peliosis hepatitis, steatohepatitis, cholestasis, and massive hepatic necrosis).
4. Haematological (thrombocytopenia, leukopenia).
5. Ocular (blurred vision; red, irritated eyes).
6. Cardiovascular (slow or fast heartbeat).
7. Respiratory (cough).
8. Endocrine.
9. Nervous system side effects (anxiety, depression and headache).
10. Immunologic (flu-like symptoms).
11. Dermatologic.
12. Others.

Aromatase inhibitors often cause muscle and joint pain, heart problems, and could also raise cholesterol levels. The most common side effects of these drugs are symptoms of menopause like hot flashes, night sweats and vaginal dryness. Aromatase inhibitors also tend to speed up bone thinning and in result this could lead to osteoporosis.

1. Metabolic (anorexia, hypercholesterolemia).
2. Nervous system side effects (headache, somnolency).
3. Dermatologic (skin rash, irritative fever).
4. Musculoskeletal.
5. Gastrointestinal.
6. Others.

### *Methods for detection of aromatase inhibitors in biological samples*

**Methods for detection of letrozole:** Schänzer et al. (German Sport University) have developed a mass spectrometric method which is incorporated into the existing screening procedures for doping substances. The fragmentation pattern shows suitable ion transitions at m/z 294/225, 294/210, 294/142 and 294/130 [3]. The characterisation of the method shows a linear and homoskedastic calibration curve with a detection limit of 0.02 ng anastrozole per millilitre as well as high accuracy and precision. For the detection of letrozole misuse screening for the letrozole metabolite bis-(4-cyanophenyl)-methanol by GC-MS is an excellent tool. The EI-MS fragmentation+pattern of the TMS derivative shows suitable ions with high intensity (m/z 217 (M-89/base peak), m/z 291 (M+-15) and m/z 306 (M +)). The validation of the method shows a linear and homoskedastic calibration curve with an estimated lower limit of detection of 4.4 ng/ml [3]. Rodríguez et al. have developed a high performance liquid chromatography method with fluorescence detection to determinate letrozole in urine samples. The method offers high precision and accuracy but also it is simple and could be successfully applied for doping control. The only sample preparation step is to dilute the urine in the mobile phase (1:2, v/v). The mobile phase is phosphate buffer 80 mM (pH 3.0) and acetonitrile (65:35, v/v) at a flow rate of 1.0 mL/min. The analytes were detected at 295 nm after excitation at 230 nm [16]. Letrozole could be detected also in human plasma by HPLC with fluorescence detection: separation has been achieved on a monolithic silica column using acetonitrile–phosphate buffer. A fluorescence detector has been used for the quantitation with excitation and emission wavelengths at 230 and 295 nm. Minimum quantification limits (LOQ): 0.5 ng $mL^{-1}$. The method involves a simple, one-step extraction procedure with complete recovery [18].

**Methods for detection of formestane:** The substance is included in WADA prohibited list in 2004. Recent studies have shown that formestane is produced endogenously in small amounts. Lower concentrations could be due to endogenous production and not due to doping use, which makes the analytical procedure very difficult. Usually there is need of further investigation to prove the exact origin through determination of the carbon isotope ratio [12]. The basic analytical methodologies developed for application in sports drug testing, are based on Gas Chromatography-Mass Spectrometry (GC-MS) or Liquid Chromatography-Mass Spectrometry (LC-MS), targeting formestane itself or 4-hydroxytestosterone [44,45]. Torre et al. have developed analytical methodologies based on GC/MS and LC/MS, targeting formestane itself. This work was a WADA project. The researchers have found that traces of formestane can be produced endogenously and detected in urine samples in low concentrations (0.5-20 ng/mL) and thus, since 2011, it is mandatory according to WADA rules to perform a confirmation based on Isotope Ratio Mass Spectrometry (IRMS) in order to assess the synthetic origin of formestane before releasing an adverse analytical finding. The IRMS developed methods require two consecutive liquid chromatographic purifications (HPLC) before obtaining extracts of adequate purity. This is a very complicated analysis and the problem is that and not all anti-doping laboratories are currently prepared to perform such IRMS analyses [11]. The researchers Polet et al. have proposed a lower concentration limit of 25 ng/mL beneath of detected formestane to be considered as endogenous, and no further investigation to be needed [12].

**Methods for detection of anatrozole:** The metabolites of anatrozole are essential for its detection in biological samples. Anastrozole is metabolised in the liver [46]. In liver it undergoes oxidation (catalyzed by CYP3A4) to form hydroxy-anastrozole, which may further undergo glucuronidation (catalyzed by UGT1A4) to yield conjugated hydroxy-anastrozole. It could also be directly glucuronidated to anastrozole N-glucuronide [47-49]. Approximately 60% of the administered dose of anastrozole is excreted as metabolites while another 10% is excreted unchanged in the urine [49,50]. Triazole, hydroxyl-anastrozole, hydroxylanastrozoleglucuronide and anastrozoleglucuronide are the major metabolites of anastrozole, which could be detected in human plasma and urine. The methods for detection of anastrozole as doping substance should be sensitive enough to detect as anastrozole and also its metabolites. Nowadays gas chromatography methods are widely used for the quantification of anastrozole in biological samples. These techniques are very productive but also labour-intensive and require extensive sample clean-up to remove the co-extracted compounds from the biological matrix prior to use. Other sensitive and specific methods for detection of anastrozole are Liquid Chromatography Mass Spectrometry (LC-MS/MS) methods based on both Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI) interfaces. The only disadvantage of these methods is the time-consuming sample preparations [50].

**Methods for detection of aminoglutethimede:** The most appropriate method for the detection of the misuse of aminoglutethimide in urine is mass spectrometry.

### *Methods for the detection of the misuse of estrogen blockers*

**Methods for detection of tamoxifen:** One of the earliest methods for detection of tamoxifen was the thin layer chromatography [51]. The method had many disadvantages and nowadays it is replaced by many different more productive sensitive analytical procedures. Between 1978 and 1987 the most popular method for detection of tamoxifen was the gas chromatography-mass spectrophotometry [52-54]. In the beginning of the 80 ties of 20$^{th}$ century was developed a HPLC method with post-column fluorescence activation for detection of tamoxifen [40,55]. In the beginning of 90 ties of 20$^{th}$ century many HPLC methods for detection of tamoifen were improved which allowed easily to handle large numbers of samples [56]. Nowadays the most appropriate and use methods for detection of tamoxifen and metabolites are the LC-MS/MS

[40,57-60]. These methods are very specific, sensitive, and fast enough. Brown et al. have synthesised a signalling molecularly imprinted polymer for easy detection of tamoxifen and its metabolites. 6-Vinylcoumarin-4-Carboxylic Acid (VCC) was synthesised from 4-bromophenol to give a fluorescent monomer, designed to switch off upon binding of tamoxifen. Clomiphene was used as a template for the imprinting. Clomiphene has the ability to quench the coumarin fluorescence. Non-imprinted and imprinted polymers were synthesised using 6-Vinylcoumarin-4-carboxylic acid, methacrylic acid as a backbone monomer and ethylene glycol dimethacrylate as cross-linker, and were ground and sieved to particle sizes ranging between 45 and 25 μm. The results of rebinding experiments showed that while the non-imprinted polymer demonstrated negligible rebinding, the imprinted polymer has a very strong affinity for clomiphene and also for tamoxifen. The fluorescence of the imprinted polymer is quenched by clomiphene, 4-hydroxytamoxifen and tamoxifen [20]. Báezl et al. have developed a very successful method for detection of tamoxifen metabolites in urine samples: hydroxymethoxytamoxifen was detected in urine by gas chromatography-mass selective detection using a selective ion monitoring [22]. The researchers have reported that unchanged tamoxifen was not excreted and using this method, the main metabolite, hydroxymethoxytamoxifen, could be detected up to 7 d after administration [22]. The urinary tamoxifen methabolites could be also successfully detected by liquid chromatography quadrupole time-of-flight mass spectrometry [23]. The weaknesses and strengths of the different analytical procedures for detection of tamoxifen, other oestrogen blockers and AIs are described on Table 4.

***Table 4.*** *Analytical procedures for detection of AIs and oestrogen blockers: difficulties and strengths.*

| Analytical procedure | Suitable for detection of | Difficulties | Strengths |
|---|---|---|---|
| Gerace et al. have developed a fast screening protocol for the simultaneous determination of aminoglutethimide, anastrozole, clomiphene, drostanolone, formestane, letrozole, mesterolone, tamoxifen, testolactone and five of their metabolites in human urine. The method was successfully tested on real samples arising from clinical treatments of hospitalized patients. The first step of the analytical procedure was an enzymatic hydrolysis. The substances were extracted simultaneously from urine with a simple liquid extraction at alkaline conditions. The analytes were subsequently analyzed by fast-gas chromatography/mass spectrometry (fast-GC/MS) after derivatization. The use of a short column, high-flow carrier gas velocity and fast temperature ramping produced an efficient separation of all analytes in about 4 min, allowing a processing rate of 10 samples/h [61]. | Simultaneous determination of aminoglutethimide, anastrozole, clomiphene, drostanolone, formestane, letrozole, mesterolone, tamoxifen, testolactone and five of their metabolites. | • An enzymatic hydrolysis should be done. | • Simultaneous determination of aminoglutethimide, anastrozole, clomiphene, drostanolone, formestane, letrozole, mesterolone, tamoxifen, testolactone and five of their metabolites in human urine.<br>• The method is fast. |
| • Liquid chromatography coupled to mass spectrometry with a time-of flight system: urinary excretion samples of three selective oestrogen receptor modulators (SERMs) with a common triphenylethylene structure: clomiphene, toremifene, and tamoxifen, obtained after oral administration of a single dose of each drug, were analysed using a time-of-flight system, after automatic tuning and calibration of the equipment, in positive full scan mode using an electrospray ionisation source [62]. | Simultaneous determination of clomiphene, toremifene, and tamoxifen. | The lack of certified reference materials does not allow an accurate determination of the Limit Of Detection (LODs) of all metabolites. | • Detection of most of all significant metabolites.<br>• Detection of very low concentration. |
| Gas chromatography coupled with quadrupole mass spectrometer detection [63]. | Clomiphene and its methabolites | | • High selectivity.<br>• LOD found at 25 ng $mL^{-1}$.<br>• $r^2$ = 0.9966. |
| Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection [64]. | Tamoxifen and N-desmethyltamoxifen,<br>4-hydroxytamoxifen, Z-4-hydroxy-N-desmethyltamoxifen in human plasma. | | • A sensitive and reproducible method.<br>• Relatively simple procedure. |
| Liquid chromatographic mass spectrometric method (LC-MS/MS) for detection of tamoxifen and metabolites in blood plasma [40]. | Tamoxifen and metabolites. | | • The procedure is relatively simple.<br>• The method is specific.<br>• The method accurate and sensitive. |

| | | | |
|---|---|---|---|
| Method for the quantitation of tamoxifen and one tamoxifen metabolite (4HT) involving a direct extraction from plasma or ion-paired extraction for whole blood [40,65]. | Tamoxifen and one tamoxifen metabolite. | • Large sample volume.<br>• Large volumes of organic solvents are required for the extractions.<br>• Time consuming method.<br>• Detect only one metabolite.<br>• Not suitable for large number of samples. | • Simple and easy method. |
| HPLC method with post-column fluorescence activation [55]. | Tamoxifen and metabolites. | • The method requires air-cooled housing unit for the fluorescent activation of tamoxifen.<br>• Not all the currently identifiable metabolites of tamoxifen could be detectable.<br>• It is not suitable for doping control.<br>• The method requires aluminium foil reflectors and the generation of ozone.<br>• Complicated procedure. | |
| Low-dispersion Liquid Chromatography. The method is based on a one-step protein precipitation with acetonitrile followed by direct column injection, with the possibility of automation of sample batches [40,66]. | Tamoxifen and metabolites. | The method is not suitable for most analytical laboratories. | |
| Ion-paired HPLC chromatographic method with fluorescence detection. The analytes are extracted from biological fluid with diethyl ether and subsequently converted to fluorescent phenanthrene derivatives by irradiation with UV light. The fluorophores are separated by paired-ion chromatography on a reversed-phase (C18) column. Spectrofluorometric monitoring of the column eluent allows quantitation of analytes as their phenanthrene derivatives to levels of 100 pg/ml of plasma [67]. | Tamoxifen and three tamoxifen metabolites. | Time consuming method: need of photochemical conversion, (20 minutes or more), and the use of a dry-ice acetone bath. | • Tamoxifen and three tamoxifen metabolites. |
| High-performance liquid chromatography coupled to photo-spray tandem mass spectrometry. The analyte and the I.S. were extracted from 200 micro of human plasma by liquid-liquid extraction using a mixture of diethyl ether: dichloromethane (70:30, v/v) solution. Extracts were removed and dried in the organic phase then reconstituted with 200 micro of acetonitrile: water (50:50; v/v). The extracts were analyzed by high performance liquid chromatography coupled with photospray tandem mass spectrometry (HPLC-MS-MS). Chromatography was performed isocratically on a Genesis, C18 4 micro analytical column (100 mm × 2.1 mm i.d). The method had a chromatographic run time of 2.5 min and a linear calibration curve ranging from 0.05-10 ng $ml^{(-1)}$ [68]. | Anastrozole | | • Rapid method.<br>• Sensitive method.<br>• Specific method;<br>• The method could detect anastrozole even in very low concentrations. |

**Detection of clomiphene:** Ganchev et al. have proposed a rapid-resolution liquid chromatography-electrospray ionization-tandem mass spectrometry for quantification of clomiphene metabolite isomers in human plasma. The method is rapid, sensitive, specific and appropriate for the quantification of (E) and (Z)-isomers of clomiphene and their putative N-desethyl, N, N-didesethyl, 4-hydroxy, and 4-hydroxy-N-desethyl metabolites, and the N-oxides in human plasma. Following protein precipitation all analytes are separated on a ZORBAX Eclipse plus C18 1.8 μm column with a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile and detected on a triple quadrupole mass spectrometer with positive electrospray ionization in the multiple reaction monitoring modes. Lower limit of quantification for metabolites ranged from 0.06 ng/mL for clomiphene-N-oxides to 0.3 ng/mL for (E)-N-desethylclomiphene [69]. Gas chromatography coupled with a quadrupole mass spectrometer detection system is also very appropriate method for detection of clomiphene and its metabolites. Researchers have found that hydroxyclomiphene, the main metabolite that is monitored for doping control disappeared relatively faster than hydroxymethoxyclomiphene from urine. Therefore monitoring this additional proposed metabolite is recommended [42].

## Conclusion

It is an undeniable fact that with the development of pharmacy and medicine the doping has also evolved. Nowadays the relationship between sport and science has become stronger

than ever, because to be created a clean of doping sport culture science would help sport. Most of the doping substances are dangerous for athlete's health and could cause many negative consequences. Oestrogen blockers and aromatase inhibitors are essential medicines, but also dangerous doping substances. Currently, urine and blood samples are the only matrices authorised for doping testing by the World Anti-Doping Agency. Most appropriate analytical procedures for their detection in biological samples are HPLC/MS and GC/MS techniques but researchers still explore new models for enhanced detection.

## References


1. Riggins RB, Bouton AH, Liu MC, Clarke R. Antiestrogens, aromatase inhibitors, and apoptosis in breast cancer. Vitam Horm 2005; 71: 201-237.
2. Macgregor JI, Jordan VC. Basic guide to the mechanisms of antiestrogen action. Pharmacol Rev 1998; 50: 151-196.
3. https://www.wada-ama.org/sites/default/files/resources/files/schanzer_development.doc_0.pdf6.
4. World Anti-Doping Agency. Prohibited list. WADA 2017.
5. Carlson HE. Approach to the patient with gynecomastia. J Clin Endocrinol Metab 2011; 96: 15-21.
6. De Ronde W, de Jong FH. Aromatase inhibitors in men: effects and therapeutic options. Reprod Biol Endocrinol 2011; 9: 93.
7. Nabholtz J-MA. Long-term safety of aromatase inhibitors in the treatment of breast cancer. Therap Clin Risk Management 2008; 4: 189-204.
8. Samojlik E, Veldhuis JD, Wells SA. Preservation of androgen secretion during estrogen suppression with aminoglutethimide in the treatment of metastatic breast carcinoma. J Clin Invest 1980; 65: 602-612.
9. Perez N, Borja J. Aromatase inhibitors: clinical pharmacology and therapeutic implications in breast cancer. J Int Med Res 1992; 20: 303-312.
10. Wiseman LR, McTavish D. Formestane. A review of its pharmaco-dynamic and pharmacokinetic properties and therapeutic potential in the management of breast cancer and prostatic cancer. Drugs 1993; 45: 66-84.
11. De la Torre PX, Parr PK, Botrè P. Confirmation of formestane abuse in sports: a metabolic approach. In Force 2015.
12. Polet M, Van Renterghem P, Van Gansbeke W, Van Eenoo P. Profiling of urinary formestane and confirmation by isotope ratio mass spectrometry. Steroids 2013; 78: 1103-1109.
13. Martin GD, Narvaez J, Marti A. Synthesis and bioconversions of formestane. J Natural Products 2013; 76: 1966-1969.
14. Mauras N, Lima J, Patel D, Rini A, Salle E, Kwok A, Lippe B. Pharmacokinetics and dose finding of a potent aromatase inhibitor, aromasin (exemestane), in young males. J Clin Endocrinol Metab 2003; 88: 5951-5956.
15. Fischer J, Ganellin CR. Analogue-based drug discovery. Chem Int 2010; 32: 12.
16. Rodríguez J, Castañeda G, Muñoz L. Rapid determination of letrozole, citalopram and their metabolites by high performance liquid chromatography-fluorescence detection in urine: Method validation and application to real samples. J Chromatography B 2013; 914: 8-12.
17. Lonning P, Geisler J. Aromatase inhibitors: Assessment of biochemical efficacy measured by total body aromatase inhibition and tissue estrogen suppression. J Steroid Biochem Mol Biol 2008; 108: 196-202.
18. Zarghi A, Foroutan SM, Shafaati A. HPLC determination of letrozole in plasma using fluorescence detection. Application Pharmacokinetic Studies Chroma 2007; 66: 747.
19. Katzenellenbogen BS, Miller MA, Eckert RL, Sudo K. Antiestrogen pharmacology and mechanism of action. J Steroid Biochem 1983; 59-68.
20. Ray JV, Mirata F, Pérollier C, Arotcarena M, Bayoudh S, Resmini M. Smart coumarin-tagged imprinted polymers for the rapid detection of tamoxifen. Analytical Bioanalytical Chem 2016; 408: 1855-1861.
21. Jordan VC. Tamoxifen (ICI46, 474) as a targeted therapy to treat and prevent breast cancer. Brit J Pharmacol 2006; 147: 269-276.
22. Báez H, Camargo C, Osorio H, Umpiérrez F. Detection of tamoxifen metabolites by GC-MSD. J Chromatographic Sci 2004; 42: 551-553.
23. Lu J, He C, He G, Wang X, Xu Y, Wu Y, Dong Y, Ouyang G. Structural elucidation of new urinary tamoxifen metabolites by liquid chromatography quadrupole time-of-flight mass spectrometry. J Mass Spectrom 2014; 49: 570-578.
24. Handelsman DJ. Indirect androgen doping by oestrogen blockade in sports. Brit J Pharmacol 2008; 154: 598-605.
25. Handelsman DJ. Clinical review: The rationale for banning human chorionic gonadotropin and estrogen blockers in sport. J Clin Endocrinol Metab 2006; 91: 1646-1653.
26. Desta Z, Ward BA, Soukhova NV, Flockhart DA. Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. J Pharmacol Exp Therap 2004; 310: 1062-1075.
27. Ahmad A, Shahabuddin S, Sheikh S, Kale P, Krishnappa M, Rane RC, Ahmad I. Endoxifen, a new cornerstone of breast cancer therapy: demonstration of safety, tolerability, and systemic bioavailability in healthy human subjects. Clin Pharmacol Therap 2010; 88: 814-817.
28. Dickey RP, Holtkamp DE. Development, pharmacology and clinical experience with clomiphene citrate. Hum Reprod Update 1996; 2: 483-506.
29. Lai AC, Crews CM. Induced protein degradation: an emerging drug discovery paradigm. Nature Rev Drug Discov 2017; 101-114.

30. Dickey RP, Holtkamp DE. Development, pharmacology and clinical experience with clomiphene citrate. Hum Reprod Update 1996; 2: 483-506.
31. Tenover JS, Dahl KD, Hsueh AJ, Lim P, Matsumoto AM, Bremner WJ. Serum bioactive and immunoreactive follicle-stimulating hormone levels and the response to clomiphene in healthy young and elderly men. J Clin Endocrinol Metab 1987; 64: 1103-1108.
32. Guay AT, Bansal S, Heatley GJ. Effect of raising endogenous testosterone levels in impotent men with secondary hypogonadism: double blind placebo-controlled trial with clomiphene citrate. J Clin Endocrinol Metab 1995; 80: 3546-3552.
33. Parker LN, Gray DR, Lai MK, Levin ER. Treatment of gynecomastia with tamoxifen: a double-blind crossover study. Metabolism 1986; 35: 705-708.
34. Maier U, Hienert G. Tamoxifen and testolactone in therapy of oligozoospermia: results of a randomized study. Eur Urol 1988; 14: 447-449.
35. Krause W, Holland-Moritz H, Schramm P. Treatment of idiopathic oligozoospermia with tamoxifen-a randomized controlled study. Int J Androl 1992; 15: 8-14.
36. Duschek EJ, Gooren LJ, Netelenbos C. Effects of raloxifene on gonadotrophins, sex hormones, bone turnover and lipids in healthy elderly men. Eur J Endocrinol 2004; 150: 539-546.
37. Uebelhart B, Herrmann F, Pavo I, Draper MW, Rizzoli R. Raloxifene treatment is associated with increased serum estradiol and decreased bone remodeling in healthy middle-aged men with low sex hormone levels. J Bone Miner Res 2004; 19: 1518-1524.
38. Mauras N, Lima J, Patel D, Rini A, di Salle E, Kwok A. Pharmacokinetics and dose finding of a potent aromatase inhibitor, aromasin (exemestane), in young males. J Clin Endocrinol Metab 2003; 88: 5951-5956.
39. Leder BZ, Finkelstein JS. Effect of aromatase inhibition on bone metabolism in elderly hypogonadal men. Osteoporos Int 2005; 16: 1487-1494.
40. Heath DD, Flatt SW, Wu AHB, Pruitt MA, Rock CL. Evaluation of tamoxifen and metabolites by LC-MS/MS and HPLC Methods. Brit J Biomed Sci 2014; 71: 33-39.
41. Lu WJ, Desta Z, Flockhart DA. Tamoxifen metabolites as active inhibitors of aromatase in the treatment of breast cancer. Breast Cancer Res Treat 2012; 131.
42. Martínez BD, Montes de OPR, Teresa CVM, Roberto SO, Rosado PA. Excretion study of clomiphene in human urine: evaluation of endogenous steroids profile after multiple oral doses. J Brazilian Chem Soc 2010; 21: 2220-2225.
43. Mikkelson TJ, Kroboth PD, Cameron WJ. Single-dose pharmacokinetics of clomiphene citrate in normal volunteers. Fertil Steril 1986; 46: 392-396.
44. De la Torre X, Colamonici C, Curcio D, Jardines D, Molaioni F, Parr MK, Botrè F. Detection of formestane abuse by mass spectrometric techniques. Drug Test Anal 2015; 6: 1133-1140.
45. Leung GNW, Kwok WH, Wan TSM, Lam KKH, Schiff PJ. Metabolic studies of formestane in horses. Drug Test Anal 2013; 5: 412.
46. Ingle JN, Buzdar AU, Schaid DJ, Goetz MP, Batzler A, Robson ME. Variation in anastrozole metabolism and pharmacodynamics in women with early breast cancer. Cancer Res 2010; 70: 3278-3286.
47. Lazarus P, Sun D. Potential role of UGT pharmacogenetics in cancer treatment and prevention: focus on tamoxifen and aromatase inhibitors. Drug Metab Rev 2010; 42: 182-194.
48. Kamdem LK, Liu Y, Stearns V, Kadlubar SA, Ramirez J, Jeter S. In vitro and in vivo oxidative metabolism and glucuronidation of anastrozole. Br J Clin Pharmacol 2010; 70: 854-869.
49. Abubakar MB, Gan SH. A review of chromatographic methods used in the determination of anastrozole levels. Indian J Pharm Sci 2016; 78: 173-181.
50. Sanford M, Plosker GL. Anastrozole: a review of its use in postmenopausal women with early-stage breast cancer. Drugs 2008; 68: 1319-1340.
51. Adam HK, Douglas EJ, Kemp JV. The metabolism of tamoxifen in human. Biochem Pharmacol 1979; 28: 145-147.
52. Gaskell SJ, Daniel CP, Nicholson RI. Determination of tamoxifen in rat plasma by gas chromatography-mass spectrometry. J Endocrinol 1978; 78: 293-294.
53. Daniel CP, Gaskell SJ, Bishop H, Nicholson RI. Determination of tamoxifen and a hydroxylated metabolite in plasma from patients with advanced breast cancer using gas chromatography-mass spectrometry. J Endocrinol 1979; 83: 401-408.
54. Murphy C, Fotsis T, Pantzar P, Adlercreutz H, Martin F. Analysis of tamoxifen, N-desmethyltamoxifen and 4-hydroxytamoxifen levels in cytosol and KCl-nuclear extracts of breast tumours from tamoxifen treated patients by gas chromatography-mass spectrometry (GC-MS) using selected ion monitoring (SIM). J Steroid Biochem 1987; 28: 609-618.
55. Brown RR, Bain R, Jordan VC. Determination of tamoxifen and metabolites in human serum by high-performance liquid chromatography with post-column fluorescence activation. J Chromatogr 1983; 272: 351-358.
56. Fried KM, Wainer IW. Direct determination of tamoxifen and its four major metabolites in plasma using coupled column high-performance liquid chromatography. J Chromatogr B Biomed Appl 1994; 655: 261-268.
57. Lu W, Poon GK, Carmichael PL, Cole RB. Analysis of tamoxifen and its metabolites by on-line capillary electrophoresis-electrospray ionization mass spectrometry employing non-aqueous media containing surfactants. Anal Chem 1996; 68: 668-674.
58. Sanders JM, Burka LT, Shelby MD, Newbold RR, Cunningham ML. Determination of tamoxifen and metabolites in serum by capillary electrophoresis using a non-aqueous buffer system. J Chromatogr B Biomed Sci Appl 1997; 695: 181-185.

59. Lim HK, Stellingweif S, Sisenwine S, Chan KW. Rapid drug metabolite profiling using fast liquid chromatography, automated multiple-stage mass spectrometry and receptor-binding. J Chromatogr A 1999; 831: 227-241.
60. Zhu YB, Zhang Q, Zou JJ, Yu CX, Xiao DW. Optimizing high-performance liquid chromatography method with fluorescence detection for quantification of tamoxifen and two metabolites in human plasma: application to a clinical study. J Pharm Biomed Anal 2008; 46: 349-355.
61. Gerace E, Salomone A, Abbadessa G, Racca S, Vincenti M. Rapid determination of anti-estrogens by gas chromatography/mass spectrometry in urine: Method validation and application to real samples. J Pharma Analysis 2012; 2: 1-11.
62. Mazzarino M, Fiacco I, De La Torre X, Botrè F. A mass spectrometric approach for the study of the metabolism of clomiphene, tamoxifen and toremifene by liquid chromatography time-of-flight spectroscopy. Eur J Mass Spectrometry 2008; 14: 171-180.
63. Ganellin J, Robin C. Analogue-based drug discovery. John Wiley and Sons 2006; 516.
64. Lee KH, Ward BA, Desta Z, Flockhart DA, Jones DR. Quantification of tamoxifen and three metabolites in plasma by high-performance liquid chromatography with fluorescence detection: Application to a clinical trial. J Chromatogr B: Anal Technol Biomed Life Sci 2003; 791: 245-253.
65. Mendenhall DW, Kobayashi H, Shih FM, Sternson LA, Higuchi T, Fabian C. Clinical analysis of tamoxifen, an anti-neoplastic agent, in plasma. Clin Chem 1978; 24: 1518-1524.
66. Lien EA, Ueland PM, Solheim E, Kvinnsland S. Determination of tamoxifen and four metabolites in serum by low-dispersion liquid chromatography. Clin Chem 1987; 33: 1608-1614.
67. Golander Y, Sternson LA. Paired-ion chromatographic analysis of tamoxifen and two major metabolites in plasma. J Chromatogr 1980; 181: 41-49.
68. Mendes GD, Hamamoto D, Ilha J, Pereira AS, De Nucci G. Anastrozole quantification in human plasma by high-performance liquid chromatography coupled to photospray tandem mass spectrometry applied to pharmacokinetic studies. J Chromatogr B: Anal Technol Biomed Life Sci 2007; 850: 553-559.
69. Ganchev B, Heinkele G, Kerb R, Schwab M, Mürdter TE. Quantification of clomiphene metabolite isomers in human plasma by rapid-resolution liquid chromatography-electrospray ionization-tandem mass spectrometry. Anal Bioanal Chem 2011; 400: 3429-3441.

## *Correspondence to

Stanislava Ivanova

Faculty of Pharmacy

Department of Pharmacognosy and Pharmaceutical Chemistry

Medical University of Plovdiv

Plovdiv

Vasil Aprilov Blvd. 15-A

Bulgaria