Steroids 138 (2018) 47–56

Contents lists available at ScienceDirect

Steroids

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

^{1}H NMR determination of adulteration of anabolic steroids in seized drugs

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ARTICLE INFO

Keywords:
Anabolic steroids
^{1}H NMR
Authenticity
Adulteration
Quantification

ABSTRACT

Counterfeiting and adulteration of pharmaceuticals is a prevalent problem worldwide and represents a major health risk to the population, with anabolic steroids being one of the main classes of drugs consumed and obtained from dubious sources. In this work, we propose the use of the ^{1}H NMR technique to evaluate formulations containing anabolic steroids, with analysis of 40 samples of anabolic drugs that are used in injectable and capsule forms. The samples analyzed presented the following active ingredients: testosterone propionate, testosterone phenylpropionate, testosterone isocaproate, testosterone decanoate, testosterone cypionate, testosterone undecanoate, stanozolol, drostanolone propionate, trenbolone acetate, oxymetholone, and methandrostenolone. The ^{1}H NMR spectroscopic measurements were performed using a 600 MHz Bruker Avance III spectrometer, with deuterated chloroform ($CDCl_3$) containing 0.1% TMS as solvent. Of the 40 samples analyzed, eight did not show the presence of the active principle stated on the label. Three types of adulteration were found in the analyzed samples: absence of the active ingredient, adulteration with other substances, and concentration values below those indicated on the label. Sildenafil citrate was found in four samples. The GC–MS technique was used to confirm the adulteration results found using ^{1}H NMR. Quantitative determination by NMR was performed using internal standard and ERETIC 2 methods, and the results obtained were statistically the same.

1. Introduction

According to the World Health Organization (WHO), a counterfeit medicine is a product that is improperly packaged and labeled, in a deliberate and fraudulent way, with its source or identity not being respected, and that can exhibit alterations and adulterations, compared to the original formula [1,2]. Drug counterfeiting and commercialization is an extremely lucrative activity, with a lower risk of repression by police authorities, relative to drug trafficking, and is directly linked to international criminal organizations [3,4]

In Brazil, the drugs most widely falsified are those most commonly sought by the population and that command higher prices on the legal market. These include erectile dysfunction, anabolic steroid, weight loss, and anti-cancer formulations. These types of medicines are easily purchased via the internet, free fairs, and clandestine drugstores [3,5]. The illegal production of medicines, with copying of their active ingredients (or simply omitting them) does not require expensive facilities or procedures. The profits are considerable, even when the products are sold at prices much lower than those of the original drugs [6].

Anabolic steroids appear at the top of the list of counterfeit substances. People who wish to make fast weight and muscle gains, achieving an athletic body in a short time, make use of anabolic steroids that are often prescribed by instructors, unscrupulous doctors, and physical education teachers with no knowledge in the area. This is due to the ease of obtaining the substances, since they can be purchased on the black market, via the internet or across borders, without medical prescription. Such substances are manufactured/falsified in clandestine laboratories and are packaged in ampoules, often unsterilized or mixed with other drugs [7–9]. Some users receive veterinary products based on steroids, for which there is no information available concerning the likely risks to humans [10].

The literature describes several methods for determination of the authenticity and quantification of anabolic steroids in different pharmaceutical formulations, highlighting chromatographic methods including gas chromatography [7,8,11,12] and liquid chromatography [9,13–15]. However, although these techniques are well established for this type of analysis, with good detection limits, they have a number of disadvantages from the operational point of view, such as the need for specific chromatographic columns, laborious sample preparation steps, and sometimes the use of derivatization reactions and toxic solvents.

Unlike other techniques, NMR generally does not require separation steps in order to quantitatively determine the analyte of interest.

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https://doi.org/10.1016/j.steroids.2018.07.002
Received 26 March 2018; Received in revised form 11 June 2018; Accepted 5 July 2018
Available online 10 July 2018
0039-128X/

Therefore, residual solvents, excipients, degraded products, and other impurities in drugs can be identified and subsequently quantified. In the case of falsifications where there is the intentional addition of impurities or diluents, the quality of the medicinal product can be easily checked because the added organic components will show characteristic signals in the ^{1}H NMR spectra. The ability of NMR to detect unknown organic impurities means that it can be considered a universal detector capable of identifying counterfeit drugs [16]. NMR also stands out for being a nondestructive technique that uses a small amount of solvent, where the analyte of interest can be recovered by the solvent drying process. The potential of this technique for drug analysis is reflected in the fact that methods of quantitative analysis of drugs using NMR have already been made official and are listed in international pharmacopoeias, such as in the American and European Pharmacopoeias [17–20].

It is vital to be able to use simple analytical methods that are rapid, effective, and reliable for the quality control of medicines [21], especially those that are widely used and of great commercial value, such as anabolic steroids. The objective of the present study was to propose the use of ^{1}H NMR for evaluation of the quality of medications containing anabolic steroids, seized by the Brazilian Federal Police, which are widely available in the national marketplace. Qualitative and quantitative analyses were performed using different analytical methods.

2. Materials and methods

2.1. Samples and reagents

Analyses were made of 40 samples of drugs containing anabolic steroids, seized and provided by the Brazilian Federal Police, with different active ingredients: testosterone propionate (TPR), testosterone phenylpropionate (TPH), testosterone isocaproate (TIS), testosterone decanoate (TDE), testosterone cypionate (TCY), testosterone undecanoate (TUN), stanozolol (STZ), drostanolone propionate (DPR), trenbolone acetate (TRA), oxymetholone (OXY), and methandrostenolone (MTH). Analytical standards for stanozolol (99.21%) and testosterone propionate (99.88%) were used in quantitative assays. The samples were solubilized using deuterated chloroform ($CDCl_3$, 99.8%) containing 0.1% tetramethylsilane (TMS). All the reagents used were analytical grade and were purchased from Sigma-Aldrich. The spectral profiles of the samples were obtained using solutions containing 5 mg mL^{-1} of each active ingredient.

2.2. ^{1}H NMR analysis

The ^{1}H NMR spectroscopic measurements were performed at 298 K with a 5 mm direct observation multinuclear probe (BBFO-Z plus SmartProbe Broadband Observe), using a Bruker Avance III HD 600 spectrometer operating at 14.1 Tesla (600.13 MHz for ^{1}H). A time of 17.15 ms was obtained for a 90° pulse. Subsequently, the longitudinal relaxation time (T1) was estimated using the inversion recovery pulse sequence; the T1 obtained for the slowest relaxing signal of interest was 3.5 s. Considering the estimated time in this experiment and an acquisition time equal to 4.7 s, the recycling time established was 24 s. The spectra were processed using an exponential function, with a spectral window of 10.0 ppm, 64 K data points, LB = 0.3 Hz, and 16 scans. The analysis time for each sample ranged from 8 to 12 min. Automatic baseline corrections and manual phase corrections were applied. The integration region around each peak was manually set outside the respective ^{13}C satellites in order to ensure maximum area recovery. Data analysis and signal integrations were performed with TopSpin 3.2 software (Bruker Biospin).

2.3. Preparation of samples

Solutions of all the commercial samples were prepared at concentrations of 5 mg mL^{-1}. For the injectable samples, aliquots of 40–70 μL of the drugs (the volume used depended on the concentration indicated on the label) were transferred to 2 mL Eppendorf tubes, followed by addition of $CDCl_3$ (with 0.1% TMS) and stirring manually until solubilization was complete. Aliquots (600 μL) of the prepared solutions were transferred to 5 mm NMR tubes. For preparation of the capsule samples, 3 capsules were weighed, ground, and a mass corresponding to 5.0 mg of the active principle was dissolved in 1 mL of $CDCl_3$ (with 0.1% TMS). The solutions were submitted to vortex agitation for 1 min in order to ensure good extraction of the active ingredient into the solvent. The solutions were then centrifuged at 14,000 rpm for 10 min and filtered through 0.45 μm polyvinylidene difluoride (PVDF) membrane syringe filters (Millipore, Milford, MA, USA). Finally, 600 μL aliquots of the prepared solutions were transferred to 5 mm NMR tubes and analyzed by ^{1}H NMR.

Table 1
Qualitative analysis of the presence or absence of the active principle in the samples.

Active ingredient	Form	Sample	Result (adulterated or authentic)
Testosterone propionate	Injectable	1	Authentic
		2	Authentic
		3	Authentic
		4	Authentic
Testosterone propionate Testosterone phenylpropionate Testosterone isocaproate Testosterone decanoate	Injectable	5	Adulterated
		6	Adulterated
		7	Adulterated
		8	Adulterated
Testosterone cypionate	Injectable	9	Authentic
		10	Authentic
		11	Authentic
		12	Authentic
Testosterone undecanoate	Capsule	13	Authentic
		14	Authentic
		15	Authentic
		16	Authentic
Stanozolol	Injectable	17	Authentic
		18	Authentic
		19	Authentic
		20	Authentic
	Capsule	21	Adulterated
		22	Adulterated
		23	Adulterated
		24	Adulterated
Drostanolone propionate	Injectable	25	Authentic
		26	Authentic
		27	Authentic
		28	Authentic
Trenbolone acetate	Injectable	29	Authentic
		30	Authentic
		31	Authentic
		32	Authentic
Oxymetholone	Capsule	33	Authentic
		34	Authentic
		35	Authentic
		36	Authentic
Methandrostenolone	Capsule	37	Authentic
		38	Authentic
		39	Authentic
		40	Authentic

2.4. Quantitative determination (NMRq)

2.4.1. Internal standard (IS) method

Quantitative determination of the analytes was based on the premise that the integrated signal strength in a ^{1}H NMR spectrum is

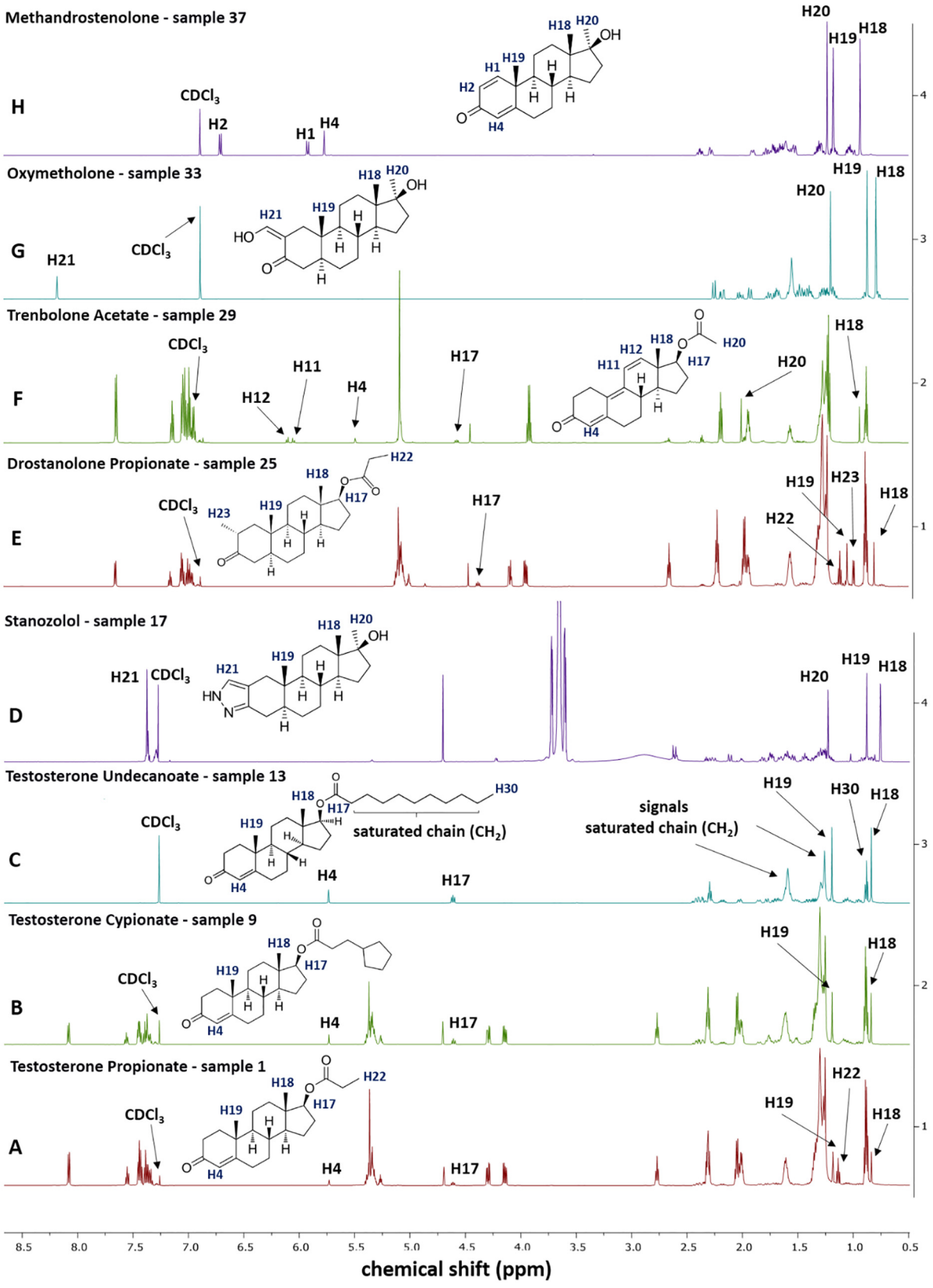

Fig. 1. ^{1}H NMR spectra (600 MHz, $CDCl_3$) of samples 1, 9, 13, 17, 25, 29, 33, and 37.

proportional to the number of nuclei responsible for that particular resonance, as well as to the molar concentration of the substance that generates the signal [22–25]. The following expression was used:

$$P_A = \frac{I_A}{I_{IS}} \frac{N_{IS}}{N_A} \frac{M_A}{M_{IS}} \frac{m_{IS}}{m} P_{IS} \quad (1)$$

where M_{IS} and M_A are the molar masses of the internal standard and the

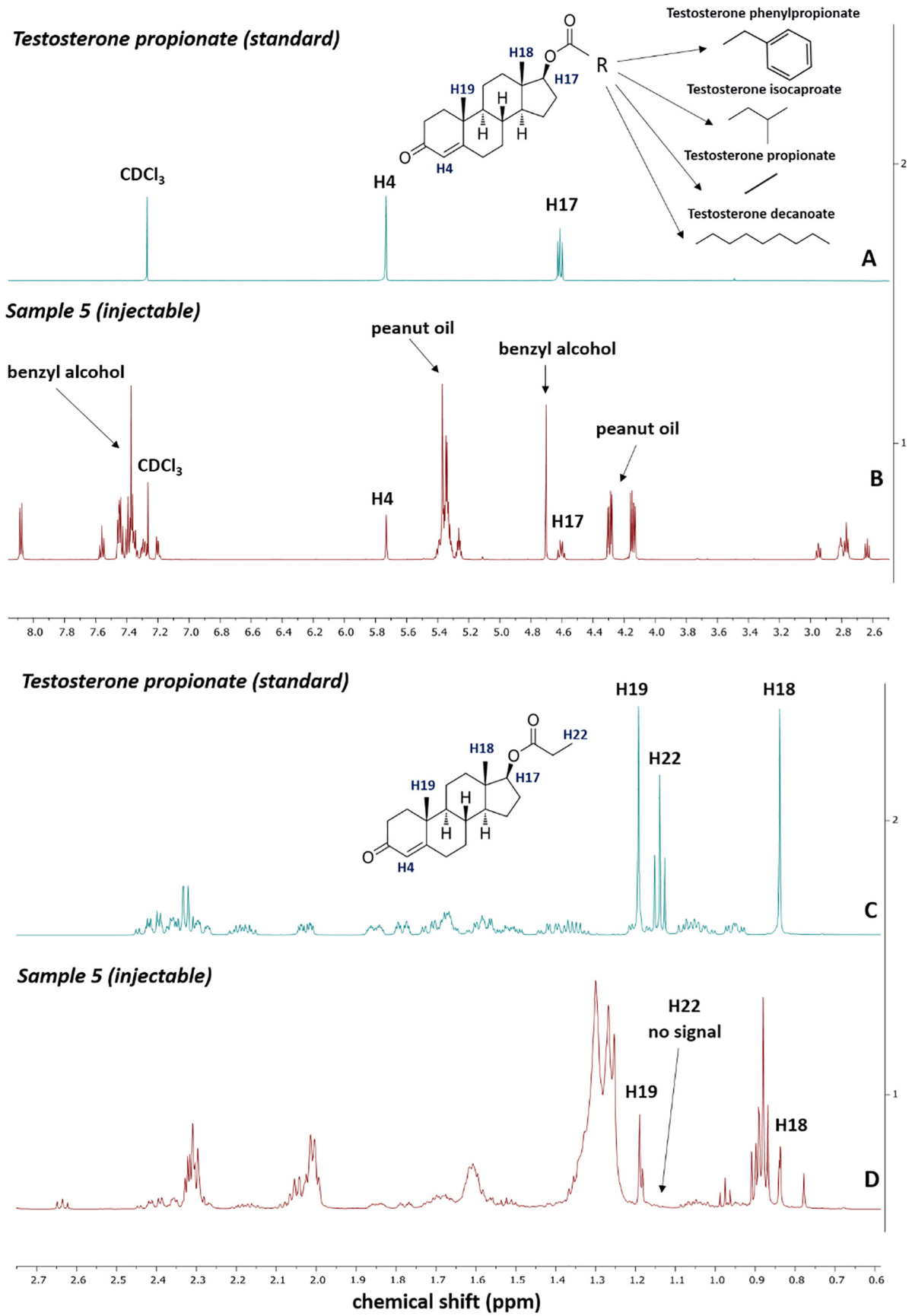

Fig. 2. Overlap of the ^{1}H NMR spectra (600 MHz, $CDCl_3$) for the TPR formulation (sample 5) and the TPR standard.

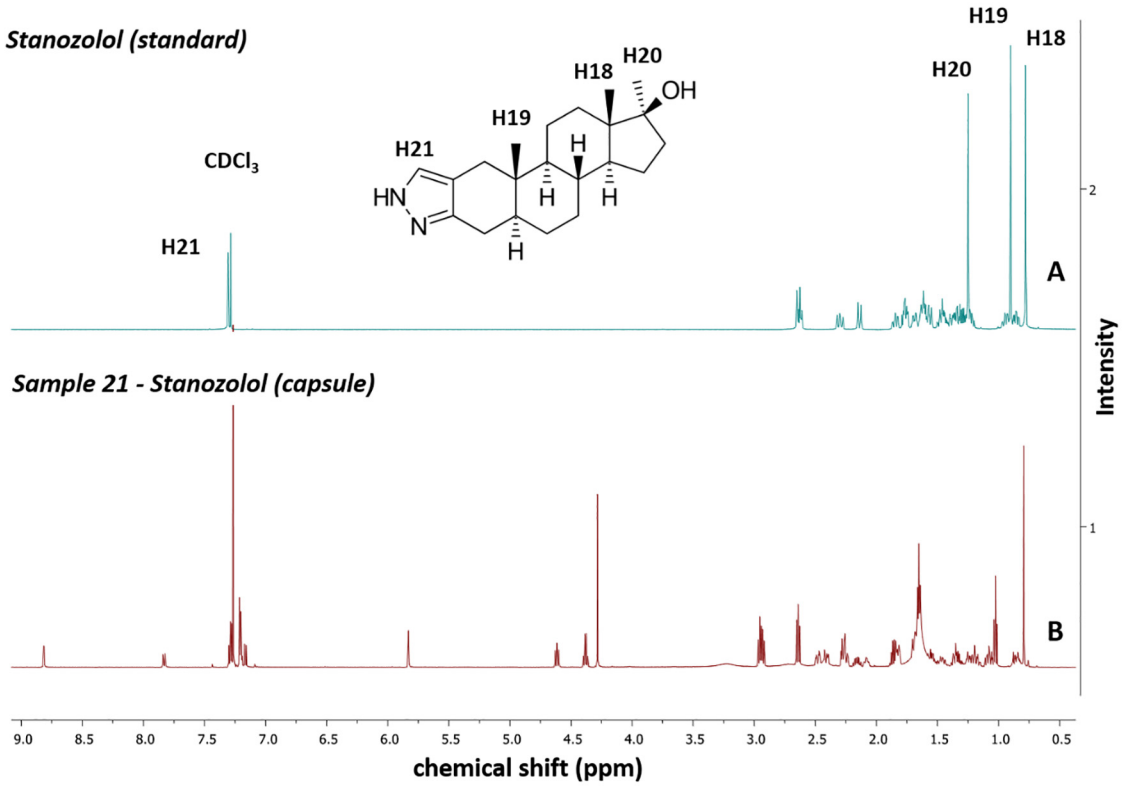

Fig. 3. Overlap of the ^{1}H NMR spectra (600 MHz, $CDCl_3$) for the STZ formulation (sample 21) and the STZ standard.

Table 2
Chemical shifts (ppm) of the compounds found in samples 21–24 (STZ capsule formulations).

Sildenafil citrate				Nandrolone phenylpropionate	
H	Signal	H	Signal	H	Signal
10	4.28 (s, 3H)	17	7.83 (dd, J = 8.8, 2.4 Hz, 1H)	4	5.83 (s, 1H)
11	2.93 (t, 2H)**	18	7.16 (d, J = 8.8 Hz, 1H)	17	4.62 (t, J = 9.2 Hz, 1H)
12	1.84 (m, 2H)**	20	4.38 (q, J = 6.9 Hz, 2H)	18	0.79 (s, 3H)
13	1.03 (t, J = 7.4 Hz, 3H)	21	1.65 (t, J = 7.0 Hz, 3H)	20	2.64 (t, J = 8.3 Hz, 2H)
15	8.84 (d, J = 2.4 Hz, 1H)	–	–	–	–

s: singlet; d: doublet; dd: double-doublet; t: triplet; q: quartet; m: multiplet.
** Overlapping signals.

analyte, respectively; m is the weighed mass of the investigated sample; m_{IS} and P_{IS} are the weighed mass and the purity of the internal standard; and N_{IS} and I_{IS} correspond to the number of spins and the integrated signal area of an NMR line of the standard, respectively [26–28]. Dimethyl sulfone ($DMSO_2$) (99.65 ± 0.08%; Sigma-Aldrich) was used as an internal reference standard.

2.4.2. ERETIC 2 method

The determination was performed using ERETIC 2 (Electronic REference To access In vivo Concentrations) (TopSpin, v. 3.0; Bruker Biospin), based on PULCON (PULse length based CONcentration determination) [29,30]. ERETIC 2 only requires a 1D spectrum obtained for a sample of known concentration, under the following quantitative conditions: a tuned and exactly matched probe, a calibrated 90° pulse, a relaxation delay time of at least 5 * T1, an acquisition time longer than T2, and a sufficient signal to noise ratio [31]. The ERETIC 2 tool allows precise concentration determination based on the fact that the 90° pulse is inversely proportional to the NMR signal intensity [32]. If the concentration of a reference sample (C_{Ref}) is known and the 90° pulses of all samples have been accurately calibrated, then the concentration of an unknown analyte (C_{UN}) in the sample can be determined using the following equation:

$$C_{UN} = KC_{Ref}\frac{A_A\, T_A\, \theta_{90}^{UN}\, n_{Ref}}{A_{Ref}\, T_{Ref}\, \theta_{90}^{Ref}\, n_{UN}} \quad (2)$$

where T is the temperature of the sample (in Kelvin); θ_{90} is the 90° pulse length; A is the value of the integral of the resonance lines; and n is the number of hydrogens corresponding to the signal in the spectrum used for measurement of the standard reference sample and the unknown sample. The correction factor (k) is related to experimental variations, such as incomplete relaxation or different receiver gains applied for measurement of the concentrations of the reference sample or the sample with unknown analyte concentration [29,31]. It should be noted that Eq. (2) is only valid when the experiments are performed with the same NMR probe, which must be properly tuned and matched [32]. In these experiments, a 5 mm probe was used and the external standard for determination of the concentration was testosterone propionate (39.00 mmol L^{-1}). Tests with the internal standard and ERETIC 2 procedures were performed in the same assays, because the two

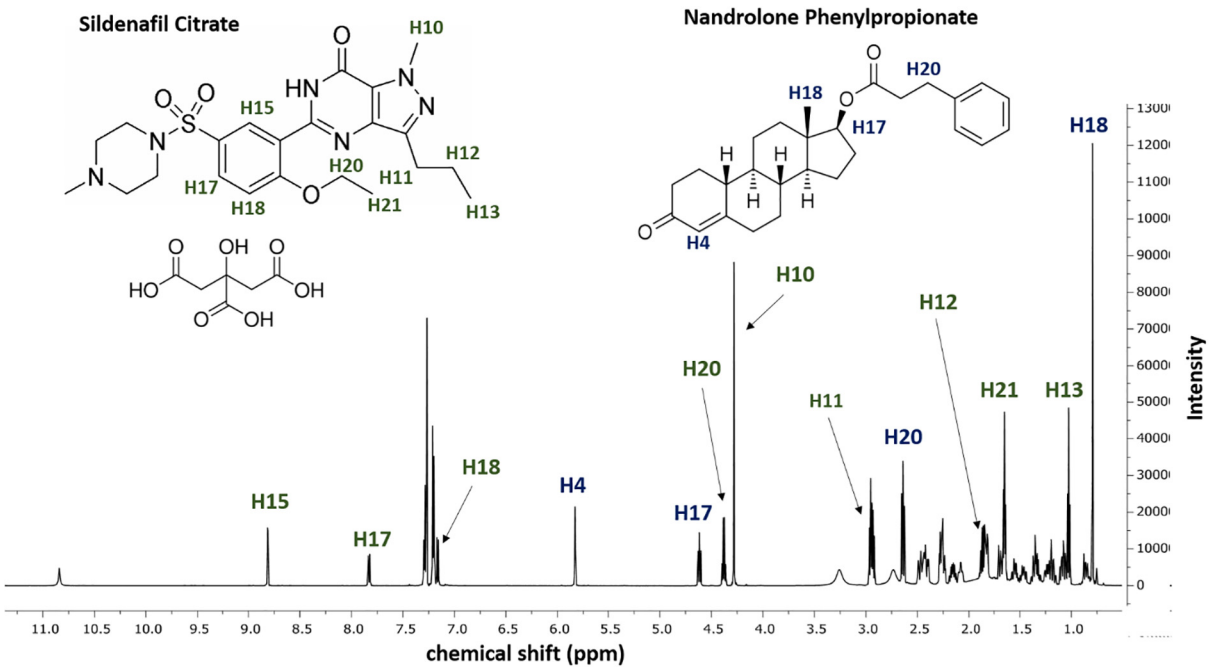

Fig. 4. ^{1}H NMR identification (600 MHz, $CDCl_3$) of the compounds found in STZ capsule samples 21–24.

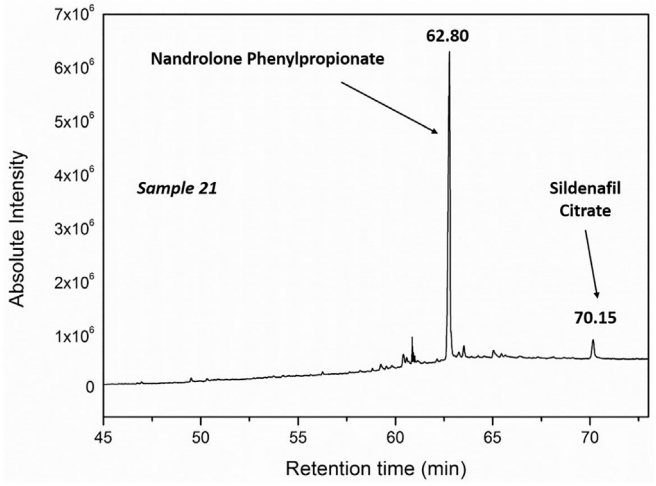

Fig. 5. GC–MS chromatogram for STZ capsule sample 21.

methods can be applied concomitantly without prejudicing the determinations. For validation of the quantitative methods, the parameters evaluated were as follows: precision (intra-day and inter-day), stability, accuracy, robustness, and limits of detection and quantification. All these validation parameters were determined in accordance with the guidelines of the International Conference on Harmonization (ICH) [33].

2.5. Determination by GC–MS

The GC–MS analyses employed a Shimadzu QP-2010 gas chromatograph equipped with an automatic AOC-5000 Shimadzu injector and interfaced with a mass spectrometer. The column was a Phenomenex ZB-5MS (30 m × 0.25 mm × 0.25 μm) and the software used for analysis of the spectra was GC–MS Solutions v. 2.5. The chromatograph operating parameters were as follows: oven temperature of 140 °C held for 3 min, followed by an increase at 3 °C min^{-1}–320 °C (held for 10 min); injector temperature of 260 °C; injection in split mode (1/50); helium (99.999%) carrier gas at a constant flow rate of 1.3 mL min^{-1}; 1 μL sample injection volume; pressure of 114.9 kPa; linear speed of 43.1 cm s^{-1}. The chromatographic run time was 73 min. The MS conditions were ion source and interface temperatures of 250 °C, electron impact mode at 70 eV, and mass acquisition in the *m/z* range 40–650 Daltons. Compounds were identified using Lab Solutions GC/MS analysis software (Shimadzu), comparing the mass spectra of the chromatographic peaks with the spectra available in the NIST 2014 database (National Institute of Standards and Technology).

Preparation of the samples for GC–MS analysis was the same as for ^{1}H NMR (Section 2.3), but with replacement of the deuterated chloroform solvent by non-deuterated chloroform (99.4%; Merck) and using 0.27 μm polyvinylidene difluoride (PVDF) membrane syringe filters (Millipore, Milford, MA, USA).

3. Results and discussion

3.1. Qualitative analysis of the samples

An initial qualitative test was applied to determine whether the samples were either authentic or adulterated, considering the presence or absence of the active principle indicated on the label. The results are shown in Table 1. Of the 40 samples analyzed, 8 did not have all or some of the components indicated on the label. These results demonstrated that the proposed methodology was effective for analysis of the authenticity of these samples.

The ^{1}H NMR spectrum of sample 1 (containing TPR) is shown in Fig. 1A, with chemical shift signals at (ppm) 5.73 (s, 1H), 4.61 (t, J = 9.1 Hz, 1H), 1.21 (s, 3H), 1.14 (t, J = 7.6 Hz, 3H), and 0.84 (s, 3H), corresponding to hydrogens H4, H17, H19, H22, and H18, respectively. The spectrum for only one of the TPR samples is shown in Fig. 1A, since the other samples (samples 2, 3, and 4) presented the same spectral profile. The same rationale was followed for the samples containing other active principles, where the ^{1}H NMR spectrum shown is representative of the behavior exhibited in the spectral profiles obtained for the other samples.

The TCP samples (Fig. 1B) also showed positive results for the presence of the analyte, with ^{1}H NMR signals at (ppm) 5.74 (s, 1H), 4.63 (t, J = 9.1 Hz, 1H), 1.21 (s, 3H), and 0.84 (s, 3H) corresponding to H4, H17, H19, and H18, respectively.

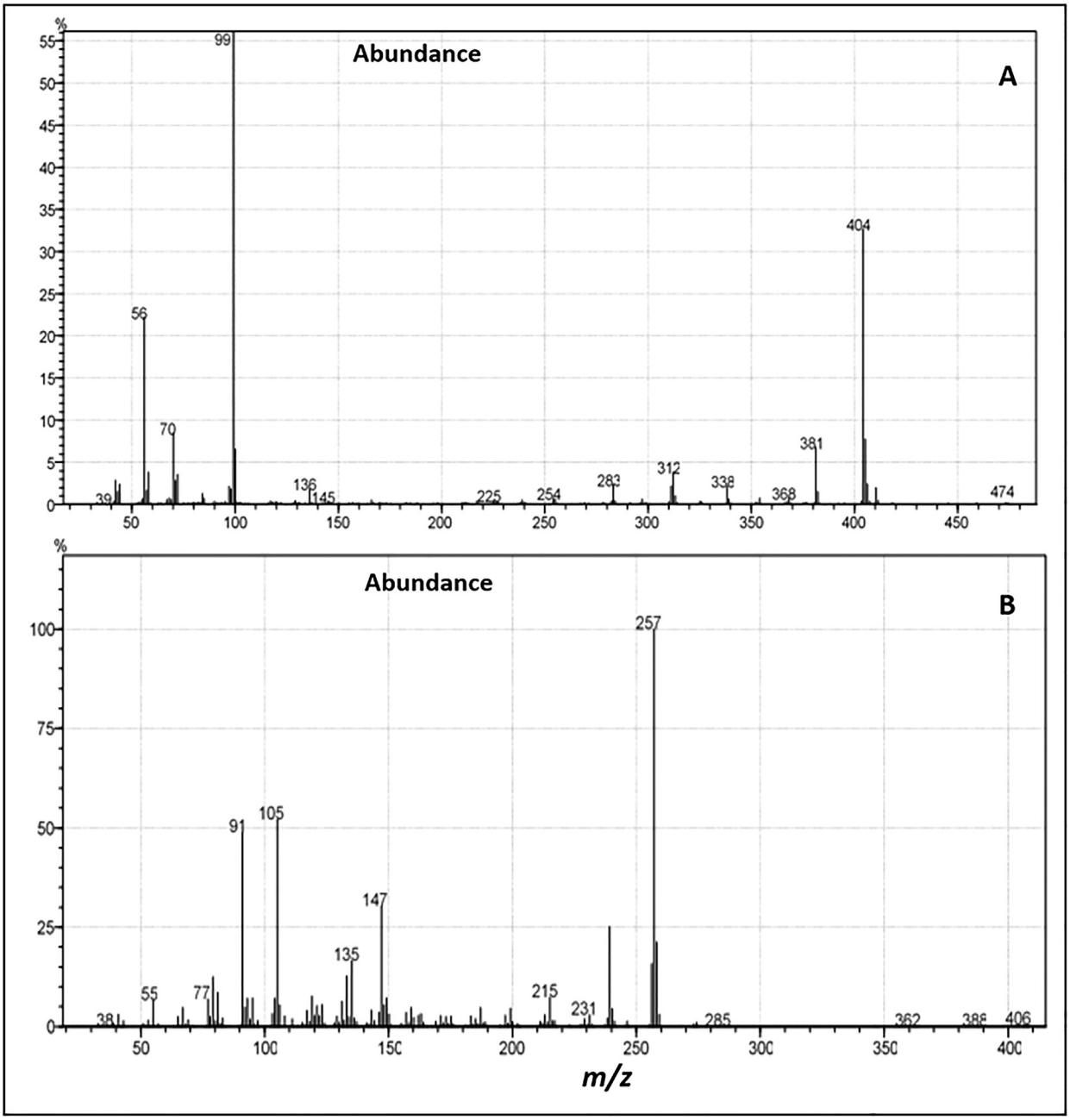

Fig. 6. Mass spectra of (A) nandrolone phenylpropionate (RT = 62.80) and (B) sildenafil citrate (RT = 70.15).

The spectra of the samples containing TUN (Fig. 1C) also indicated that the formulations were authentic, since they presented ^{1}H NMR signals at (ppm) 5.73 (s, 1H), 4.61 (t, J = 9.1 Hz, 1H), 1.19 (s, 3H), 0.88 (t, J = 7.0 Hz, 3H), and 0.84 (s, 3H), corresponding to H4, H17, H19, H30, and H18, respectively. Analysis of the injectable STZ samples (Fig. 1D) revealed no adulterations, since the signals at (ppm) 7.27 (s, 1H), 1.23 (s, 3H), 0.88 (s, 3H), and 0.76 (s, 3H), corresponding to H21, H20, H19, and H18, respectively, were in agreement with those of the analyte indicated on the label.

The DPR samples (Fig. 1E) showed positive results for the presence of the analyte, with ^{1}H NMR chemical shifts at (ppm) 4.60 (t, J = 9.1 Hz, 1H), 1.13 (t, J = 7.6 Hz, 3H), 1.06 (s, 3H), 1.00 (d, 6.5 Hz, 3H), and 0.81 (s, 3H), corresponding to H17, H22, H19, H23, and H18, respectively.

Analysis of the TRA samples (Fig. 1F) showed that they were authentic and contained the analyte, since the ^{1}H NMR spectra showed signals at (ppm) 6.43 (d, J = 9.9 Hz, 1H), 6.37 (d, J = 9.9 Hz, 1H), 5.78 (s, 1H), 4.81 (t, J = 9.3 Hz, 1H), 2.08 (s, 3H), and 0.94 (s, 3H), corresponding to H12, *H*11, H4, H17, H20, and H18, respectively.

Fig. 1G shows the ^{1}H NMR spectrum for the OXY samples. The presence of the active principle indicated on the label was confirmed by chemical shift signals at (ppm) 8.64 (s, 1H), 1.22 (s, 3H), 0.87 (s, 3H), and 0.79 (s, 3H), corresponding to H21, H20, H19, and H18, respectively.

Finally, analysis of the MTH samples (Fig. 1H) confirmed that they were authentic, since the spectra showed ^{1}H NMR signals with chemical shifts at (ppm) 7.07 (d, J = 10.1 Hz, 1H), 6.24 (d, J = 10.1 Hz, 1H), 6.08 (s, 1H), 1.25 (s, 3H), 1.20 (s, 3H), and 0.94 (s, 3H), corresponding to H2, H1, H4, H20, H19, and H18, respectively.

For all the samples showing positive for the presence of the analyte, quantitative analyses were performed to investigate whether the amount measured was in accordance with the values stated on the product labels. The results are discussed in Section 3.4.

3.2. Analysis of adulterated samples

The analyses revealed three types of adulteration: absence of the active principle in the formulation, addition of other substances, and quantitative values well below those indicated on the label. These results showed that in only a single spectrum, the NMR analysis provided valuable structural level information about the nature of the chemical compounds present in the samples.

After the qualitative procedures, detailed analyses were made of the samples that showed negative for the presence of the active ingredient. Samples 5, 6, 7, and 8 were formulations containing 4 active principles (TPR, TPH, TIS, and TDE) that have very similar chemical structures, differing from H21 (Fig. 2A). These samples were checked for the absence of TPR, for which the main marker chemical shift signal was at 1.14 (t, $J = 7.6$ Hz, 3H), corresponding to H22 (Fig. 2C and D). The spectra of the samples were compared with that of a TPR standard (Fig. 2A and C). It is noteworthy that in addition to the identification of the analytes in the samples, it was possible to observe the presence of peanut oil and benzyl alcohol (Fig. 2B), which are excipients used in the manufacture of this type of injectable formulation.

Samples 21–24 presented negative results for the presence of the STZ analyte (Fig. 3B). In contrast to the results for the TPR samples (Fig. 2), the spectral profiles were completely different from that of the active ingredient indicated on the labels, since they presented several signals that were not compatible with those for the STZ standard (Fig. 3A). An interesting observation was that the spectra of the samples analyzed were suggestive of the presence of different spin systems, indicative of a highly complex substance or even a mixture of compounds. Since these were capsule samples, the presence of excipients was very low because the great majority was retained in the filtration process during the treatment of the sample.

Detailed analysis of the ^{1}H NMR spectra of samples 21–24 revealed the presence of chemical shift signals for the compounds nandrolone phenylpropionate and sildenafil citrate (Table 2, Fig. 4).

The discovery of sildenafil citrate in the anabolic steroid samples was a surprise, because the compound is not an anabolic steroid, unlike nandrolone phenylpropionate. The association of these two drugs in a single formulation is quite worrying, since the substances can cause irreversible damage to the body of the user, in addition to the risk of developing new diseases that can sometimes lead to death.

These results demonstrated that it is increasingly necessary to develop effective analytical methodologies for the detection of adulterations in medications, alerting the population and health agencies to the indiscriminate use of these substances. In addition to being prohibited, they are frequently produced without the minimum conditions of hygiene and quality control.

The accuracy of the results obtained by ^{1}H NMR was evaluated by performing analyses using the GC–MS technique. The results obtained by the two methods were in excellent agreement. The chromatogram (Fig. 5) for STZ sample 21 showed signals for nandrolone phenylpropionate and sildenafil citrate at retention times of 62.80 and 70.15 min, respectively.

The identification of compounds by GC–MS (Fig. 6) was confirmed by the presence of their molecular ions with m/z 474 (sildenafil citrate) and m/z 406 (nandrolone phenylpropionate). The sildenafil spectra additionally showed a prominent ion with m/z 99, attributed to the methyl piperazine moiety, as well as ions with m/z 311 and m/z 283, derived from the subsequent cleavages of the C–S bond and the ethyl group on the ethoxy substituent of the phenyl ring, respectively. The analysis of nandrolone showed characteristic ions with m/z 257 (prominent signal), attributed to breaking of the C-O bond of the ester moiety and the cyclopentane ring, m/z 105, attributed to the phenylpropanoid ester moiety followed by decarboxylation, and m/z 91, corresponding to the tropylium ion. All the mass spectral data of the identified compounds were compared to the NIST 2014 library and literature data [34], obtaining great similarity (over 90%).

An important point is that the ^{1}H NMR technique has some advantages over GC–MS analysis, such as lower reagent consumption and a shorter analysis time.

3.3. Validation parameters

The validation parameters were determined in order to validate the values found in the quantitative assays. All tests were performed according to the ICH procedures [33].

The precision values showed that measurements performed on the same day (intra-day) and on different days (inter-day) did not present any significant differences, since the relative standard deviation (RSD) values were less than 5% (Table 3). The limits of detection (LOD) and quantification (LOQ) were calculated using the signal/noise ratio (S/N) of the chemical shift chosen for the quantification. A value of S/N greater than 150 confirmed that the analysis at that concentration could be performed without prejudicing the determination. The LOQ

Table 3
Values obtained for precision and limits of detection and quantification.

		Precision			LOD	LOQ
Sample	Signal (ppm)	Concentration (mg mL^{-1})	Intra-day RSD (%)	Inter-day RSD (%)	(mg mL^{-1})	(mg mL^{-1})
01	5.73	5.00	3.02	3.11	0.22	1.16
09	4.63	5.00	2.39	3.16	0.32	1.28
13	0.84	4.00	1.90	2.25	0.53	1.69
17	0.88	5.00	3.22	4.06	0.43	1.89
25	4.61	6.00	2.93	3.55	0.29	1.68
29	6.37	4.00	1.92	2.72	0.41	1.77
33	8.64	5.00	2.44	2.90	0.39	1.85
37	0.94	5.00	3.09	3.26	0.51	1.92

RSD: relative standard deviation; LOD: limit of detection; LOQ: limit of quantification.

Table 4
Values obtained for the accuracy and robustness of the ^{1}H NMR method.

Accuracy					Robustness			
Sample signal (ppm)	Level (%)	Added (mg mL^{-1})	Recovered (mg mL^{-1})	Recovery[a] (%) n = 3	Sample signal (ppm)	Parameter	Modification	Recovery[a] (%) n = 3
1 (5.73)	50	2.0	5.8	96.6 (± 0.1)	37 (0.94)	Number of scans (16)	8	99.1 (± 0.1)
	100	4.0	7.9	98.7 (± 0.3)			32	98.6 (± 0.2)
	150	6.0	10.1	101.1 (± 0.3)		Spectral width	10	99.8 (± 0.3)
17 (0.76)	50	2.5	7.7	102.6 (± 0.2)		(15 ppm)	20	101.3 (± 0.3)
	100	5.0	9.7	97.0 (± 0.1)		Relaxation delay	15	99.7 (± 0.1)
	150	7.5	12.8	102.4 (± 0.2)		(20 s)	25	98.5 (± 0.4)

[a] Average ± standard deviation (SD), n = 3.

Table 5
Comparison of the internal standard and ERETIC 2 methods.

Analyte (sample)	Labeled	Signal (ppm)	Method[c] (Internal standard) n = 3	Method[c] (ERETIC 2) n = 3	*t*-test[d]	Analyte (sample)	Labeled	Signal (ppm)	Method[c] (Internal standard) n = 3	Method[c] (ERETIC 2) n = 3	*t*-test[d]
TPR (1)	100[a]	5.73	62.82 (± 0.14)	62.97 (± 0.17)	3.40	DPR (25)	100[a]	4.61	81.45 (± 0.08)	83.05 (± 0.04)	2.70
		1.14	63.11 (± 0.12)	62.55 (± 0.13)	2.30			0.81	82.10 (± 0.11)	83.11 (± 0.07)	1.64
TPR (3)	100[a]	5.73	62.78 (± 0.10)	63.01 (± 0.15)	2.88	DPR (27)	100[a]	4.61	81.09 (± 0.14)	82.01 (± 0.02)	3.16
		1.14	62.90 (± 0.11)	62.72 (± 0.11)	3.25			0.81	81.72 (± 0.16)	82.46 (± 0.05)	2.92
TCP (9)	250[a]	5.74	204.73 (± 0.09)	202.15 (± 0.02)	2.45	TRA (29)	75[a]	6.37	74.25 (± 0.18)	74.04 (± 0.03)	2.02
		4.63	203.96 (± 0.07)	203.08 (± 0.03)	1.10			5.78	73.88 (± 0.12)	74.36 (± 0.08)	1.88
TCP (11)	250[a]	5.74	204.55 (± 0.11)	202.99 (± 0.02)	3.05	TRA (31)	75[a]	6.37	74.17 (± 0.15)	74.81 (± 0.07)	2.30
		4.63	204.01 (± 0.09)	203.82 (± 0.04)	2.13			5.78	73.64 (± 0.13)	74.90 (± 0.09)	1.97
TUN (13)	50.0[b]	5.73	44.21 (± 0.08)	45.18 (± 0.09)	3.22	OXY (33)	50.0[b]	8.64	29.01 (± 0.15)	30.25 (± 0.05)	2.59
		0.84	44.79 (± 0.06)	46.01 (± 0.04)	2.28			0.87	28.75 (± 0.13)	29.72 (± 0.04)	3.16
TUN (15)	50.0[b]	5.73	45.03 (± 0.06)	45.64 (± 0.06)	2.72	OXY (35)	50.0[b]	8.64	29.29 (± 0.10)	30.41 (± 0.08)	2.89
		0.84	44.88 (± 0.09)	45.12 (± 0.03)	1.79			0.87	28.66 (± 0.07)	29.02 (± 0.04)	2.11
STZ (17)	100[a]	0.88	36.94 (± 0.20)	37.43 (± 0.18)	4.03	MTH (37)	10.0[b]	6.08	9.33 (± 0.07)	9.75 (± 0.01)	1.39
		0.76	37.12 (± 0.17)	37.08 (± 0.10)	3.77			0.94	9.52 (± 0.10)	9.81 (± 0.02)	2.17
STZ (19)	100[a]	0.88	36.89 (± 0.16)	37.19 (± 0.14)	2.95	MTH (39)	10.0[b]	6.08	9.41 (± 0.09)	9.70 (± 0.03)	2.02
		0.76	37.12 (± 0.11)	36.82 (± 0.09)	2.04			0.94	9.67 (± 0.11)	9.87 (± 0.02)	1.99

[a] g in 100 mL;
[b] mg in 250 mg;
[c] average ± standard deviation (SD), n = 3;
[d] values of *t* (95% confidence level; critical *t* = 4.303).

concentration values were calculated for all the substances having the active ingredient indicated on the label, and their values are given in Table 3. The LOD values (Table 3) represent the minimum concentrations required to be able to qualitatively show whether or not a particular compound is present in the sample, without being able to determine its concentration.

The accuracy tests were performed at three concentration levels (50, 100, and 150%), with the amount of analyte added being compared to the measured content. The results, expressed in terms of recovery (%), provided an indication of the effect of the matrix on the determination. The values indicated that satisfactory recoveries were obtained for all the levels studied (Table 4), being within the limits recommended by ICH [33]. The robustness tests were performed by evaluating the effects of variations in different parameters during the analysis, considering the number of scans, the spectral window, and the relaxation delay. The results, evaluated in terms of recovery, showed that small variations in these parameters did not shift the values from those obtained previously using the optimized parameters (Table 4).

Evaluation was also made of the stability of the stock solutions over time. Analyses were performed at different times to determine whether changes occurred in the spectra and in the analyte concentration values. It was found that the solutions remained quite stable for up to 24 h, after which the concentrations began to vary due to evaporation of the $CDCl_3$ solvent used to prepare the solutions, hence leading to errors in the measurements.

3.4. Quantitative analysis by ^{1}H NMR using the internal standard and ERETIC 2 methods

The two methods evaluated for determination of the active ingredient contents of the samples were addition of an internal standard and the use of ERETIC 2. The results are shown in Table 5.

The main requirement for quantitative determination by the internal standard method is to use a standard that presents a ^{1}H NMR chemical shift signal that does not overlap with the signals of the sample to be analyzed and is easy to integrate, hence avoiding errors in the determination. Therefore, it was decided to use dimethyl sulfone ($DMSO_2$) as the internal reference standard, because it is a very stable compound that presents a single chemical shift signal at 3.00 ppm (s, 6H), which did not overlap any signal from the analyzed samples (Fig. 1). It should be pointed out that the use of a substance added to the samples is disadvantageous, relative to the ERETIC 2 method, because for some matrices it is very difficult to find regions that do not contain signals. In the determination using ERETIC 2, the sample concentration is calculated from the spectrum of an external standard at a known concentration.

Table 5 compares the results obtained by the internal standard and ERETIC 2 methods. The *t*-test values showed that the results found for the two methods were not significantly different, hence validating the values obtained. It was observed that for the great majority of the drugs analyzed, the concentration values were much smaller than indicated on the label, showing that the consumer would be prejudiced by the consumption of these products and evidencing the dubious nature of this type of medication. Irrespective of the cost associated with the maintenance of the equipment, the use of NMR spectroscopy to screen a large volume of drug samples may be commercially more feasible than identification by other analytical techniques (such as HPLC or GC–MS). This is due to the low cost of obtaining a single spectrum, considering the consumables costs, as well as the short time required to acquire a spectrum. Furthermore, NMR analysis provides information about the compounds at the structural level.

4. Conclusions

The NMR technique proved to be a powerful tool for evaluating the quality of samples containing anabolic steroids, since only a single spectrum was needed in order to obtain valuable information about the structures of the compounds present, the authenticity of the substances analyzed, and the presence of impurities. The ^{1}H NMR quantification (using an internal standard or ERETIC 2) of the active ingredients could be performed without the use of analytical reference standards, which are often expensive and difficult to obtain, hence offering an advantage over chromatographic and photometric methods. Another advantage of the NMR method, compared to other techniques, is the ability to quantify the analytes using different signals of the same molecule, which is very useful in the analysis of complex structures. There was evidence of adulteration in 80% of the samples analyzed, as indicated by absence of the analyte, presence of other substances not indicated on the label, or active ingredient content below the value indicated on the packaging. These findings confirmed the importance of the development of new analytical methodologies for this type of application.

Acknowledgment

The authors are grateful to CAPES for a scholarship awarded to M.V.M. Ribeiro, and to the Brazilian Federal Police for providing samples for analysis.

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