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Validation of qualitative chromatographic methods: strategy in antidoping control laboratories☆

C. Jiménez[a], R. Ventura[a,b,*], J. Segura[a,b]

[a]*Unitat de Farmacologia, Institut Municipal d'Investigació Mèdica, Doctor Aiguader 80, 08003 Barcelona, Spain*
[b]*Universitat Pompeu Fabra, Doctor Aiguader 80, 08003 Barcelona, Spain*

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Abstract

An experimental approach for the validation of chromatographic qualitative methods and its application in an antidoping control laboratory is described. The proposed strategy for validation of qualitative methods consists of the verification of selectivity/specificity, limit of detection (LOD), extraction recovery and repeatability (intra-assay precision). A one-day assay protocol, based on the analysis of five blank samples obtained from different sources and four replicates of control samples at two different concentrations of the analytes, has been defined to evaluate the validation parameters. The following evaluation criteria have been applied: absence of interfering substances at the retention time of the analytes in the blank samples to check the selectivity/specificity of the method, the LOD recommended by international sports authorities has to be attained, and for repeatability, the relative standard deviation should be $<25\%$ for the low concentration control sample and $<15\%$ for the high concentration control sample. Qualitative screening procedures are able to detect a great number of analytes so that extraction and analysis conditions are always a compromise for the different analytes. For this reason, no minimum acceptance criteria have been defined for data of extraction recoveries. The proposed protocol has been used for the validation of the screening and confirmation qualitative methods included in the scope of the accreditation of an antidoping control laboratory according to ISO quality standards.

Keywords: Antidoping control

1. Introduction

One important step to ensure the quality and acceptability of the analytical results released by a laboratory is the use of analytical methods with performance capabilities consistent with the application requirements. The objective demonstration that the particular requirements for a specific intended use are fulfilled is achieved by means of method validation [1]. This demonstration is usually carried out through a series of laboratory experiments in which different performance characteristics of the

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*Corresponding author. Unitat de Farmacologia, Institut Municipal d'Investigació Mèdica, IMIM, Doctor Aiguader 80, 08003 Barcelona, Spain. Tel.: +34-93-221-1009; fax: +34-93-221-3237.

E-mail address: rventura@imim.es (R. Ventura).

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method are assessed. Guiding principles and general requirements for validation of analytical methods have been proposed by different national and international organizations and regulatory authorities [2–9]. Validation approaches described in the literature are specially addressed to quantitative methods [5–21]. However, less guidance is available for validation of qualitative methods in spite of their wide use in some areas of analytical chemistry. In general, validation of a quantitative analytical method includes evaluation of accuracy, selectivity/specificity, linearity, range, sensitivity and limit of detection (LOD), limit of quantitation, precision (repeatability and reproducibility), ruggedness and recovery. However, the differences in the aims of the analysis between quantitative and qualitative methods (quantitation vs. identification) considerably reduce the list of validation parameters for qualitative methods. Selectivity/specificity and LOD are the most important parameters recommended by different organizations for the validation of qualitative methods (Table 1).

Antidoping control is a two-step process. In the first step, a series of screening procedures are applied to all samples to eliminate ''true negative'' specimens. If the presence of a compound or metabolite is suspected in some sample, a second confirmatory test, specific for the compound, is applied. Qualitative methods are mainly used for both screening and confirmation purposes, and the techniques used depend on each specific group of compounds. The physico-chemical properties of the parent compounds or their metabolites and the sensitivity and specificity required are the most important factors to take into account. In general, most methods are based on chromatographic techniques, i.e. capillary gas chromatography (GC) coupled with nitrogen phosphorus detection (NPD) or mass selective detection (MSD) are the common techniques used for screening purposes. GC–MSD is the most widely used method for confirmation [22].

In this study, an experimental approach defined for the validation of chromatographic qualitative methods and its application in an antidoping control laboratory is described.

Table 1
Validation parameters for qualitative methods according to the requirements of different organizations

Validation parameter	ENAC[a] [3]	ICH[b] [5]	UN[c] [8]
Accuracy	–	–	X
Precision/repeatability	–	–	X
Specificity/selectivity	X	X	X
Range	–	–	–
Linearity	–	–	–
Limit of detection	X	X	X
Limit of quantitation	–	–	–
Ruggedness	–	–	X
Recovery	–	–	–

[a] ENAC: Entidad Nacional de Acreditación (Spanish Accreditation Body).
[b] ICH: International Conference on Harmonization.
[c] UN: United Nations Drug Control Programme.

2. Validation of qualitative methods

2.1. Validation parameters

2.1.1. Selectivity/specificity

Selectivity (or specificity) is the ability of a method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test [7].

To demonstrate the selectivity of the analytical procedure, five blank urine samples (negative control samples, NC) obtained from different healthy volunteers were analysed. The evaluation was made by verifying the absence of interferences (peaks with a signal-to-noise ratio $<2:1$) at the retention times of the analytes in the chromatograms obtained in GC–NPD analyses, or in the chromatograms of the three diagnostic ions monitored for a given compound in GC–MSD analyses.

2.1.2. Limit of detection (LOD)

The limit of detection (LOD) of an individual analytical procedure is the lowest concentration of analyte in a sample that can be reliably differentiated from background noise [6].

An estimation of the LOD was obtained using one of the two following methods: by analysis of four replicates of blank urine samples spiked with the

analyte at the lowest concentrations recommended by international sports authorities [23] (positive control low concentration, PC-low) and checking that the analyte can be reliably detected; or by analysis of spiked blank urine samples at different concentrations near the expected LOD and determining the minimum concentration at which the analyte can be reliably detected.

Signal-to-noise ratios >3:1 for the chromatographic signal in GC–NPD analyses, and for two of the three diagnostic ions in GC–MSD analyses, had to be obtained to accept the LOD.

2.1.3. Extraction recovery

Extraction recovery is a measure of the efficiency of the extraction of the analyte from the sample matrix. It is expressed as the ratio of the response obtained when the analyte is submitted to the extraction procedure to that measured when it is determined without the extraction step.

The extraction recovery was studied by analysis of four replicates of a positive control sample at high concentration (PC-high). PC-high consists of a blank urine sample spiked with the analyte at a concentration from five to 10 times higher than PC-low. The response obtained with the PC-high sample was compared to that obtained with a sample where the analytes were added after extraction of the blank matrix, representing 100% extraction recovery (PC-100).

2.1.4. Repeatability (intra-assay precision)

Repeatability (also termed intra-assay precision) is the closeness of agreement between a series of measurements obtained from different aliquots of the same homogeneous sample under the same operating conditions.

PC-low and PC-high samples were used to measure the repeatability, which was expressed as the relative standard deviation (RSD) of the responses obtained. RSDs of 25 and 15% were accepted for PC-low and PC-high samples, respectively.

2.2. Experimental protocol

The experimental strategy consisted of a one-day assay protocol (see Table 2) in which five negative control samples (NC1 to NC5), four replicates of PC-low, four replicates of PC-high and four replicates of PC-100 samples were analysed in the same analytical batch.

In PC-low and PC-high samples, the analytes were added to blank urine samples before the application of the extraction procedure. A volume of a methanolic solution of the analyte was added to blank urine samples or evaporated to dryness and then redissolved in blank urine. The final percentage of organic solvent added to each sample never exceeded 5% of the total volume.

PC-100 samples were prepared by extracting blank urine samples and adding the analytes to the sample extract at a concentration equivalent to that of PC-high. Then, the extracts were evaporated to dryness, and redissolved or derivatized according to the corresponding procedure.

When an internal standard (ISTD) was used, it was added to PC-low, PC-high and PC-100 samples at the beginning of the analytical procedure, before

Table 2
Protocol for validation of qualitative analytical methods

Sample code	No addition of analytes	Addition of analytes before sample extraction	Addition of analytes after sample extraction	Validation parameters
NC	NC_1 NC_2 NC_3 NC_4 NC_5			Selectivity/specificity
PC-low		4		LOD, repeatability
PC-high		4		Extraction recovery, repeatability
PC-100			4	Extraction recovery

Sample codes: NC_1 to NC_5, negative control samples; PC-low, positive control sample at low concentration; PC-high, positive control sample at high concentration; PC-100, positive control sample representing 100% extraction recovery.

application of the extraction step; ISTD was not added to NC samples. In some cases, an external standard (ESTD) was used to calculate the extraction recovery; the ESTD was added to the PC-high and PC-100 samples after the extraction step.

High-quality reference materials were used. When the solutions used to prepare spiked samples were not freshly prepared, the identity and concentration of the analytes in the methanolic solutions were verified using UV–Vis spectrophotometry or GC–MSD analysis. Once verified, a solution was considered suitable for use for a 6-month period.

2.3. Calculations

The extraction recovery was calculated by comparing the detector response of the analyte obtained in the extracted spiked samples (PC-high) with that obtained with the mean value of the PC-100 samples. The peak areas of the analyte or the ratios of the peak areas of the analyte to ISTD or ESTD were used for calculations.

The repeatability was measured as the RSD of the peak area ratio of the analyte to the ISTD in four replicates of PC-low and PC-high samples. Outliers in the replicates of both concentrations (PC-low and PC-high) were detected, if present, by applying the Dixon outliers test [24].

The results of the validation process were suitably documented. A validation report was generated with the corresponding chromatograms and complete statistical analysis of the validation data obtained from the experimental studies.

2.4. Analytical method validated

The validation protocol was applied to 11 qualitative chromatographic methods used for screening or for confirmation purposes included in the scope of the accreditation of an antidoping control laboratory according to EN45001 and ISO Guide 25 criteria. A summary of representative screening and confirmation procedures is presented in Table 3.

Table 3
Summary of the sample preparation and chromatographic analysis conditions for representative screening (A, B and C) and confirmation (D, E and F) procedures used in human doping control

	Procedure					
	Screening			Confirmation		
	A	B	C	D	E	F
Detected substances:	Stimulants, narcotics, local anaesthetics	Anabolic steroids (free+conjugated), β_2-agonists, others (narcotics, stimulants, diuretics, etc.)	Diuretics, probenecid	Analgesics-narcotics, stimulants, β-adrenergic agonists and antagonists	Clenbuterol, Salbutamol	β-Adrenergic agonists
Analytes validated	52	50	19	54	2	9
Internal standard	Diphenylamine	Methyltestosterone, testosterone-d_3	7-Propyltheophylline	Codeine-d_3, MDMA-d_5	Penbutolol	Penbutolol
Sample preparation						
Hydrolysis	–	Enzymatic	–	Enzymatic	Enzymatic	Enzymatic
Extraction	Liquid–liquid	Solid-phase	Liquid–liquid	Solid-phase	Solid-phase	Solid-phase
Derivatization reagent	–	MSTFA:NH_4I: 2-mercaptoethanol	Acetone/K_2CO_3 anh./CH_3I	MSTFA and MBTFA	Trimethyl-boroxine	MSTFA
Instrumental analysis	GC–MSD, GC–NPD	GC–MSD	GC–MSD	GC–MSD	GC–MSD	GC–MSD
Ref.	[30,31]	[31]	[32,33]	[34]	[35]	[36]

TBME, *tert*.-butyl-methyl ether.

3. Results and discussion

3.1. General aspects of the validation protocol

According to international quality standards [1], the validation shall be as extensive as necessary to meet the needs of the given application. The extent of the validation depends on the aim of the analytical method, and the first step is to decide which performance parameters must be studied and then design the validation procedure accordingly [12]. When available, it is recommended to follow the validation requirements specified in guidelines within a particular field of chemical analysis relevant to the method. Different organizations and regulatory authorities provide guidance to enable the laboratory to design its own validation strategies [2–8].

As mentioned before, in antidoping control, most of the analytical methods used for screening and confirmation purposes are qualitative methods, which allow the identification of compounds on the basis of their chemical or physical properties. According to the recommendations of different national and international organizations (Table 1), the essential parameters needed to evaluate the overall performance of a qualitative analytical method are selectivity/specificity and LOD. Additionally, extraction recovery and repeatability have been included in the strategy for validation of qualitative analytical methods in our laboratory. Ruggedness (reproducibility of the method under different conditions), not considered in the validation protocol, is assessed through the internal quality control procedures applied in the laboratory [17,25].

3.2. Evaluation criteria

The characteristics of the analytical methods evaluated have also been taken into account to define the evaluation criteria and the acceptance range of the validation parameters proposed. Thus, the most restrictive requirements were set for selectivity/specificity and LOD.

In the analysis of biological samples, many substances (endogenous compounds, degradation products, etc.) can often interfere in the determination of the analyte of interest. As recommended by different authors [6,17,19,20], selectivity (specificity) was verified by analysis of biological matrices obtained from different sources and checking the absence of interferences in the chromatographic regions of the analyte and the ISTD. In GC–MSD analysis, where three diagnostic ions are used for identification of the compounds of interest according to the IOC recommendations [26], all three ions are evaluated for selectivity/specificity.

Several approaches for determining the LOD in qualitative chromatographic methods have been internationally accepted [5]. They are based on the analysis of different samples with known concentrations of the analyte and by establishing the minimum level at which the analyte can be reliably detected. The evaluation of the reliability of detection can be performed visually, by measurement of the signal-to-noise ratio or by measurement of the standard deviation of the response of blank samples. A signal-to-noise ratio between 3:1 and 2:1 is generally considered acceptable [5].

Due to the large number of analytes that must be detected by the procedures applied for antidoping purposes, estimating the LOD could become rather costly and time-consuming. Therefore, an approach to the LOD was obtained by analysis of samples spiked with the analyte at low concentrations according to the recommendations of international sports authorities [23], and demonstrating that the analyte can be detected with a signal-to-noise ratio >3. In general, only one concentration was tested for each analyte; when the procedure showed better sensitivity, lower concentrations were studied. Furthermore, and according to internal quality control criteria [25], LODs were only accepted when the peaks obtained met acceptable chromatographic criteria, which included peak symmetry, peak shape and baseline resolution [27,28]. In GC–MSD analysis, where three diagnostic ions were usually monitored for each compound, a minimum of two out of the three ions had to meet the acceptance criteria. According to the ICH criteria [5], the presentation of the relevant chromatograms was considered adequate for justification of the LOD accepted.

Recovery is the percentage of the analyte originally in the specimen that reaches the end of the procedure

[8]. However, in some cases (i.e. in GC methods when a derivatization procedure is used) it is only possible to determine a relative recovery or extraction recovery [12,29]. Due to the large number of banned substances which must be detected in routine antidoping control, for most of the qualitative procedures compromise conditions are used for sample preparation and chromatographic analysis. Thus, not all of the compounds are extracted under their optimal conditions. For this reason, no minimum acceptance criteria for extraction recovery have been defined as long as the acceptance criteria for selectivity/specificity, repeatability and LOD are met. These criteria have also been accepted by other authors considering that, although desirable recoveries must be as high as possible, small values can be accepted if adequate detection is attained [17,19].

To calculate the extraction recovery, the response of the extracted analyte was compared to that obtained when it was not submitted to the extraction process. An important factor to take into account when evaluating the extraction recovery is the matrix effect. When a derivatization procedure is needed and the analyte possesses multiple functional groups amenable to derivatization, the presence of the matrix affects the formation of the analyte derivatives and, for some analytes, different derivatives are obtained when the derivatization is performed in the presence or absence of a biological matrix. The simultaneous occurrence of the analyte with other compounds present in the matrix for the derivatizing agent gives rise to fewer derivatizating groups attached to the molecules of the analytes in the presence of a urine matrix. This was observed for some β_2-agonists (procedures D and F, Table 3) and for diuretics (procedure C, Table 3). In procedure D, a different proportion of N-TFA-tris-O-TMS vs. tris-O-TMS derivatives for salbutamol and terbutaline was obtained in the absence or presence of the sample matrix. The formation of N-TFA-tris-O-TMS was favoured in the absence of the sample matrix. For this reason, if the signal of the N-TFA-tris-O-TMS derivative is used to calculate the extraction recovery and the 100% response is established using samples without a matrix, the extraction recovery will be underestimated. In contrast, the extraction recovery will be overestimated if the signal of the tris-O-TMS derivative is used. In procedure F, the tris-O-TMS derivative was the main product when pure formoterol was subjected to derivatization with MSTFA, while the bis-O-TMS derivative was mainly obtained in urine extracts; similar behaviour was observed for salmeterol and α-hydroxysalmeterol. In procedure C, the formation of the methyl derivatives of the diuretics was favoured in the absence of sample matrix.

In contrast, in procedure E (Table 3) the signal of clenbuterol, salbutamol and penbutolol cyclic methylboronate derivatives was observed to be lower when the analytes were analyzed without sample matrix. In this procedure, the presence of sample matrix affects the chromatographic behaviour of the compound and increases the response obtained.

In summary, the results obtained show that the matrix effect has to be considered in order to obtain reliable values for the extraction recovery. For this reason, samples corresponding to 100% extraction recovery (PC-100) were prepared with a sample matrix by extracting a blank urine sample and adding the analytes to the sample extract. Nevertheless, the use of a sample matrix was not always possible. In procedure B, blank urine samples free from endogenous steroids were not available and the validation of these compounds was carried out using water as a matrix. In this case, PC-100 samples were also prepared without a sample matrix.

Different mechanisms were applied to reduce the variability that is not associated with the extraction procedure in the calculation of the extraction recovery. First of all, a minimum of four replicates was used for a good estimation of the response corresponding to 100% recovery. ISTDs or ESTDs were also used to reduce the variability. Ideally, ISTD or ESTD should be as similar as possible to the analytes in terms of physico-chemical properties; when a derivatization step is needed, the ISTD or ESTD should have the same functional groups amenable for derivatization as the analytes. However, due to the large number of compounds analysed in most screening and confirmation procedures, and the possible wide differences in chemical structures between them, a compromise has also to be reached in the selection of the ISTD or ESTD. Thus, for some of the analytes, not all sources of variability can be corrected by the use of ISTD or ESTD.

The precision of an analytical procedure can be expressed as the variance, standard deviation, or RSD of a series of measurements [5]. The RSD of the area ratio of the analyte to the ISTD was the method of choice to calculate the repeatability. As explained above, due to the different nature of the compounds detected, the ISTD used in some procedures is not the best for all of them. For this reason, not all possible sources of variability can be corrected by the use of ISTD for a given analyte. Since repeatability is generally dependent on analyte concentration, different acceptance criteria were applied to the different concentrations. As commonly accepted [6,9,18,29], a range for repeatability RSDs of ±15% was used as the acceptance criterion for samples at high concentrations of the analytes (PC-high). However, due to the special characteristics, the complexity and the objectives of the qualitative methods evaluated, a wider acceptance criterion was proposed for the low concentration sample (PC-low), and RSD values of ±25% were accepted. For the same reason, a wider acceptance range (±25%) was accepted for PC-high samples as long as the RSD value for PC-low samples complied with the proposed limits.

3.3. Application to antidoping control procedures

The proposed experimental protocol was applied to the validation of 11 qualitative chromatographic methods used for screening or for confirmation purposes in antidoping control, with more than 200 analytes. It is worth noting the complexity of the sample preparation step for some of the procedures evaluated, involving, in almost all cases, hydrolysis, liquid–liquid or solid-phase extraction, and derivatization steps (see Table 3). As examples, the chromatographic results obtained for the validation of bumetanide using procedure C are presented in Fig. 1, and the results obtained in the validation of procedure B are listed in Table 4.

The proposed protocol of qualitative method validation has been demonstrated to be feasible in terms of costs and time required, in situations where a

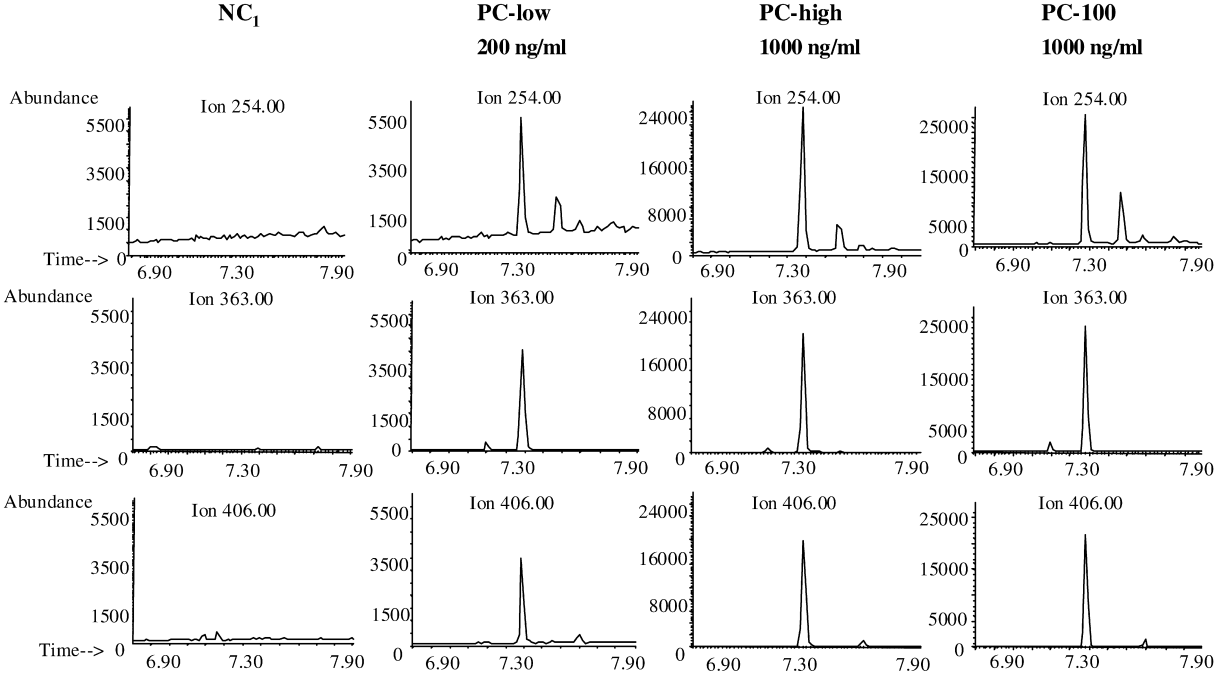

Fig. 1. Chromatograms of the characteristic ions of bumetanide-methyl derivative (m/z 254, 363, 406) obtained after the analysis of a negative control urine (NC_1), a positive control containing 200 ng/ml bumetanide (PC-low), and positive controls containing 1000 ng/ml bumetanide (PC-high and PC-100), using procedure D.

Table 4
List of results obtained for the validation of screening procedure B

	Detected analytes[a]	LOD (ng/ml)	Repeatability				Extraction recovery (%)
			Conc. (ng/ml)	RSD%	Conc. (ng/ml)	RSD%	
Endogenous steroids							
1	11β-Hydroxyandrosterone	10	10	14.9	400	6.6	60.4
2	11β-Hydroxyethiocholanolone	10	10	2.7	200	5.4	58.0
3	5α-Androstane-3α,17β-diol	5	5	7.2	80	8.1	55.2
4	5α-Androstane-3β,17β-diol	5	5	14.2	50	5.9	41.9
5	5α-Androstan-17β-ol-3-one (DHT)	5	5	20.3	20	18.1	46.5
6	4-Androstene-3,17-dione	1	1	10.1	20	9.0	48.3
7	5α-Androstane-3α-ol-17-one	1	1	16.1	2000	4.3	54.7
8	Epiandrosterone	5	5	14.9	50	6.2	52.2
9	Epitestosterone	2	2	2.4	20	3.5	59.9
10	Estradiol	5	5	14.3	50	6.0	51.7
11	Estriol	5	5	1.4	50	11.0	64.5
12	Estrone	5	5	18.5	50	7.3	52.5
13	Prasterone (DHEA)	1	1	4.8	400	6.4	57.4
14	Pregnandiol	10	10	8.9	1000	16.1	40.5
15	Testosterone	1	1	1.2	120	5.7	54.7
Exogenous steroids							
16	Bolasterone-met1	10	10	10.6	50	7.3	66.8
17	Boldenone	5	5	3.3	50	5.3	51.7
18	Boldenone-met1	10	10	13.5	50	4.3	64.2
19	Clostebol-met1	10	10	20.1	50	15.3	32.4
20	4-Chlorometandienone-met1	10	10	11.0	50	1.5	72.6
21	Dromostanolone-met1	10	10	21.5	50	5.3	26.1
22	Epimetendiol	5	5	1.6	50	6.5	86.0
23	Epitrenbolone	10	10	15.4	50	6.6	62.5
24	Ethisterone (Danazol-met1)	10	10	2.2	50	6.0	54.7
25	Fluoxymesterone	10	10	6.2	50	12.4	55.4
26	Fluoxymesterone-met3	10	10	7.7	50	0.9	72.1
27	Formebolone-met1	10	10	14.4	50	21.9	68.8
28	Mesterolone-met1	5	5	8.0	50	11.4	49.5
29	Metandienone-met1	10	10	11.7	50	11.5	71.2
30	Metenolone	10	10	4.2	50	3.5	62.9
31	Metenolone-met1	10	10	3.2	50	1.6	60.7
32	Methyltestosterone-met1	5	5	4.2	50	15.9	71.5
33	Methyltestosterone-met2	5	5	7.4	50	13.5	72.1
34	Mibolerone	10	10	2.4	50	2.7	56.2
35	Norandrosterone	2	2	10.5	20	7.0	93.4
36	Norethandrolone-met1	10	10	14.0	50	6.1	47.7
37	Noretiocholanolone	2	2	7.2	20	9.0	86.1
38	Oxandrolone	10	10	8.7	50	6.1	51.8
39	Oxymesterone	10	10	13.2	50	4.8	54.7
40	Stanozolol-met1	10	10	12.4	50	7.6	55.8
β_2-Agonists							
41	Clenbuterol	10	10	16.6	50	5.3	65.9
42	Salbutamol	10	10	17.2	100	18.2	10.9
43	Terbutaline	10	10	13.2	100	13.4	11.1
Others							
44	THC-COOH	5	5	12.6	20	3.1	55.2
45	Buprenorphine	10	10	12.2	50	10.6	63.6
46	Codeine	50	50	18.1	500	2.3	62.8
47	Morphine	50	50	15.4	500	3.2	31.9
48	Ethamivan	50	50	4.7	200	1.8	51.4
49	Pemoline	50	50	4.2	500	8.2	74.5
50	Triamterene	50	50	2.9	200	4.9	27.5

[a] met, metabolite.

large number of analytes have to be evaluated using complex analytical procedures as in the case of antidoping control. On the other hand, this protocol provides all the information required to demonstrate that the procedures are adequate for the intended purpose according to the quality standards to demonstrate the competence of testing laboratories [1–3,37].

The results obtained in the validations indicated that the criteria for the evaluation of the validation parameters also have to be defined in accordance with the intended purpose. Thus, in the case of qualitative screening methods, where compromise extraction and analysis conditions are used, a wide range of extraction recoveries is expected to be obtained. Nevertheless, in spite of the low extraction recoveries obtained for some analytes, LODs and repeatabilities within the accepted range, and similar to those obtained for analytes with high extraction recoveries, were obtained for most of them. As mentioned before, in accordance with the recommendations the most relevant validation parameters in qualitative methods are the selectivity/specificity and the LOD, so that low extraction recoveries were accepted when the detection method was reproducible and sufficiently sensitive.

An example was found in the results obtained for the validation of screening procedure B (see Table 4). This procedure was optimised for the detection of anabolic steroids (endogenous and exogenous), however it is also able to detect other groups of compounds at the required LODs. Extraction recoveries of up to 50% were obtained for most anabolic steroids and metabolites, whereas lower extraction recoveries were obtained for other groups of compounds, such as β_2-agonists, depending on the structure of the analyte. The highly hydrophilic β_2-agonists salbutamol and terbutaline presented poor extraction recoveries, in contrast to the results obtained for the most lipophylic clenbuterol. Similar results were obtained for the narcotics. Buprenorphine and codeine presented extraction recoveries of up to 60%, whereas the more polar morphine presented a lower extraction recovery. Repeatabilities in the accepted range were obtained for most of the analytes, even for those with low extraction recoveries.

In procedure A, extraction recoveries of up to 50% were obtained for most of the stimulants, except for the most polar substances with extraction recoveries of about 30–40%. In spite of the poor extraction recoveries, adequate LODs in the range 0.1–0.5 μg/ml and acceptable repeatabilities were obtained for most analytes. As expected, the use of a NPD provided better repeatabilities for most of the analytes at both concentrations, especially for the most volatile compounds, and better sensitivity, allowing the detection of some analytes at LODs lower than those attained by GC–MSD. Furthermore, the results obtained for the anaesthetics analysed by procedure A illustrate the differences in the extraction efficiency among analytes of the same pharmacological group with different molecular structures: extraction recoveries obtained for anaesthetics such as lidocaine and mepivacaine (with an amide group) were up to 80%, whereas those obtained for benzocaine, procaine, propoxycaine and tetracaine (containing an ester function and a primary amino group) were about 30%.

In procedure D, the extraction recoveries obtained were up to 80% for most of the compounds according to the results previously reported for some of the analytes [34]. The formation of different derivatives was found to be especially problematic in the validation of some stimulants (e.g. amphetamine, ephedrines), narcotics (e.g. hydrocodone, hydromorphone, nalbuphine), β_2-agonists (e.g. salbutamol, terbutaline) and also for most of the analytes belonging to the β-blocker group. The formation of more than one derivative for these analytes resulted, in most cases, in lower repeatabilities when compared with the other analytes detected by this procedure.

4. Conclusions

In this paper, a protocol for the validation of qualitative chromatographic methods has been defined, meeting the requirements of different quality standards, and guidelines to method validation provided by different international organizations. The protocol is based on the evaluation of selectivity/specificity, LOD, extraction recovery and repeatabili-

ty. The experimental work has been designed so that all the validation parameters are considered simultaneously in only one day of assay. The criteria for data evaluation and for acceptability of the results, and the factors affecting the validation process (i.e. the matrix effect), have been examined and discussed.

The experimental strategy was applied successfully to the validation of the qualitative methods included in the scope of the accreditation of an antidoping control laboratory meeting the requirements of the quality standard ISO 17025, and can also be applied in other fields of chemical analysis.

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