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A simple toxicological analysis of anabolic steroid preparations from the black market

Analyse toxicologique simple de stéroïdiens anabolisants provenant de marchés parallèles

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Abstract – Objectives: A simple and rapid gas chromatography (GC) method with mass spectrometry (MS) detection was developed for the identification and quantification of anabolic steroids in pharmaceutical preparations from the black market. **Material and Methods:** After a liquid-liquid extraction of pharmaceutical products at acidic, neutral and basic pH with chloroform-isopropanol (9:1, v/v), the different steroids were separated by fused silica capillary column and detected by electron impact (EI)-MS in positive ionization mode. **Results and Conclusion:** The assay was validated in the range from 10 mg to 250 mg/g powder preparations and 0.02 mg to 200 mg/mL liquid preparations with good determination coefficients ($r^2 \geqslant 0.99$) for the calibration curves. At three concentrations spanning the linear dynamic ranges of the calibration curves, mean recoveries were always higher than 90% and intra-assay and inter-assay precision and accuracy were always better than 15%. This method was successfully applied to the analysis of 15 pharmaceutical preparations sold by illegal sources. In only two cases the content was the one reported on the labels. In the other cases, no substances at all, or steroids different from those reported on the labels or different amounts from those declared were found.

Key words: Anabolic steroids, ccounterfeits, black market, analysis

Résumé – Objectifs : Une méthode d'identification de stéroïdes anabolisants obtenus sur les marchés parallèles par chromatographie en phase gazeuse simple et rapide avec détection par spectrométrie de masse a été développée. **Matériel et Méthodes :** Après une extraction liquide-liquide des principes actifs à pH acide, neutre et basique par le mélange chloroforme-isopropanol (9:1, v/v), les différents stéroïdes ont été séparés sur une colonne capillaire et détectés par spectrométrie de masse (impact électronique en mode d'ionisation positive). Le dosage a été validé sur la gamme 10–250 mg/g de préparations en poudre et sur la gamme 0,02–200 mg/mL de préparations liquides ($r^2 \geqslant 0{,}99$ pour les courbes d'étalonnage). **Résultats et Conclusion :** À trois concentrations comprises dans les gammes des courbes d'étalonnage, les recouvrements moyens étaient toujours supérieurs à 90 %, et les précisions intra-essai et inter-essai étaient toujours supérieures de 15 %. Cette méthode a été appliquée avec succès à l'analyse de 15 produits pharmaceutiques vendus par des sources illégales. Dans deux cas seulement, le contenu correspondait à ce qui était indiqué sur les étiquettes. Dans les autres cas, aucune autre substance n'a été trouvée, ou les stéroïdes détectés étaient différents de ceux inscrits sur les étiquettes ou comportaient des quantités différentes de celles indiquées.

Mots clés : Stéroïdes anabolisants, contrefaçons, marché parallèle, analyse

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1 Introduction

Anabolic steroids are synthetic substances related to the male sex hormones [1]. They have a number of physiological effects, most notably an anabolic effect that promotes the growth of skeletal muscle and androgenic effects that foster the development of male sexual characteristics. Abuse of anabolic steroids is motivated in most cases by a desire to build muscles, reduce body fat, and improve sports performance. Abuse

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is estimated to be very high among competitive bodybuilders and spread among elite and recreational athletes [2]. Anabolic steroids are taken orally as tablets or capsules, by injection into muscles, or as gels or creams that are rubbed into the skin. Doses taken by abusers can be up to 100 times greater than doses used for treating medical conditions. Anabolic steroids are often taken in combination in a practice called "stacking", in which the abusers mix oral and/or injectable types of anabolic steroids. Steroid abusers often also "pyramid" stacked compounds in cycles of 6 to 12 weeks, meaning that they gradually increase doses then slowly decrease them to zero. Since steroids must be medically prescribed, a black market has developed due to the increasing popularity of bodybuilding [3,4].

After opening the borders to Eastern Europe an explosion of the black market for anabolic steroids occurred. Most of the hormone products in the European black market nowadays come from countries within the European Union, or from Eastern European countries such as Russia, Poland, Bulgaria and Romania, but also, sometimes, from Turkey and Egypt. Another very popular country of origin for anabolic steroids is Thailand, and sometimes these preparations are also produced in Pakistan, India and even in Korea [5].

During the last 10 years, numerous products that supposedly support athletic performance and muscle growth were obtained from different websites, but also confiscated in house searches. Several of those were identified as counterfeit substances [6]. The World Health Organization (WHO) defines a counterfeit drug as one that is "deliberately and fraudulently mislabeled with respect to identity and/or source". A counterfeit substance may contain inappropriate quantities of active ingredients, or none, may be improperly processed within the body (*e.g.*, absorption by the body), may contain ingredients that are not on the label (which may or may not be harmful), or may be supplied with inaccurate or fake packaging and labeling. Low-quality counterfeit medication may cause any of several dangerous health consequences, including side effects or allergic reactions, in addition to their obvious lack of efficacy due to having less or none of their active ingredients [7].

In industrialized countries and to some extent in poorer countries, internet-based sales of pharmaceuticals are a major source of counterfeit medicines, threatening those who seek cheaper, stigmatized or unauthorized treatments. Illegal internet pharmacies sell medications that have an unknown or vague origin without prescriptions and use unapproved or counterfeit products. In this concern, in the past year the Italian Anti-Adulteration and Safety Bureau (Carabinieri per la tutela della salute – NAS) seized several pharmaceutical preparations sold via websites or through illegal venues (private doctors, fitness centers and gyms, odd stores) as "anabolic steroids" with the high suspicion that those preparations contained counterfeit unauthorized substances and requested specific analyses of the seized products.

Different methodologies have been reported to date to detect anabolic steroids in pharmaceutical preparations [8–11] and some specifically analyzed illegally distributed products [12–15]. The reported methodologies generally include some sort of sample treatment of extraction before the analytical step, which always includes a separation by liquid or gas chromatography (LC) coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS).

Nonetheless, in the case of seized products of unknown origin, a toxicological analysis of all possible pharmacologically active substances (drugs of abuse, doping agents, pharmaceuticals, etc.) that could be used illegally in these preparations is recommended [16]. Furthermore, an assay which requires a simple gas chromatograph coupled to a mass spectrometer which can be routinely used by all control laboratories (including those of the police forces) is preferable.

The *Drug Abuse and Doping* Unit of the National Institute of Health in Rome proposes a simple toxicological analysis of pharmaceutical preparations from the black market suspected of containing anabolic steroids.

2 Experimental

2.1 Chemicals and reagents

All steroid standard solutions (nandrolone decanoate, methenolone enanthate, stanozolol, testosterone enanthate, testosterone isocaproate, testosterone decanoate, testosterone cypionate, testosterone undecanoate, testosterone propionate, testosterone phenylpropionate, trenbolone acetate, oxymethanolone, boldenone, methadienone, oxandrolone, drostanolone and 17 methyltestosterone used as an internal standard) were supplied by LGC Standards (Milano, Italy). The derivatization reagent, a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and dithioerythritol (DTE) (MSTFA/NH_4I/DTE 1000:2:6 v:v:v), was supplied by Sigma-Aldrich (Milano, Italy). All reagents of analytical grade were obtained from Carlo Erba (Milano, Italy).

2.2 Seized products

Fifteen different pharmaceutical preparations (4 tablet-preparations, 7 oily solutions and 4 suspensions for intramuscular injection) seized by the NAS following alerts from consumers were received at the *Istituto Superiore di Sanità* to be analyzed for the presence of active compounds.

The products analyzed are shown in Table I.

Before sample preparation, 500-mg pulverized tablets or 500-µl oily solution or suspension for intramuscular injection were dissolved in 5 mL of methanol with the support of an ultrasonic bath for 15 min.

Calibration standards (from 10 mg to 250 mg/g powder preparations and 0.02 mg to 200 mg/mL liquid preparations) with different mg amounts of the analytes under investigation were prepared for each analytical batch by adding suitable amounts of standard stock solutions to 1 g or to 1 mL blank pharmaceutical products (powder or oily suspensions). Calibration samples were treated and processed as unknown samples. Several aliquots of quality control (QC) samples (low, medium and high, respectively) were prepared in blank pharmaceutical products to be used for the calculation of validation parameters. In detail, QC samples were at 15, 150 and 200 mg

Table I. Retention times and qualifying and quantifying (in bold) ions for the anabolic steroids under investigation.

Steroid	Retention time (min)	Qualifying and quantifying (in bold) ions
Testosterone	17.1	**432**, 417, 209, 73
Boldenone	19.7	**430**, 415, 325, 206
Drostanolone	19.8	**448**, 433, 405, 141
Methadienone	20.5	**444**, 429, 339, 206
Testosterone isocaproate	20.7	**458**, 443, 343, 73
Trenbolone acetate	20.8	472, 412, 397, **332**
Testosterone propionate	21.6	**416**, 401, 209, 193
Oxandrolone	21.7	363, 308, **143**, 73
Testosterone enanthate	21.7	**472**, 457, 343 ,73
Oxymethanolone	22.8	**548**, 533, 4, 5, 281
Stanozolol	24.9	472, 457, 342, **143**
Nandrolone decanoate	25.6	**500**, 485, 207, 73
Testosterone decanoate	27.1	**514**, 499, 343, 73
Testosterone phenylpropionate	27.7	**492**, 477, 91, 73
Methenolone enanthate	28.5	**486**, 471, 195, 73
Testosterone undecanoate	29.6	**528**, 513, 343, 73
Testosterone cipionate	33.8	**484**, 469, 343, 73
17 methyltestosterone (Internal Standard)	20.65	**446**, 431, 301, 73

steroid per g of powder preparations and 0.05, 10 and 150 mg steroid per ml liquid preparations.

2.3 Instrumentation and conditions

Analyte separation was achieved on a fused silica capillary column (HP-5MS, 30 m × 25 mm i.d, film thickness 0.25 m: Agilent Technologies, Palo Alto, CA, USA). The oven temperature was programed at 100 °C for 2 min, increased to 290 °C at 10 °C/min. Split injection mode (15:1) was used. Helium (purity 99%) with a flow rate of 1 mL/min was used as carrier gas. The injection port, ion source, quadrupole and interface temperatures were: 260, 230, 150 and 280 °C, respectively.

The electron-impact (EI) mass spectra of the analytes and internal standard were recorded in total ion monitoring mode (scan range 40–550 *m/z*) to determine retention times and characteristic mass fragments.

Retention times (RT) and qualifying and quantifying ions for the anabolic steroids under investigation are reported in Table I.

2.4 Sample preparation

One mL of methanol-diluted samples was put in 2 mL 0.1 M phosphate buffer at three different pH values: acid (pH = 2.5), basic (pH = 10–12) and neutral (pH = 7)

pH in an ultrasonic bath for 15 minutes, then the solutions were extracted with two different aliquots of 2 mL chloroform/isopropanol (9:1, v/v). After centrifugation the organic layer was divided into two aliquots of 2 mL and evaporated to dryness at 40 °C under a nitrogen stream. For GC–MS screening analysis of pharmacologically active substances including steroids, one 1-µL aliquot was injected after dilution in ethylacetate, the other one after derivatization in capped test tubes with 100 of N,O-bis-trimethylsylyl-trifluoroacetamide (BSTFA)+1% (trimethylsilyl (TMS) at 70 °C for 30 min.

A second extraction, specific for anabolic steroids in pharmaceutical preparations, was also performed according to the procedure described by Geyer *et al.* [13]. Briefly, 500 µL of methanol-diluted samples in alkaline buffer were extracted with n-pentane with the addition of 200 µL isoamyl alcohol to break the emulsion formed between the two layers, thus facilitating the separation of the organic layer. This last layer was evaporated to dryness at 40 °C under a nitrogen stream, derivatized with 100 µL of a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH_4I) and dithioerythritol (DTE) (MSTFA/NH_4I/DTE 1000:2 :6 v:v:v), and a 1-µL aliquot was injected into the chromatographic system.

2.5 Validation procedures

Prior to application to real samples, the method was tested in a 5-day validation protocol. Selectivity, linearity, limits of detection (LOD) and quantification (LOQ), recovery, precision, accuracy, and stability were assayed as previously reported [17].

3 Results and discussion

3.1 Chromatography and validation results

A chromatographic run was completed in 34 min, and afterward initial conditions were restored in 2 min. The temperature program was set up in order to let drugs of abuse and pharmaceutical substances with low molecular weight appear before the anabolic steroids, whose peaks started to appear 17 min from the beginning of the run. No additional peaks due to other substances that could have interfered with the detection of compound of interest were observed. No pharmacologically active substances (principal drugs of abuse, doping agents, most diffused pharmaceutical products) were detected in the products under investigation. Blank samples injected after the highest point of the calibration curve did not present any traces of carryover. Nonetheless, an injection of methanol was introduced between each injection of the batch.

Linear calibration curves were obtained with a determination coefficient (r^2) higher than 0.99 for the different steroids. The analytical recoveries obtained at three QC concentrations after liquid–liquid extraction were always higher than 90% for the different analytes. Limits of detection and quantification were always below the first point of the calibration curves and satisfactorily met the internationally established acceptance criteria [18]. Intra-assay and inter-assay precision and accuracy were always better than 15%. No relevant degradation was observed in QC samples after any of the three freeze/thaw cycles, with differences in the initial concentration of less than 10%. Similar results (differences to the initial concentration always lower than 10%) were obtained in the case of the mid-term stability test (QC samples analyzed once a month during a four-month period), assuring the feasibility of stored sample analysis.

3.2 Analysis of products

The results from the analysis of pharmaceutical products sold as anabolic steroids through illegal venues are listed in Table II.

Of 15 preparations with presumptive presence of steroidal compounds, just two (N. 4 and 13) contained what was declared on the respective labels. Of the other 13 preparations, which did not contain what was declared on the label, two (N. 1 and 5) did not contain any anabolic steroid compound nor other active substances at all; three (N. 8, 11, 12) contained the steroid reported on the label but at concentrations that were significantly lower than those declared (7.6%, 1.4% and 0.5%, respectively) and the other 8 contained steroids different from those shown on the labels. Differences in concentration of measured steroids from tablet to tablet or from one vial to another of the same product were always less than 10%. The presented results were in accordance with what was found by the study group of Thevis *et al.*, who successfully analyzed steroids from the black market and nutritional supplement products with skilled chromatographic – mass spectrometric approaches [14,15].

4 Conclusion

Smuggling of anabolic steroids and other related hormonal substances is big business, but up to now not a crime to be pursued in most of the world. Indeed, it is possible to buy this kind of pharmaceutical without a prescription in most countries through illegal sources (*e.g.* Asia, Africa and in South America and Europe). The internet is the other principal open source for people interested in this kind of product. There are no official statistics, but naive information (often reported on websites of bodybuilders or product users) show that these products are usually consumed by students and recreational athletes of different ages (principally from 20 to 45 years of age) and sport disciplines (bodybuilding, cycling, swimming) who desire to increase their performance with no medical advice or medical prescriptions for these products.

Because the demand is high, the black market is still growing. In many cases the products are substances different from the ones declared on the labels or they do not contain any of the purported ingredients, as demonstrated in our study, which applied a simple GC-MS assay which can be used by all control laboratories of police forces, customs and public health

Table II. Analysis of pharmaceutical products sold as anabolic steroids through illegal venues.

Item	Pharmaceutical preparation	Type of formulation	Content as declared on the label	Manufacturer	Major compounds determined
1	Methandienone	Tablets (n = 100)	10 mg Methandienone per tablet	Biotech Pharmaceuticals: Sydney, Australia	No active compounds
2	Masteron-200	10 mL oily solution for i.m.*application	100 mg/mL Drostanolone Propionate	Biotech Pharmaceuticals: Sydney, Australia	0.02 mg/mL Testosterone 0.03 mg/mL Testosterone Propionate
3	Oxandrolone	Tablets (n = 50)	10 mg Oxandrolone per tablet	Biotech Pharmaceuticals: Sydney, Australia	40.6 mg/tablet Stanozolol
4	Winstrol	3 vials of 1 mL suspension for i.m. application	50 mg/mL Stanozolol	Desma: Madrid, Spain	43.3 mg/mL Stanozolol
5	Nandrolone-200	Oily solution for i.m. application	100 g/mL Nandrolone Decanoate	Biotech Pharmaceuticals: Sydney, Australia	No active compounds
6	Primabolan-200	Suspension for i.m. application	100 mg/mL Methenolone enanthate	Biotech Pharmaceuticals: Sydney, Australia	0.46 mg/mL Boldenone Undecylenate
7	Enanthate-500 ANDROLONE-200	Oily solution for i.m. application	250 mg/mL Testosterone enanthate	Biotech Pharmaceuticals: Sydney, Australia	2.8 mg/mL Testosterone 17.2 mg/mL Testosterone Propionate
8	Trenbolone-150	Oily solution for i.m. application	150 mg/mL Trenbolone-acetate	Biotech Pharmaceuticals: Sydney, Australia	11.4 mg/mL Trenbolone-acetate
9	Oxymethanolone	Tablets (n = 50)	50 mg/tablet Oxymethanolone	Biotech Pharmaceuticals: Sydney, Australia	1.4 mg/tablet Methandienone
10	Testomix-300	Oily solution for i.m. application	1 mL contains: 50 mg Testosterone-propionate, 70 mg Testosterone-phenylpropionate 80 mg Testosterone-isocaproate, 100 mg Testosterone-decanoate	Biotech Pharmaceuticals: Sydney, Australia	32.8 mg/mL Testosterone Phenylpropionate 62.8 mg/mL Testosterone isocaproate
11	Propionaat 200	Oily solution for i.m. application	200 mg/mL Testosterone-propionate	Biotech Pharmaceuticals: Sydney, Australia	20.8 mg/mL Testosterone-propionate
12	Boldone	Oily solution for i.m. application	100 mg/mL Boldenone	Biotech Pharmaceuticals: Sydney, Australia	0.5 mg/mL Boldenone
13	Testex Prolungatum 250	Suspension for i.m. application	100 mg/mL Testosterone cypionate undecanoate	Biotech Pharmaceuticals: Sydney, Australia	119.2 mg/mL Testosterone cypionate
14	MITGAN 50	Suspension for i.m. application	50 mg/mL Boldenone	Compania California: Bogotà, Colombia	1.04 mg/mL Testosterone Phenylpropionate
15	Naposim	Tablets (n = 10)	10 mg/tablet Methadienone	Not reported	10.8 mg/tablet Methyltestosterone

*intramuscular.

laboratories still not equipped with the latest hyphenated mass spectrometric techniques.

Conflicts of interest: The authors declare that there are no conflicts of interest.

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