Forensic Science International 275 (2017) 272–281

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

GC–MS quantitative analysis of black market pharmaceutical products containing anabolic androgenic steroids seized by the Brazilian Federal Police

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ARTICLE INFO

Article history:
Received 16 December 2016
Received in revised form 10 March 2017
Accepted 22 March 2017
Available online 5 April 2017

Keywords:
Anabolic steroids
GC–MS
Method validation
Counterfeiting
Medicine
Dietary supplement

ABSTRACT

The use of counterfeit or substandard medicines can have an important health impact, resulting in therapeutic failure, be toxic or even cause death. Anabolic steroids are a frequent target for counterfeiters worldwide, being the second most frequent counterfeited class in Brazil. The aims of this work were to optimize and validate a GC–MS method for the quantitative determination of anabolic steroids in tablet, aqueous suspension and oil solution forms, and to analyze pharmaceutical products sent to Brazilian Federal Police (BFP) for forensic analysis. Sample preparation included extraction with methanol in ultrasonic bath followed by centrifugation. The method was successfully validated and 345 samples of pharmaceutical products were analyzed (328 medicines and 17 dietary supplements). About 42% of the medicines were counterfeits, 28.7% of tablets, 12.0% of suspensions and 65.2% of oil solutions; 11% were considered substandards. Five dietary supplements contained undeclared anabolic steroids, including two containing methandrostenolone at 5.4 and 5.8 mg/capsule, equivalent to levels found in medicines. The proposed method is suitable for implementation in routine analysis for identification of counterfeits and substandard products. The analytical results show the need to raise awareness of consumers over the risks from the consumption of anabolic steroids from the clandestine market and for more incisive actions from government agencies aiming at decreasing the availability of these products.

1. Introduction

Substandard, spurious, falsely labelled, falsified and counterfeit (SSFFC) medical products are a serious concern worldwide, reaching any therapeutic class [1]. SSFFC medicines can have no effect at all, result in therapeutic failure, be toxic or even cause death [1,2]. Substandard products, also known as out-of-specification products, are genuine medicines produced by authorized manufacturers that do not meet their quality specifications. Counterfeit (spurious, falsely labelled and falsified products, SFFC) are those deliberately and fraudulently mislabeled with respect to identity and/or source, both branded and generic products, with the wrong ingredients, without active ingredients, with insufficient quantities of correct ingredient(s) or with fake packaging [3]. SFFC products are not easily identified, since they are designed to appear identical to genuine products.

In Brazil, pharmaceutical products under suspicion of being SSFFC are routinely sent for forensic analysis at the Brazilian Federal Police (BFP). In conjunction actions with the National Health Surveillance Agency (ANVISA) during the period of 2007–2011, the BFP seized 115 thousand units of SSFFC products, mainly those for erectile dysfunction and anabolic androgenic steroids (AAS) [4]. Data obtained from forensic reports issued by the BFP from 2007 to 2010 showed that 69% of 610 cases of seized counterfeit medicines were for erectile dysfunction and 26% declared the presence of AAS [5]. Another study showed that about one third of the 3537 AAS-declaring medicines analyzed by the BFP from 2006 to 2011 was considered counterfeit [6]. Almost half of these counterfeits (48.6%) had no active ingredient, 28.3% had different ingredients from those stated on the label and 16.1% declared an inexistent manufacturer. Since not all products were chemically analyzed, and analysis were only qualitative, this counterfeit rate may be severely underestimated.

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http://dx.doi.org/10.1016/j.forsciint.2017.03.016
0379-0738/

The abusive use of anabolic androgenic steroids by amateur and professional athletes is well documented in several countries. Recent estimates indicate that the prevalence of AAS use in Brazil varies between 2.1% and 31.6%, depending on the region studied and sample characteristics [7]. Considering data from 271 studies from all continents, MPhil et al. [8] estimated a 3.3% overall prevalence of AAS use in the general population (6.4% for males and 1.6% for females). It is reasonable to hypothesize that several of these users acquire their products on the black market, since AAS are controlled substances in most countries.

The presence of undeclared anabolic steroids in medicines and dietary supplements is well documented on the literature [9–11]. Pellegrini et al. [12] found eight of the 15 medicine samples seized by the Italian Anti-Adulteration and Safety Bureau containing an AAS not stated on the label. Cho et al. [13] analyzed 19 tablets, injectable medicines and dietary supplements obtained from the market or websites in South Korea, finding nine medicines adulterated with AAS. Van Poucke et al. [14] found 11 of the 19 supplement samples intercepted by the Belgian pharmaceutical inspection at the post office containing AAS. Abbate et al. [15] analyzed 24 bodybuilding supplements sold in fitness equipment and online shops in the United Kingdom, 16 of which contained steroids not declared on the label. In Italy, Odoardi et al. [16] found prasterone, androstenedione, methandienone, stanozolol and/or testosterone present in most of the 30 supplements analyzed; although the levels of methandienone, stanozolol and testosterone were below those required for biological activity, their consumption could lead to a positive anti-doping exam, and be a health hazard if consumed at high amounts or continually.

Different methods are used for the analysis of AAS in pharmaceutical products, including HPLC-DAD [17], LC–MS/MS [11,13,14], GC–MS with derivatization [11,12], or association of different methods [11,19]. Some methods using these techniques, however, gave only qualitative results [9,18–20].

The aims of the present work were to develop and validate a quantitative GC–MS method suitable for the routine analysis of AAS in pharmaceutical products, with a simple sample preparation procedure, and no derivatization step, and apply this method to analyze pharmaceutical products seized by the BFP.

2. Material and methods

2.1. Reagents and standards

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany) and Tedia (Fairfield, OH, USA). Cellulose, lactose and starch, used as a blank tablet matrix, were purchased respectively from Merck (Darmstadt, Germany), Sigma–Aldrich (St. Louis, MO, USA) and J. T. Baker (Phillipsburg, NJ, USA). Manitol, also used as a blank tablet matrix, was a chemically characterized material sent for forensic analysis by the BFP. Water used as blank matrix for aqueous suspensions was produced by a Milli-Q Direct-Q system (Millipore, Bedford, MA, USA).

Reference standards of prasterone (PR; 99.9% purity), testosterone (T; 97.8% purity), methandrostenolone (ME; 96.7% purity), testosterone propionate (TP; 99.9% purity), stanozolol (ES; 98.4% purity), testosterone isocaproate (TI; 99.8% purity) and nandrolone decanoate (ND; 96.0% purity) were purchased from LGC Standards (Luckenwalde, Germany). Oxandrolone (OXA; 98.0% purity), boldenone undecylenate (BU; 98.0% purity) and nandrolone phenylpropionate (NF; 96.0% purity) standards were purchased from Toronto Research Chemicals (Toronto, Canada). Testosterone enanthate (TE; 99.0% purity, determined by Nuclear Magnetic Resonance in the context of this study) was purchased from the European Directorate for the Quality of Medicines & HealthCare (Strasbourg, France) and testosterone cypionate (TC; 100.0% purity) from the United States Pharmacopoeia.

Drostanolone propionate (PD) and testosterone phenylpropionate (TF), used as internal standards (IS), were prepared from bulk materials seized by the BFP that had their identity confirmed by Mass spectrometry and Infra-Red spectrometry. Spectrometric information did not indicate the presence of any substance that could interfere with the analysis.

2.2. Standard solution preparation

Methanol stock solutions at 1000 μg/mL of each AAS were used to prepare the methanol AAS working solutions at 50 μg/mL, with exception of ES (100 μg/mL), which showed a less intense signal in the GC–MS. Stock solutions at 1000 μg/mL of PD and TF in methanol were used to prepare the IS intermediate solutions at 200 μg/mL, which were added to all calibration and sample vials to a final concentration of 9.52 μg/mL. PD was used as an internal standard for PR, T, ME, OXA, TP and ES, and TF as internal standard for TI, TE, TC, ND, NF and BU. Quantitation of each analyte was performed by determining the ratio between the AAS peak area and the respective IS peak area.

2.3. Samples

A total of 328 medicine and 17 dietary supplement samples sent to forensic analysis by the BFP and for which label information or GC–MS qualitative analysis indicated the presence of any investigated AAS were retrieved for this study; compound identification during qualitative analysis was performed by comparison of the mass spectra obtained with the National Institute of Standards and Technology (NIST) electronic library. Samples were seized from 2011 to 2016, with declared expiry date of at least 2012. The medicine samples included 87 tablets (13 different medicines plus two samples without identification), 83 aqueous suspensions (8 different medicines) and 158 oil solutions (39 different medicines). All dietary supplements were in tablet/capsule form and included 10 different products. Samples had 17 different countries of declared origin, mainly Paraguay (N = 154), Brazil (N = 30), United States (N = 24), Argentina (N = 22), Australia (N = 19) and Spain (N = 13); 21 samples did not declare their origin.

Medicine tablets stated the presence of ES, ME or OXA (5 or 10 mg/tablet), and some did not declare its contents. Suspensions declared the presence of T (100 mg/mL) or ES (50 or 100 mg/mL). Oil solutions had several declared formulations, containing BU, ME, ND, NF, PD, TP, TF, TI, TD, TC, TE, methenolone enanthate, trenbolone acetate and trenbolone enanthate, alone or in different associations. Total AAS contents in these products ranged from 50 to 300 mg/mL. Dietary supplements declared the presence of PR (25, 50 or 100 mg/tablet or capsule; two did not specify the amount) or the prohormones methasterone (10 mg/capsule) or methasterone plus halodrol (15 and 25 mg/capsule, respectively). All formulations are described in the Supplementary material.

2.4. Sample extraction method

Sample preparation varied according to the pharmaceutical form, declared concentration and previous results obtained during qualitative analysis. Mean weights of tablets and capsules were determined and five tablets or the contents of five capsules were ground together and homogenized. In cases when there were fewer than five units available, all units where ground together and homogenized. Aqueous suspensions and oil solutions were manually homogenized.

An amount corresponding to 1/10th the mean weight of the tablet/capsule, 50 or 100 μL of aqueous suspensions or 20 to 100 μL

of oil solutions (depending on their nominal concentrations) was transferred to falcon tubes, and diluted to 5 mL with methanol. Falcon tubes were vortexed for 10 s, sonicated for 10 min and centrifuged for 5 min at 3000 rpm. Sonication and centrifuge were not necessary if the sample was completely homogenized after vortexing. Extracts were diluted with methanol to a final volume of 1 mL; the volume of the extract aliquot varied according to the nominal concentration of the sample (from 20 to 250 μL for tablets/capsules, 50 μL for aqueous suspensions and from 20 to 100 μL for oil solutions).

Aliquots of 50 μL of the IS working solutions were added to all sample and calibration solutions prior to GC–MS analysis (final concentration of 9.52 μg/mL).

2.5. Equipment

GC–MS analysis was performed on a GC System 7890A, coupled with a 5975C Mass Spectrometer (operating at 70 eV) and an automated sample injector system CTC PAL G 6509-B (Agilent Technologies, Santa Clara, California, USA). Chromatography was performed on a HP5-MS capillary column (Agilent Technologies; 25 m × 0.20 mm i.d. × 0.33 μm film thickness). Temperatures of the MS ion source and GC–MS interface were 230 and 280 °C, respectively. MS detector was used in Selected Ion Monitoring (SIM) mode. Two ions were monitored for each AAS and IS, based on their relative abundance and on their absence in neighbor peaks, in case they were too close. The optimized GC–MS conditions of the method were: injector temperature = 280 °C, injection volume = 1.0 μL, splitless, helium flow of 2.5 mL/min. The oven temperature program was: Initial temperature = 200 °C, 30 °C/min until 250 °C, hold for 16 min, 30 °C/min until 300 °C, hold for 8.5 min, with a total run time of 27.83 min. A chromatogram with all AAS included in the study and both internal standards is shown in Fig. 1. Table 1 shows the chemical structure, the molar mass and the monitored ions. Total response was the sum of both ion signals.

2.6. Method validation

Method validation was performed following ANVISA guidelines for medicines [21] and MAPA (Brazilian Ministry of Agriculture) guidelines for veterinary drugs [22]. A mixture of lactose, cellulose, starch and mannitol in equal proportions was used as a tablet blank for all validation studies, and purified water as a suspension blank. A counterfeit medicine sample in oil form, for which previous forensic analysis showed to contain no AAS, was used as oil solution blank.

Linearity of the calibration curves was evaluated preparing three solution sets at 2.5, 5.0, 10.0, 25.0 and 50.0 μg/mL (two times higher for ES) and injecting each solution two times into the GC–MS (total of six replicates per level). Data were evaluated for a possible linear or quadratic relationship, and the quality of the regressions assessed considering: the correlation coefficient, heteroscedasticity by Cochran and F tests, analysis of variance (ANOVA) to evaluate lack-of-fit, sum of relative errors, graphic evaluation of the randomness of the residuals, and residual standard deviation.

Selectivity was assessed by analyzing blank matrices and investigating any response at the AAS or IS retention times for possible interferences.

Matrix effects were evaluated by analyzing *in matrix* and methanol control samples fortified at 2.5, 10 and 50 μg/mL [22] and quantified against a methanol calibration curve.

Precision and recovery studies were conducted together. Aliquots of blank matrices were fortified at four levels (only three for aqueous suspensions), four replicates for each level and were quantified using freshly prepared calibration curves in methanol. Repeatability was accessed by the RSD_r of the four intraday replicates and intermediate precision by the RSD_p of eight replicates from two days (acceptable values were up to 13.3% for RSD_r and 20% for RSD_p [22]). Acceptable recovery range was from 80 to 120% and recovery was calculated as the mean result of the four intraday replicates. The method limit of quantification (LOQ) for each analyte was defined as the smallest concentration with acceptable repeatability, intermediate precision and recovery.

Robustness of the instrument parameters was evaluated by comparing the outcome of the methanol standard solutions (at 2.5, 10 and 50 μg/mL) after altering different parameters (injector temperature, gas flow, injector temperature, injection volume, initial temperature, temperature program), with a total of 14 altered final settings. Different ultrasound times during sample preparation were also tested (0, 5 or 10 min). Samples were quantitated and results compared with those obtained from the validated method.

Stability of the standard solutions at 2.5 and 10 μg/mL in methanol and *in matrix* under different conditions was tested (n = 3). The solutions were kept at the GC tray and at the refrigerator, and analyzed on the day they were prepared, and

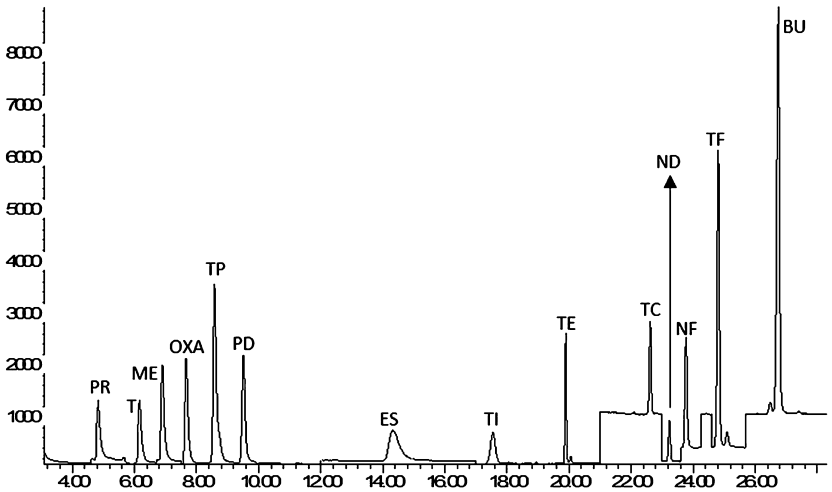


Fig. 1. Cromatogram with all AAS and internal standards. PR = Prasterone; T = Testosterone, ME = Methandrostenolone; OXA = Oxandrolone, PD = Drostanolone Propionate (Internal Standard), TP = Testosterone Propionate, ES = Stanozolol, TI = Testosterone Isocaproate; TE = Testosterone Enanthate, TC = Testosterone Cypionate, ND = Nandrolone Decanoate, NF = Nandrolone Phenylpropionate, TF = Testosterone Phenylpropionate (Internal Standard), BU = Boldenone Undecylenate.

Table 1
Chemical structure of the anabolic androgenic steroids and internal standards with the respective monitored ions.

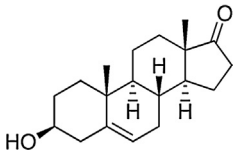
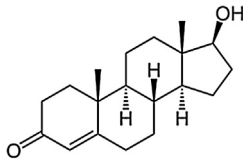
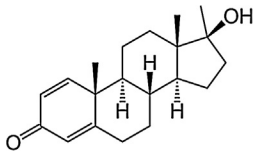
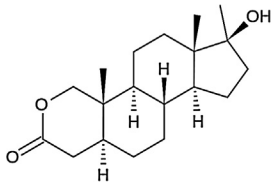
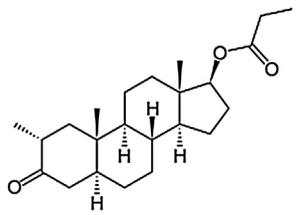
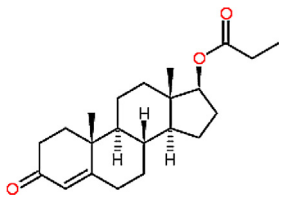
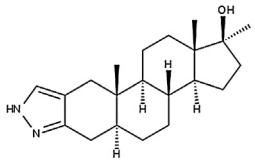
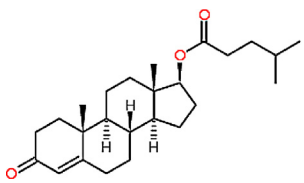
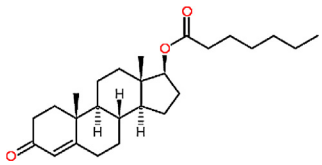
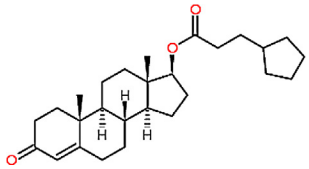
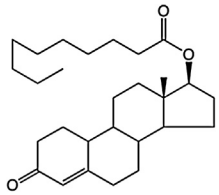
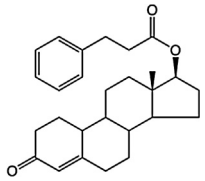
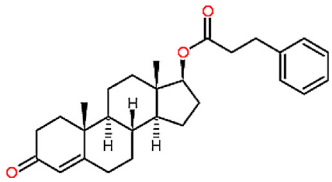
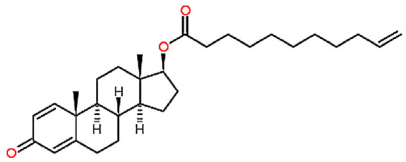
Compound	Chemical structure	Molecular mass	Ions (m/z)
Prasterone (PR)		288.4	91; 105
Testosterone (T)		288.4	79; 124
Methandrostenolone (ME)		300.4	91; 122
Oxandrolone (OXA)		306.4	71; 291
Drostanolone Propionate (PD)		358.5	149; 286
Testosterone Propionate (TP)		344.5	57; 124
Stanozolol (ES)		328.5	96; 328
Testosterone Isocaproate (TI)		386.6	81; 124

Table 1 (*Continued*)

Compound	Chemical structure	Molecular mass	Ions (m/z)
Testosterone Enanthate (TE)		400.6	113; 124
Testosterone Cypionate (TC)		412.6	124; 147
Nandrolone Decanoate (ND)		428.6	110; 155
Nandrolone Phenylpropionate (NF)		406.6	91; 257
Testosterone Phenylpropionate (TF)		420.6	91; 271
Boldenone Undecylenate (BU)		452.7	122; 147

after three, seven, ten and fourteen days, every time with a freshly prepared calibration curve (all vials septa were changed after each analysis to prevent solvent evaporation). Calibration curves were stored in the freezer and were also analyzed on the subsequent days of analysis.

3. Results

3.1. Method validation

Linearity studies indicated that data were quadratic and heteroscedastic; several regressions were calculated comprising all combinations between two options of data transformation (square root and log 10) plus untransformed data and six weighting factors (1; 1/variance; 1/x; $1/x^2$; 1/y; $1/y^2$). For all analytes, the best results were obtained using non-weighted regressions with log 10 data transformation.

The method showed to be selective as no peaks were found near the AAS eluting times for tablet and suspension blanks. For the oil solutions, some peaks near the retention times of T, ME and NF were detected, however only NF was found in oil preparations, and later it was demonstrated that this blank peak did not interfere with the NF signal. All results obtained *in matrix* were within ±10% of those obtained in methanol, and this effect was not considered significant. Hence, quantitation was performed against a calibration curve prepared in methanol.

Table 2 shows the data for recovery, repeatability, intermediate precision and LOQ for each analyte. Threshold values were 13.3%

Table 2
Validation parameters for the analysis of anabolic androgenic steroids (AAS) in different formulations by GC–MS.

AAS	Conc. (μg/mL)	% recovery (N = 4)	Repeatability RSD_r, % (N = 4)	Intermediate precision, RSD_p % (N = 8)	LOQ[b]
Tablets					
PR	2.56	103.0	10.5	11.6	0.5 mg/tablet
	4.96	100.0	4.4	11.3	
	10	96.7	2.1	2.6	
	50	103.6	1.8	2.6	
T	2.56	131.5	21.9	27.4	2 mg/tablet
	4.96	150.1	42.0	31.2	
	10	99.1	2.57	17.5[a]	
	50	103.6	3.3	4.4	
ME	2.56	110.0	16.6	19.8	0.5 mg/tablet
	4.96	96.7	11.0	12.8	
	10	95.3	1.6	6.1	
	50	107.3	2.5	4.3	
OXA	2.56	100.2	10.5	13.3	0.5 mg/tablet
	4.96	82.3	8.5	20.3	
	10	91.6	2.0	2.8	
	50	102.6	3.2	3.5	
TP	2.56	108.4	3.0	13.4	0.5 mg/tablet
	4.96	101.0	7.7	9.4	
	10	88.0	3.7	9.3	
	50	97.8	3.5	3.5	
ES	5.12	103.5	7.6	12.7	1 mg/tablet
	9.96	91.0	6.0	13.6	
	20	94.9	1.4	5.3	
	100	102.5	3.1	3.9	
Suspensions					
T	2.56	105.2	5.7	17.2	2.56 mg/mL suspension
	10	108.8	3.5	9.8	
	50	108.3	1.6	5.1	
ES	5.12	95.5	5.6	5.3	5.12 mg/mL suspension
	20	104.9	3.1	2.4	
	100	96.6	0.9	4.1	
Oil solutions					
TP	2.56	122.0	4.2	9.4	2.48 mg/mL solution
	4.96	111.1	4.7	9.6	
	10	105.8	3.0	6.0	
	50	92.3	2.4	5.9	
TI	2.56	97.5	2.2	10.1	1.28 mg/mL solution
	4.96	91.8	7.1	8.6	
	10	94.0	2.7	6.0	
	50	87.1	4.2	5.9	
TE	2.56	109.8	5.6	15.6	1.28 mg/mL solution
	4.96	97.9	6.3	11.4	
	10	96.8	4.5	7.8	
	50	87.1	5.3	5.7	
TC	2.56	93.7	6.3	11.9	1.28 mg/mL solution
	4.96	90.3	4.9	9.8	
	10	90.6	4.3	5.9	
	50	85.6	3.4	6.0	
ND	2.56	104.9	6.3	14.1	1.28 mg/mL solution
	4.96	100.7	6.6	5.7	
	10	100.7	4.5	8.5	
	50	90.8	2.2	9.6	
NF	2.56	124.6	13.0	22.4	2.48 mg/mL solution
	4.96	117.3	10.0	10.6	
	10	118.2	7.9	10.7	
	50	97.8	0.8	5.4	
BU	2.56	95.7	3.0	8.8	1.28 mg/mL solution
	4.96	92.3	4.1	6.7	
	10	93.2	3.4	5.1	
	50	87.5	2.5	6.0	

PR = Prasterone; T = Testosterone, ME = Methandrostenolone; OXA = Oxandrolone, TP = Testosterone Propionate, ES = Stanozolol, TI = Testosterone Isocaproate; TE = Testosterone Enanthate, TC = Testosterone Cypionate, ND = Nandrolone Decanoate, NF = Nandrolone Phenylpropionate, BU = Boldenone Undecylenate.
[a] N = 7.
[b] In the sample.

for repeatability, 20% for intermediate precision [22] and 80–120% for recovery.

The experiments showed that the method was robust regarding the instrumental conditions for most parameters, with exception to the final temperature, whose changes lead to important alterations in retention time. To monitor for any change in retention time on different days, a calibration solution containing all AAS and IS was injected in the GC–MS prior to analysis to adjust

for any change in retention time. The use of sonication (10 min) during sample extraction provided a slight gain compared with no sonication (around 5%), so this step was kept in the sample preparation.

All analytes were considered stable (90–110% of the initial value) after 3 and 7 days of storage, either at room temperature at the GC tray (~22 °C) or under refrigeration (~4 °C). Most analytes were stable after 10 days, but after 14 days most of them were present at levels below 70% of the initial concentration, with stanozolol being the least stable compound (16.4% remaining at room temperature). In general, the stability of the AAS was not affected by the presence of the matrices. AAS calibration solutions were kept frozen, and were shown to be stable for a maximum of 10 days (at least 90% of the initial concentration).

3.2. Analysis of medicines and dietary supplements

The validated method was used to analyze 328 samples of medicines and 17 dietary supplements sent for forensic analysis by the BFP. All samples were previously qualitatively analyzed by GC–MS for forensic purposes so their composition was known. For every batch of analysis, new calibration curves were prepared, and a fortified blank matrix at 10 μg/mL was analyzed as a quality control sample.

Quantitative results for each sample analyzed are shown in the Supplementary material. Trade names were omitted, the medicines received a letter code (such as Tablet A) and different batches of the same medicine were numbered sequentially (A.1, A.2 . . .). Samples were classified as original, counterfeit or substandard, following the criteria shown in Table 3. Most samples were analyzed after the stated expiry date, and such information was considered in the classification. Packaging analysis includes comparison with other similar products and evaluation of lot numbers and security codes (when information was available). An example of package comparison is shown in Fig. 2.

The overall counterfeiting rate detected for medicines was 42.1% (138 of 328 samples), 28.7% of tablets, 12.0% of suspensions and 65.2% of oil solutions, with different kinds of counterfeit products detected (Table 4). Among the counterfeit products, 21 declared to be manufactured by inexistent companies (none of them contained any declared substance).

Fig. 2. Original (left) and counterfeit (right) samples of Decaland Depot®. Counterfeit flask is slightly larger, the label font is thinner and the overall label is of lower quality.

Some products were more prone to counterfeiting than others. For example, all the thirteen samples of Tablet M (no content declared) contained TP (alone or associated with PR), OXA or no AI at all; this product has been previously declared as a counterfeit by

Table 3
Criteria adopted for medicine and dietary supplements classification according to the GC–MS analysis and packing characteristics.

Classification	Criteria
Original	• Qualitative formulation detected fully matches the one declared on the label[a];
	• Levels of active pharmaceutical ingredients (AI) detected are between 80–130% of the declared formulation if product is not expired; if expired, levels detected are at least 50% of what is declared and/or are similar to levels detected in other products with the same expiry year.
Substandard	• Qualitative formulation detected fully matches the one declared[a];
	• Levels of AI detected are not on the acceptable range defined for original products, but packaging is authentic.
Counterfeit	• Qualitative formulation detected does not match the label (no AI present; different AI, or not all active ingredients);
	• Qualitative formulation detected matches the one declared but at very low concentrations (<50% of declared formulation) and/or differing significantly from similar products with the same expiry year;
	• Fake packaging;
	• Product presents significant differences from other products with the same lot number (such as the mean weight and tablet dimension);
	• Product was declared as inexistent by ANVISA;
	• Product declares an inexistent/unregistered manufacturer (“underground” products).
No specification	• Product was not sent in its original package or package did not state the contents of the product; no information available regarding the identity and concentration of AAS present.

[a] Should the product declare an AAS not included on the study, identification by its mass spectrum on full scan mode was considered enough.

Table 4
Amount and kinds of counterfeit medicines detected.

Matrix	Total N	Number of counterfeits (%)	Kinds of counterfeits
Tablet	87	25 (28.7%)	13 inexistent medicines; 5 contained a different AI; 4 contained no AI; 2 contained lower doses of the correct AI and different physical properties; 1 contained an additional AI.
Aqueous suspension	83	10 (12.0%)	6 contained lower doses of the correct AI; 3 contained no AI; 1 contained a different AI.
Oil solution	158	103 (65.6%)	65 contained no AI; 22 contained a different AI; 9 contained lower doses of the correct AI; 6 did not contain all AI declared; 1 contained an additional AI

AI = active ingredient.

ANVISA. Three counterfeits and two substandard products were detected among the 16 samples of Tablet K (OXA 5 mg/tablet) analyzed. On the other hand, out of the 26 samples of Tablet B analyzed (manufactured by the same industry as Tablet K and declaring the presence of 10 mg ES/tablet), only one counterfeit was detected. (Supplementary material, Table S1). Product D (ES 50 mg/mL) was the suspension product most seized (47), but only one counterfeit was detected (no ingredient). All four Suspension F (ES 50 mg/mL) analyzed were counterfeits (Supplementary material, Table S2).

Oil products had the highest rate of counterfeiting (Table 4), with a variety of products being analyzed (Supplementary material, Table S3). Oil K (ND 200 mg/mL) was the most analyzed product (27 samples), with 3 counterfeits detected. All 13 samples of Oil J (ND 50 mg/mL) analyzed were counterfeits, containing no active ingredient or low levels of TP (one sample contained 39.5 mg/mL, the others were $<$10 mg/mL). All 15 samples of Oil Q (declared TP, TF, TI and TD, total of 250 mg/mL), which informed the same manufacturer as Oil J, were counterfeits (no AI or low levels of TP). Samples of Oil J and Oil Q were frequently seized together (data not shown) and have a similar aspect; their results and contents were alike, which might indicate that these products were manufactured together.

Four tablets, six suspensions and 26 oil solutions (almost 11% of all medicine samples) were considered substandard products. The quantitative analysis did not match the label, but no counterfeiting evidences were found on the packages. Two tablets, one suspension and 18 oils had much more active ingredient (AI) than the concentration stated (up to 170% of the declared dose in tablets, 142% in suspension and 221% in oil solution), whereas two tablets, five suspensions and eight oils had lower AI levels than declared (as low as 58% of the declared dose in tablets, 21.6% in suspensions and 16.4% in oil solution) (Supplementary material, Tables S1, S2 and S3 respectively). This is probably due to poor quality control practices or substandard raw materials, but the possibilities of fraudulent package reutilization or deviation from the manufacturing line cannot be excluded. Some noteworthy cases are Suspension G, for which four out of five analyzed samples were substandard, containing lower doses than what was stated, and Oil K, of which all samples contained less than the 200 mg/mL of ND declared, but since most were analyzed after their expiry date, they were considered originals (Table 3). Five Oil K samples, however, were analyzed prior to their expiry date and still contained less AI than described (average of 112.9 mg/mL for these five samples), so they were considered substandard.

From the 17 dietary supplement samples analyzed, 12 declared the presence of prasterone and were considered original (Supplementary material, Table S4). The other five samples were counterfeits, a conclusion that had already been reached after the previous qualitative analysis due to differences in the substances declared and effectively detected. Quantitative analysis showed that two samples contained methandrostenolone (ME) at 5.4 and 5.8 mg/capsule. The product label recommends the intake of one to two capsules a day, which corresponds to up to approximately 11 mg of ME per day, a dose similar to medicines declaring ME.

Three counterfeit supplements declaring to contain methasterone and halodrol, had instead prasterone (4.3 to 6.5 mg/capsule; Supplementary material, Table S4). Additionally, during the period of the study, one supplement containing undeclared oxymetholone and two containing undeclared chlorodehydromethyltestosterone were detected, but no quantitative analysis was performed.

The final classification of all samples is shown in Fig. 3. Considering counterfeits and substandard products, 53% of the medicines were SSFFC products.

4. Discussion

A GC–MS method for the quantitation of anabolic androgenic steroids in medicine and dietary supplement products using a simple sample preparation procedure and no derivatization was developed, validated and used to analyze 345 samples sent to forensic analysis by the BFP. The sample preparation was similar to that used by Musshoff et al. [18] for qualitative analysis of medicines containing anabolic steroids in oil, tablet and aqueous suspension pharmaceutical forms. The proposed method was validated at a LOQ of 0.5 to 2 mg/tablet (2.5 to 10 mg/g for a 200 mg tablet) and of 1.3 to 5.1 mg/mL for suspension and oil products. Pellegrini et al. [12] reported a similar LOQ for AAS in solid samples using GC–MS after sample derivatization, but the method was more sensitive for liquid samples (LOQ of 0.02 mg/mL). The LOQs reported here are compatible with the levels usually found in original or counterfeit medicines and supplements. The method is of low cost and less time-consuming, has a high throughput of samples and is suitable to detect counterfeit and substandard products in forensic and quality control laboratories, especially those with fewer resources.

This large number of analyzed samples, and the fact that they were collected during a five-year period (2011–2016), most likely

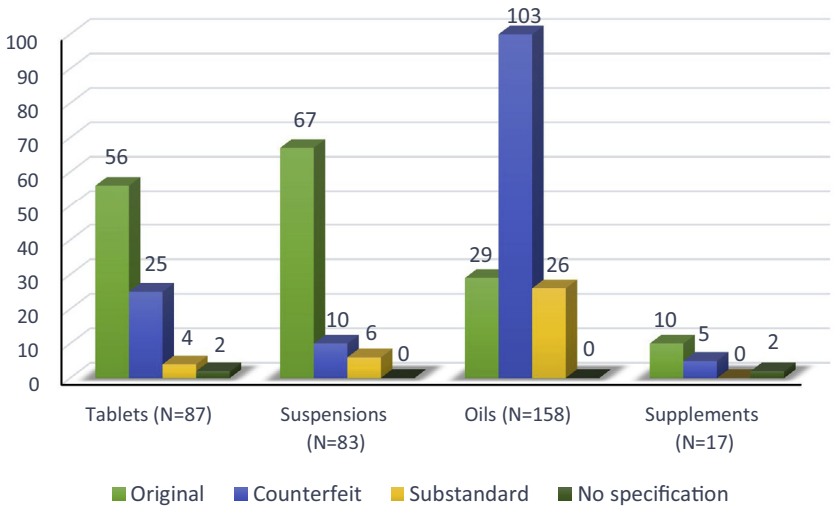


Fig. 3. Final classification of samples after quantitative analysis.

reflects the real situation of clandestine medicines circulating in Brazil. These data complement a previous study conducted by our research group based on package analysis and GC–MS qualitative data for AAS-containing medicines seized by BFP from 2006 to 2011 [6]. In this study, a counterfeiting rate of 31.7% was detected, but since only qualitative analysis was performed, an under counterfeit detection hypothesis was raised. This has proven true since quantitative information was decisive for detecting 17 low dose counterfeits (12.3% of 138 counterfeits) and all 36 substandard products, which would not be identified without a quantitative analysis, leading to the overall SSFFC rate of 53%.

Many studies published in the literature analyzed less than 20 samples [12–17], but the establishment of partnerships between public authorities and researchers allows the evaluation of a larger number of samples, which enables the determination of more precise estimates of the incidence of SSFFC products on the black market. Weber et al. [23] recently published an example of preliminary results from such kind of cooperation, evaluating 960 postal packages seized at the Swiss border in 2013 and 2014 containing 1825 doping products, mostly declaring anabolic agents (main substances were testosterone esters, methandienone and nandrolone esters); however, no chemical analyses were performed on the products to check for counterfeits. Hullstein et al. [24] analyzed 296 black market products seized by the Norwegian police and sent to the Norwegian Doping Control Laboratory for further investigations, and found that 18% of these products did not contain any of the declared substances. In Germany, Krug et al. [25] evaluated 337 black market products, most of which containing AAS and 57% did not contain the substances stated on the labels. Geyer et al. [26] analyzed 634 non-hormonal supplements purchased in 13 different countries (United States and Europe) and found 15% of the samples containing prohormones not declared on the labels.

The consumption of AAS *per se* is associated to several adverse events, including reproductive, hepatic, cardiovascular, musculoskeletal, endocrine, neuropsychiatric and renal effects, among others [27,28]. Most adverse effects are dose-dependent, and abusive consumers of AAS are more prone to manifest these effects, since they use the products at much higher doses that what is recommended for therapeutical purposes [27,29]. SSFFC products represent additional risks since there is no guarantee of which AAS is present and at which levels — different AAS have different characteristics, such as different anabolic-to-androgenic ratios, and lead to different adverse effects [29].

Some limitations of the present study must be pointed out. Most of the products were seized on Brazilian borders (such as Foz do Iguaçu, border with Paraguay and Argentina), which means that they may be representative of black market products entering the country, but not products manufactured in Brazil for local distribution. Some samples were intercepted by post offices and they might account for the "internal market" products, but they may also be foreign products that successfully entered the country and are just being distributed. Some relevant AAS were not included in this study, such as methyltestosterone, trembolone and methenolone, due to difficulties during the standard importing process in Brazil. Furthermore, oxymetholone could not be quantitatively analyzed in this study due to the low quality of the standard acquired, which contained a large proportion of mestanolone compared to the oxymetholone itself. Samples that showed to contain oxymetholone also showed a small amount of mestanolone on their chromatograms, probably originated from oxymetholone degradation at the injector. Qualitative data showed 46 tablet samples containing oxymetholone (6 of which did not declare its presence), 12 tablets with methyltestosterone (all undeclared), 10 oil samples with trembolone acetate (two undeclared) and 4 with methenolone enanthate (one undeclared, data not shown). Additionally, most samples were evaluated after their declared expiry date, and their quality specifications regarding acceptable contents were not available. To take this into account, as no information on the long-term stability of the AAS in the matrices was available, large concentration ranges were accepted as classification criteria, and some substandard samples might have been regarded as originals.

5. Conclusion

This study has two major advantages that should be highlighted. First, the validated quantitative GC–MS method involved a simple extraction procedure with no derivatization step, which is suitable for routine analysis in forensic and quality control laboratories. Secondly, this is one of the largest available studies concerning the quantitative analysis of medicines and dietary supplements containing or suspected to contain AAS. The high rate of counterfeiting found in this study, mainly products of non-Brazilian origin, demands more incisive actions from government agencies aiming at decreasing the availability of black market products worldwide. Furthermore, health authorities should raise awareness among consumers of black market-originated AAS

regarding the potential health risks associated with these products.

Acknowledgements

The authors would like to thank Professor Natalija Nakov, from the Faculty of Pharmacy at the Saints Cyril and Methodius University of Skopje, Macedonia, for helping with weighted regressions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.forsciint.2017.03.016.

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