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A confirmatory method for detection of a banned substance: The validation experience of a routine EU laboratory

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Abstract

The Commission Decision 2002/657/EC is a fundamental reference document for the UE laboratories involved in residue analysis although its implementation has caused some difficulties in the requirements interpretation. In this work a pragmatic validation approach of a quantitative confirmatory method for the detection of 17-alpha-(α-NT) and 17-beta-19-nortestosterone (β-NT) in bovine urine by gas chromatography mass spectrometry is proposed. The 19-nortestosterone is a banned anabolic steroid for which no minimum required performance limit (MRPL) has been laid down, therefore the limit reported in Italian Residue Monitoring Plan (2 μg L^{-1}) has been considered the reference level to evaluate the method performances. The decision limit (CCα) and the detection capability (CCβ) were obtained by the calibration curve procedure. The minimum required performance level (*mrpl*), which represents the starting concentration of the calibration curves, was preliminary fixed estimating the results dispersion of blank urine samples fortified at 2 μg L^{-1} for each isomer. The found CCα and CCβ were 1.5 and 1.9 μg L^{-1} for α-NT and 1.2 and 1.4 μg L^{-1} for β-NT. The precision (repeatability and within-laboratory reproducibility) and recoveries were suitable for the investigated concentration range (1–3 μg L^{-1}). Finally, the method ruggedness (minor and major changes) has been also demonstrated.

Keywords: Decision 2002/657/EC; Validation; Confirmatory method; 19-Nortestosterone

1. Introduction

The validation is required for the analytical methods used in the official control of foods. Recently, the European Decision 2002/657/EC revised the technical criteria that must be applied in the screening and confirmatory analysis of veterinary drug residues in food of animal origin [1] and its practical application has generated several discussions and different interpretations [2–5]. Particularly, the decision limit (CCα) and the detection capability (CCβ) determination for banned substances has caused misunderstanding and difficulties to the EC laboratories so much that in the 2004 the European Commission has published the document SANCO/2004/2726 which is a Community Reference Laboratory guideline to the implementation of the 2002/657/EC [6].

In this work a confirmatory method for the determination of nortestosterone residues in bovine urine was validated according to the 2002/657/EC. For this banned hormonal growth promoter, no MRPL (minimum required performance limit) has yet been established, therefore the CCβ should be as low as reasonably achievable (ALARA). The Limit reported in Italian Monitoring Plan (2 μg L^{-1}), which was considered here as a national MRPL (NMRPL), in substance represents the reference concentration for the final judgment about fitness for purpose of the method performance characteristics.

The aim of this study was to investigate some critical point in the way of obtaining the validation parameters following the EU Decision criteria and so practically contributing to the debate on its interpretation and application.

2. Experimental

2.1. Materials and reagents

The α-19-nortestosterone was purchased from RIVM (European Union Community Reference Laboratory, Bilthoven, The Netherlands), β-19-nortestosterone from Fluka and β-d_3-19-nortestosterone from Tecna (Trieste, Italy). The individual stock and work solutions were prepared in ethanol.

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Table 1
Monitored ions and retention times (RT)

Analyte	HFBA derivative		MSTFA derivative	
	Ions[a] (*m/z*)	RT (min)	Ions[a] (*m/z*)	RT (min)
α-NT	133–306–453–**666**	14.48	194–313–**418**–419	19.72
β-NT	133–306–453–**666**	15.60	194–313–**418.5**–419	20.74
β-NT-d_3	**669**	15.56	**421**	20.20

[a] The base peaks are in bold.

The β-glucuronidase from Helix pomatia (H2 type), the heptafluorobutyric anhydride (HFBA) and the *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) were obtained from Sigma–Aldrich (Milan, Italy). The C_{18} (EC) (500 mg/6 mL) and NH_2 (500 mg/6 mL) cartridges were purchased either by J.T. Baker (Deventer, The Netherlands) or by International Sorbent Technology (IST, Mid-Glamorgan, UK).

2.2. GC conditions

The analyses were performed on a Agilent 6890N (Palo Alto, CA, USA) gas chromatograph coupled to a Agilent 5973N mass selective detector (MSD) operated in both scan and selected-ion-monitoring (SIM) modes. The chromatographic separation was achieved injecting 1 μL of sample in a split–splitless injector ($T = 225\,^{\circ}C$, split vent opening at 1.5 min). Helium was the carrier gas (1 mL min^{-1}) and the temperature program was: 1 min at 100 °C, increased to 240 °C at 20 °C min^{-1}, and then to 280 °C at 2 °C min^{-1}. The separation was achieved on a Rtx-5MS Integra Guard column from Restek Corporation (Bellefonte, PA, USA) (bonded phase, 5% diphenyl–95% dimethyl polysiloxane, 0.25 μm film thickness, 30 m × 0.25 mm).

The analyses were carried out in the electron impact (EI) ionization mode at 70 eV. The MD source and the quadrupole temperature were set at 230 and 150 °C, respectively. Two different derivatization reagent were used: HFBA (main) and MSTFA (confirmatory). The most suitable ions to be monitored for each analyte (high ion intensity, high mass and low background) are reported in Table 1. The MSTFA reagent was used only in the confirmatory analysis for the samples resulted "suspect" after the main derivatisation with HFBA.

2.3. Sample extraction and SPE clean-up

The analytical procedure, already described elsewhere [7], was applied with some changes. An aliquot (10 ± 0.1 mL) of a urine sample, previously centrifuged at 2000 rpm for 10 min, was adjusted to pH 5.0 ± 0.1 with 6 mL of acetate buffer (pH = 5.0 ± 0.1, 0.1 mol L^{-1}). After the spiking of each sample with the internal standard (20 μL of a solution at 1 μg mL^{-1} in ethanol), the enzymatic hydrolysis was performed by adding 50 μL of β-glucuronidase and incubating at 37 °C overnight.

The clean-up was performed on a C_{18} cartridge preconditioned with 5 mL of methanol followed by 5 mL of deionized water. The hydrolysed urine was slowly loaded on the cartridge which was then washed with a solution of 45% of methanol and dried under vacuum for 10 min. In the mean time, a second NH_2 SPE cartridge was conditioned with 3 mL of methanol and connected underneath the C_{18} cartridge. The analytes were eluted in a 10 mL test-tube with 8 mL of ethyl acetate. The eluate was brought to dryness under nitrogen stream at 40 °C and the residue, dissolved in 1 mL of ethyl acetate, transferred in a derivatizing vessel. The derivatization reaction can be performed with two different reagent: HFBA or MSTFA. In the first case, 100 μL of freshly prepared HFBA/acetone (1/3, v/v) solution was added to the reaction vessel and allowed to react at 60 °C for 30 min. The derivatized extract was dried under nitrogen at 40 °C, dissolved in *iso*-octane (100 μL), transferred to a tapered vial and capped. The sample was then analysed on the GC–MS system in electron ionization mode. In the second case, 100 μL of MSTFA/NH_4I/DTE (1000/2/4, v/w/w) solution were added to the reaction vessel and allowed to derivatize at 60 °C for 1 h. Before the GC injection, the derivatized extract was treated like in the HFBA case.

2.4. Validation study

2.4.1. CCα, CCβ, precision and recovery

For the estimation of CCα, CCβ, precision and trueness, blank urine samples were fortified at three different concentrations in equidistant steps: 1, 2 and 3 μg L^{-1}. Six urine portions, at each of the three levels, were analysed. The 18 replicate analysis (six for each level) where repeated in three separate days giving $n = 54$ independent determinations. Precision (repeatability and the within-laboratory reproducibility) was assessed by variance analysis (ANOVA) and the decision limit (CCα) and the detection capability (CCβ) were obtained via the calibration curve procedure detailed in EU Decision 2002/657/EC [1].

2.4.2. Selectivity/specificity

To establish the selectivity/specificity of the method, representative blank samples (20 urines from different animals and in different storing conditions) were analysed and checked for interferences (signal, peaks, ion traces) in the region where the target analyte might elute.

2.4.3. Ruggedness (minor and major changes)

The ruggedness was tested at 1 μg L^{-1} by the introduction of seven small but deliberate changes in the operating parameters (factors or variables) and by the consequent assessment of their influence on the method results. These deliberate changes reflect those that can occur when a method is transferred between different laboratories. The modified factors were: C_{18} and NH_2 SPE