ISSN 0974-3618 (Print)
0974-360X (Online)

www.rjptonline.org

RESEARCH ARTICLE

Development of the method for determining Methandienone in Toxicology and doping-analysis

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ABSTRACT:

Anabolic-androgenic steroids are ones of the most frequently detected drugs in amateur and professional sports. Doping control laboratories have developed numerous assays enabling the determination of administered drugs. However, it is relevant today to improve detection methods that are accurate, fast and require small amounts of reagents. The identification and quantification of methandienone from body fluids has been insufficiently developed. The gas chromatography-mass spectrometry (GC-MS) method for the determination of methandienone traces in body fluids was developed and validated in the toxicology laboratory in order to control a non-medical use of an anabolic steroid-methandienone. Extracting procedures were optimized in order to obtain entire amount of the drug and methandienone and its metabolites were determined by sensitive and specific reactions for the compound identification, as well as the system for carrying out the TLC method have been proposed, and conditions for identifying a test sample from the biomaterial by IR spectrophotometry have been selected. The GC-MS method is found to be simple, fast, sensitive, and the strategy proposed can be effortlessly and advantageously applied for the routine analysis of biological fluids for determining methandienone and its metabolites at a level less than 30 ng in toxicology and doping-analysis.

KEYWORDS: Methandienone, Doping, Toxicology Analysis, Gas Chromatography, Pharmaceutical Analysis.

INTRODUCTION:

The problem of non-medical use of anabolic steroids is acute all over the world. According to the statistics provided by the World Anti-Doping Agency, anabolic steroids are in the first place by the frequency of their use as doping in the world, and methandienone is among the most widely abused anabolic androgenic steroid[1,2].

The review of the special literature conducted allows us to conclude that the problem of doping contains not only a medical and (or) toxicological context, but has a pronounced social character[3].

Methandienone (dianabol, [(17-b)-17-hydroxy-17-methylandrosta-1,4-dien-3-one] (Figure 1) is an anabolic androgenic steroid drug, which is widely used to treat many health problems, such as aplastic anemia, burns, delayed puberty, and early climacterium. In addition to its medical uses, methandienone has popular non-medical uses in fitness and bodybuilding industries to increase the physical activity and muscle volume[4,5].

Received on 23.11.2020 Modified on 28.12.2020
Accepted on 31.01.2021
Research J. Pharm. and Tech. 2021; 14(10):5169-5174.
DOI: 10.52711/0974-360X.2021.00899

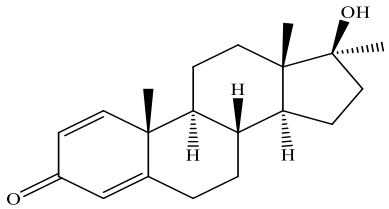

Figure 1: The chemical structure of Methandienone

Since the routine analyses used in doping control are based on the previous knowledge of the target analytes, some steroids are designed to evade the anti-doping detection. Thus, they have been named as "designer steroids"[6].

Due to the limited or even absent information about the metabolism and toxicity of such drugs, they might pose an extremely high risk to the health of their users. Methandienone was included in the World Anti-Doping Agency Prohibited List in 2006. This drug is prohibited as other steroids and has already been reported to cause serious liver damage in some subjects[7,8].

Considering the widespread use of methandienone, its toxic properties, the presence of cases of lethal poisonings, and the absence of highly sensitive methods for studying biological fluids for the presence of the drug it should be noted that the development of methods for its isolation, identification and the quantitative determination in biological fluids is one of the relevant current problems.

To isolate methandrostenolone from the biological material, the use of classical methods based on its isolation with water acidified with oxalic acid (A.A. Vasilyeva's method), water acidified with sulfuric acid (V.F. Kramarenko's method), and ethyl alcohol acidified with tartaric acid (Stas-Otto`s method) is currently described. It has been found that the use of classical isolation methods does not allow achieving a high degree of isolation of methandienone (up to 30%); in addition, they are relatively insensitive[9,10].

A number of the known variants of studying methandienone in the biological material based on isolation with methanol and acetonitrile does not provide for a sufficiently high degree of purification, which does not allow to fully applying the capabilities of modern highly sensitive methods of analysis[11].

The identification and quantification of methandienone in biological fluids has been insufficiently developed. Nowadays there aren`t generally accepted protocols in toxicology and doping controls, that is why this topic is actually[12].

Therefore, the aim of this study is to develop accurate and rapid methods that would allow the detection and identification of traces of methandienone in biological fluids.

MATERIAL AND METHODS:

The object of the research was "Danabol" tablets containing 10mg of methandienone (S.C. Balkan Pharmaceuticals S.R.L., Republic Moldova).

All studies were carried out in an accredited laboratory at the Hon. Prof. M. S. Bokarius Kharkiv Research Institute of Forensic Examinations. The plasma sample was purchased from the Red Cross Society, Ukraine, and urine samples were taken from four volunteers. All the subjects provided written informed consent for participating and sample collection under the present study[13].

The reagents were met the requirements of the European Pharmacopoeia, the United States Pharmacopoeia and the State Pharmacopoeia of Ukraine. The "Sartorius" MC 210 S balance and the ISO volumetric glassware of class A were used.

To identify methandienone in tablets and biological fluids (urine and plasma) the chemical reactions and thin layer chromatography were used.

The experiments were carried out on model mixtures of methandienone with the human blood plasma and urine, which were kept for 1.5 hours at a temperature of 16-18°C in accordance with the methods previously proposed[14,15].

The method of extracting (isolating) methandienone from biological fluids:

To a part of the model mixture of a biological object with methandienone 50ml of acetone is added, adjusted to pH = 2, and the mixture is acidified with sulfate acid, and kept at room temperature for 2 hours. After settling acetone is decanted, and the biomaterial is extracted twice more with acidified acetone, 50ml each, for 1 hour. The acetone extracts obtained are combined, evaporated and concentrated to a volume of several milliliters. About 50ml of distilled water is added to the resulting residue and evaporated with a stream of warm air until the odor of the solvent evaporates. The solution is filtered through a paper filter. The solution of sodium hydroxide is added to the filtrate and adjusted to pH = 11, then transferred to a separation funnel and extracted three times with chloroform (20, 20, 10ml). The resulting chloroform extracts are combined and filtered through a paper filter. Further, with the filtrate obtained, the identification of methandienone is carried out.

Reactions on the keto group and the unsaturated cyclopentanoperhydrophenanthrene with next reagents: the mixture of the concentrated sulfuric acid with methyl alcohol in the volume ratio of 7:3 (reagent No. 1), the solution of vanillin 1% in 70% sulfuric acid (reagent No. 2); the mixture of formaldehyde and the concentrated sulfuric acid in the volume ratio of 1:10 (reagent No. 3).

The method of the methandienone determination by the method of thin-layer chromatography. "Silica gel 60" chromatographic plates (Merk Company No. 1.05553) are used. As a test solution use 1 "Danabol" tablet is mixed with ethanol 96% and the volume of the solution is diluted with the same solvent to 100.0ml. As the reference solution the chloroform extract of methandienone from the biological fluid (urine or plasma) is used. On the starting line of the chromatographic plate 1cm strips of 10µl of the test solution and 10µl of the reference solution are applied.

The plate is dried in air and placed in a chamber with a mixture of solvents. The composition of the mobile phases is selected in such a way that the results obtained are as effective as possible and well reproduced. Thus, the following systems of TLC are selected: petroleum ether - diethyl ether (6: 4) – system No. 1; benzene - ethanol - diethylamine (9: 1: 1) – system No. 2, benzene - ethanol (98: 2) – system No. 3.

When the solvent front is 15cm from the start line the plate is removed from the chamber, dried in air for 15 minutes and then chromatographic zones are detected by fluorescence quenching at 254nm while fixing their position. Subsequently, the chromatographic zones identified are shown by visualizing reagents. Visualization of the chromatographic zones is performed with Dragendorff reagent, and they are again revised in daylight.

On the chromatogram of the test solution the main spots should be detected at the level of the spots on the chromatogram of the reference solution corresponding to the color. On the chromatogram of the test solution additional spots are allowed[16].

The IR spectrophotometry method for identifying the drug is contained in the monographs of almost all steroids used in medicine. A feature of methandienone is the manifestation of polymorphism, the absorption bands of the test and standard samples may not coincide when analyzing in different conditions. In this case, for identification, the spectra of solutions of the objects from chloroform extracts studied are compared by evaporating the solvent to dryness, and the spectra of solid residues (in tablets with potassium bromide) are compared[17].

In our study the IR-spectra were recorded on a "Nicolet 380 FT-IR" infrared spectrometer by Thermo Fisher Scientific using the attachment "Smart Perfomer" with ZnSe crystal (certificate No.84389 dated 08.11.2019).

The GC-MS research was performed with the aim of qualitatively and quantitatively determining methandienone, its metabolites in the study objects[18]. The GC-MS analysis was performed on a GCMS-QP2020 NX: SHIMADZU (Shimadzu Corporation, inventory No. 101450011, the calibration certificate dated 11.08.2020 No. 08/0643K). The GC parameters were as follows: the carrier gas was helium at 1.5mL min-1 in a constant flow mode; the capillary column – 100% polymethylsiloxane (30m × 0.25mm × 0.25µm; J&W Scientific, Agilent Technologies Inc., California, USA); the injector temperature – 250ºC; the injection volume – 1µL; the purge flow – 3.0mL/min; the split ratio – 1:20. The GC oven temperature was programmed to increase from 0 to 60ºC (followed by a hold for 3 min), and then was set to increase from 60 to 280ºC (rate of 20ºC min-1), and finally, to increase to 280ºC, followed by a hold for 28 min. The MS parameters were as follows: Detector Gain: 0.85kV+0.00 kV; Ion Source Temp: 220.00°C; Interface Temp.: 250.00°C. Mass spectra were acquired in full scan modes in the range of m/z 41-500. The data were analyzed on GCMS solution, LabSolutions Insight (Shimadzu Corporation, Tokyo, Japan).

RESULTS AND DISCUSSION:

The method was proposed for isolating methandienone from biological fluids. Urine and blood plasma are the most common objects for chemical and toxicological research. The dependence of the degree of extraction of methandienone from biofluids with acetone on the infusion rate, its duration time, and the quantitative ratio of the isolating agent and the biological material were studied.

The optimal conditions for extracting methandienone with acetone was selected; they were double infusion with acetone, the ratio of the insulating liquid and the biological object at each stage of infusion was at least 2: 1, the duration of contact between acetone and biofluids in each case was at least 2 hours. The study of the extraction of methandienone showed that it was extracted to the greatest extent by hydrophobic solvents from alkaline solutions (pH = 11). Thus, the optimal parameters for the isolation of methandienone from biological materials with acetone were found.

For selection the most sensitive methods of the qualitative and quantitative determination of methandienone were proposed: chemical and physicochemical (chemical reactions and TLC) and

instrumental methods (IR-spectrophotometry and GC-MS).

The choice of qualitative color reactions to anabolic steroids was due to the presence of the keto group O = C = in the structure of methandienone and an unsaturated cyclopentanoperhydrophenanthrene. As a result of the experimental research, the most sensitive and selective reactions for methandienone were found; they were reactions with reagents based on the concentrated sulfuric acid.

The results of reactions were the same in a standard sample and samples isolating from biofluids: with reagent No. 1 – a light yellow color of the solution, which in 30 minutes turned into orange, was observed; with reagent No. 2 – a red-orange color of the solution was observed, turning into brown-red in 30 minutes and with reagent No. 3. – an orange-green color of the solution was observed, turning into light brown.

Thin layer chromatography is commonly used as screening method for the forensic toxicological analysis of different samples like urine, stomach contents, blood and liver tissues etc. TLC is a presumptive screening method based on which we can selectively go for the other final confirmation methods[19].

The research method of methandienone in biology simples using chromatography in a thin layer of a sorbent was chosen taking into account the fact that it was highly sensitive (in the presence of a reference sample), cost-effective and did not require special technological equipment. The results of the study using chromatography in a thin layer of a sorbent with various mobile phases are presented in Table 1.

Table 1: Chromatographic mobility (Rf) values of the samples zones and "Danabol" drug

	Chromatographic mobility (Rf) of the samples zones and "Danabol" drug		
	System No. 1	**System No. 2**	**System No. 3**
Methandienone (Test solution)	0.19	0.79	0.33
Methandienone (Reference solution)	0.19	0.79	0.33

Analyzing the results of chromatographic mobility shown in the table we can conclude that the most positive separation characteristics were obtained using system No.3. As it meets the requirements of optimal Rf value in range 0, 3 – 0, 7, if other compounds/ metabolites will be present in the mixture, they will be visible on the TLC plate when the Rf is in this range. And in chosen system, the values of Rf in the system used are close to each other, therefore, to increase the selectivity of this method, it is necessary to have samples for the comparative study of these substances.

In addition to thin layer chromatography, it is advisable to use the IR-spectroscopy method[20] (Figure 2).

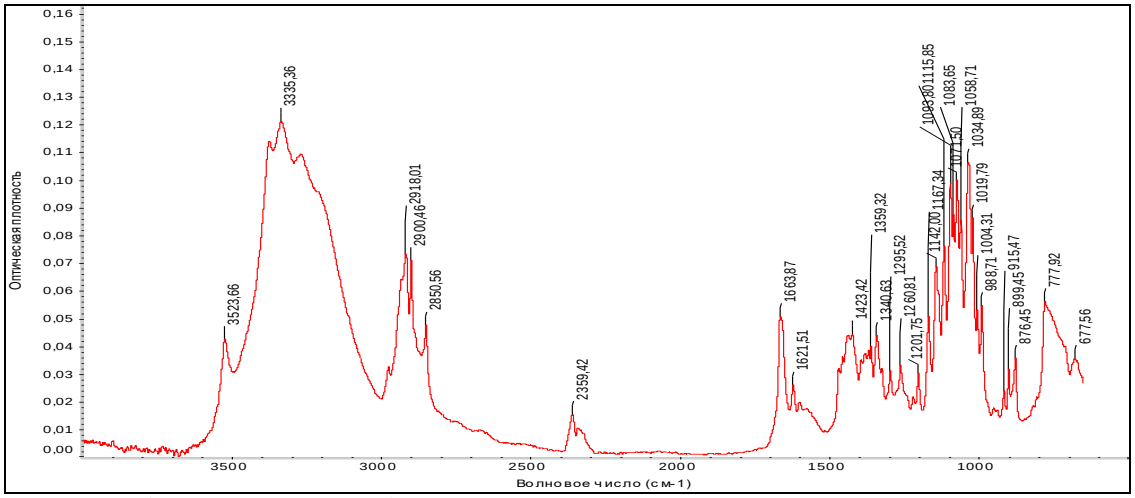

Figure 2: IR-spectra of methandienone (isolated from the blood plasma)

The IR spectra of methandienone isolated from the biomaterial (Figure 2) had characteristic absorption maxima at 1660, 1621, 876, 1663, 1142, 1260 cm^{-1}. Decryption was performed using the library of IR spectra from the Omnic 9.1 software package.

Anabolic androgenic steroids can be improved through more selective detection techniques, such as GC/MS analysis[21,22]. This is one of the main methods used to detect the presence of steroids and determine their structure.

There are the following possible thermostatting options used in determining anabolic steroids:

Mode 1: The initial column temperature is 180ºC, then the gradient temperature is at a rate of 10ºC/min to 300 ºC, the delay is 50 min.
Mode 2: The initial column temperature is 190ºC, the delay is 3 min, then the gradient temperature is at a rate of 5ºC/ min to 300ºC, the delay is 50 min.
Mode 3: The initial column temperature is 190ºC, then the gradient temperature is at a rate of 2єC/min to 235 ºC, then at a rate of 12ºC/min to 300ºC, the delay is 20 min.
Mode 4: The initial column temperature is 190ºC, then the gradient temperature is at a rate of 2 єC/min to 235 ºC, then at a rate of 30ºC/min to 300ºC, the delay is 20 min.

Experimentally, thermostating mode 4 for methandienone was selected. The retention time of the solution of the sample with methandienone was 18.0 min (Figure 3a).

Signals of a number of fragments with characteristic m/z 43, 79, 91, 122, 147, 161, 179, being the most intense in the mass spectrum of the compound, were chosen as characteristic ions for methandienone (Figure 3c).

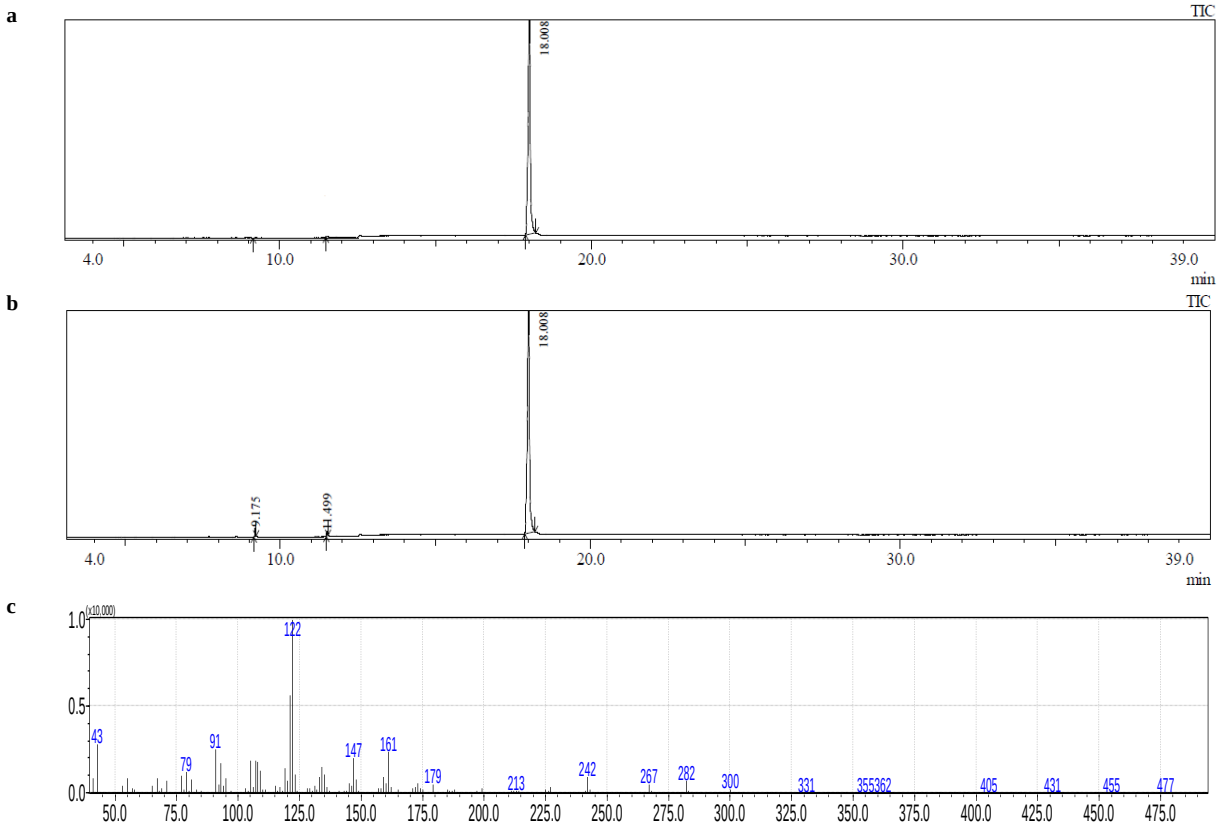

Figure 3: The gas chromatograms and mass spectra: a – the chromatogram of the solution of "Danabol" sample, b – the chromatogram of the solution of the sample isolated from the biological fluid, c – the mass spectrum of "Danabol" sample.

The combination of signals from characteristic fragments in the mass spectrum and the retention time in the column provided a sufficiently high selectivity for the identification of the compound under consideration by GC-MS[23].

The chromatograms obtained after isolation of methandienone from the blood plasma and urine had a peak at 18.1 min and minor peaks at 9.2 min and 11.5 min, which were specific for methandienone metabolites; their structures were identified as 1-epi-methandienone and 6β-hydroxy-17-epi-methandienone. These metabolites were exclusively excreted in the unconjugated form. At least two more metabolites were extractable from the free fraction of the urine, but no measurable amounts of methandienone itself were found. The rate of metabolism and urinary excretion seemed to be reasonably fast. The total amount of the recovered methandienone in the form of its metabolites – 1-epi-methandienone and 6β-hydroxy-17-epi-methandienone was about 2% (Figure 3)[24].

In addition to identifying and detecting traces of methandienone, this method can be used to quantify the active pharmaceutical ingredient and its metabolites in biological fluids in toxicology and doping-analysis.

The detection limits were determined and were at the level of 30 ng for methandienone, 10 ng for 17–epi-methandienone and 6β-hydroxy–17–epi-methandienone.

CONCLUSION:

The present research proposes a simple isolation method for methandienone and its metabolites from biological fluids, which allows us to isolate a sample without the loss and give consistent and reproducible recoveries.

Sensitive and specific reactions for the compound identification, as well as the system for carrying out the TLC method have been proposed, and conditions for identifying a test sample from the biomaterial by IR spectrophotometry have been selected.

A highly sensitive, specific, reproducible, rapid and high-throughput GC-MS has been developed to determine methandienone traces and its methabolites in biological materials (blood plasma, urine) at the Forensic Research Laboratory of Hon. Prof. M. S. Bokarius Kharkiv Research Institute of Forensic Examinations.

The results obtained demonstrate that the strategy proposed can be effortlessly and advantageously applied for the routine analysis of biological fluids for determining methandienone and its metabolites at a level of 30 ng for methandienone and 10 ng for its methabolites in toxicology and doping-analysis.

ACKNOWLEDGEMENT:

The authors are grateful to the authorities of National University of Pharmacy.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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