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# Determination of clostebol residues in the urine of slaughter animals using liquid chromatography–tandem mass spectrometry

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## Abstract

**Introduction:** Synthetic anabolic hormones, which may pose a potential risk to human health, should not be used in fattening food-producing animals. Because of the hormonal effects they cause, growth-promoting compounds are banned by legislation in the EU for use in animal husbandry. Consequently, all EU member states are required to conduct monitoring tests on the content and residues of these compounds in prescribed biological matrices to ensure the safety of food consumers. The aim of this research was to develop a liquid chromatography–tandem mass spectrometry method for the detection of the residue of one such anabolic hormone, clostebol in food animal urine. **Material and Methods:** Clostebol and its marker metabolite residues were determined by a method involving enzymatic hydrolysis, isolation of compounds from urine on a C18 solid-phase extraction (SPE) column, purification of the extract by liquid–liquid extraction using n-pentane and a $NH_2$ SPE column, and detection by liquid chromatography–tandem mass spectrometry. **Results:** No traces of this anabolic steroid hormone or its metabolites were found in any of the samples tested. The method was validated in accordance with the current requirements for confirmatory methods, and the determined parameters of the decision limits necessary for assessing sample compliance met the specified criteria. **Conclusion:** In 2023, the method was introduced for testing under the National Control Plan in Poland. Up to July 19, 2024, 53 urine samples from different animal species had been tested.

**Keywords:** urine, clostebol, hormone residue, LC-MS/MS.

## Introduction

Clostebol (17β-clostebol, 4-chlorotestosterone), first described and synthesised in 1956, is a synthetic anabolic-androgenic steroid (AAS) that can be used for fattening purposes in animals (19, 28, 31). In the past, it was mainly used for fattening cattle and increasing the performance of racehorses, and today it is still used for fattening cattle in China and Japan (21, 28). The clostebol molecule has the form of a 4-chloro derivative of the natural sex hormone testosterone. The chlorine substituent at the ring prevents the conversion of the molecule to dihydrotestosterone and also prevents the conversion to the structure of oestrogen. Clostebol is supplied in preparations available on the black market most often as an ester, examples of which are clostebol acetate (*e.g.* Macrobin, Steranabol, Alfa-Trofodermin and Megagrisevit), clostebol caproate (Macrobin-Depot), or clostebol propionate (Yonchlon); however, unmodified/non-esterified clostebol is also reported to be marketed, under the brand name (Trofodermin-S) in Mexico and Italy (6). In the 1990s, positive results for clostebol acetate were recorded in France, Belgium various other parts of Europe, especially in samples of biological material from injection sites of animals, which initiated detailed studies on the metabolism of this steroid in various species of animals and also humans (19, 20, 23). In the first stage of metabolism, clostebol is reduced by 5-α and 5-β reductase. It then undergoes 17-oxidation of the 17β-hydroxy group in the body. This is an enzymatic oxidation reaction by 17β-hydroxy-steroid dehydrogenase and is how the metabolites of the major steroid hormones testosterone, boldenone and nandrolone are formed (27). Clostebol as well as many other steroid hormones is heavily metabolised after oral and intramuscular administration, but the metabolites formed depend on the form of administration and can basically be divided into two groups (3). In animals

treated intramuscularly with clostebol acetate, the major urinary metabolites formed are epi-clostebol (17α-clostebol, 4-chloroepitestosterone), 4-chloro-4-androsten-3,17-dione (4-chloroandrostenedione, CLAD) and 4-chloro-4-androsten-3α-ol-17-one. In animals treated orally with clostebol ester, the metabolites listed above are formed and in addition some among 4-chloro-4-androsten-3α,17β-diol, 4-chloro-4-androstane-3β-ol-17-one, 4-chloro-androstane-3β-17α-diol and 4-chloro-androstane-3α-17β-diol may also be, different substances being formed in different species of animal (3, 4, 6, 19, 21, 22, 29, 31). By analogy to other, and not only steroid, hormonal growth promoters, the metabolites present in the urine are typically bound to glucuronic or sulphate acid (in the case of clostebol acetate only less than 5% of the metabolites is excreted unconjugated); therefore, a deconjugation step is needed prior to analysis of biological samples.

The most common use of *Helix pomatia* for enzymatic hydrolysis, containing both β-glucuronidase and arylsulfatase activity, effects the transformation of clostebol acetate metabolites with 3-OH-4-ene structure (4-chloro-4-androsten-3α-ol-17-one and 4-chloro-4-androsten-3α,17β-diol) into metabolites with 3-oxo-4-ene-structure (CLAD and clostebol (4-chloro-testosterone) in two epimeric forms, one with α and the other with β at position 17 of the ring of the molecule) by an oxidation reaction (3, 4). Clostebol is a questionable agent, considered rather a weak anabolic substance. This opinion is confirmed by the magnitudes of the numerical indicators describing androgenic efficacy – 25 – and anabolic efficacy – 46. Clostebol ointments are still available on the commercial market, and only in this form can it find potential use as a performance-enhancing drug. Occurring in such preparations in the form of clostebol acetate, it is used, among other things, to treat skin diseases or lesions on the genitals; for cervicitis, post-surgical inflammation or vaginal ulceration. A case report concerning a human male has confirmed that it is possible to accidentally "use doping" by having sexual intercourse with a clostebol acetate user (24). Typical side effects of clostebol use include alteration of the lipid profile of the blood, inhibition of testosterone production in the testes, and stimulation of hormones produced by the pituitary gland, causing acne and oily skin. The detection of clostebol in human samples taken for routine inspection has led to the suspension of many athletes in various sports (baseball, volleyball and football, for example). One of the high-profile cases in the media in recent years was that of Therese Johaug, a cross country skier, whose urine tested positive for the presence of a banned substance (clostebol) in 2016. Clostebol is currently listed in group S1 of anabolic agents and subgroup 1 of AAS by the World Anti-Doping Agency (WADA) as a compound banned for use by athletes (33). Cases of the use of drugs available in the form of pharmaceutical preparations in sports have been detected in various countries around the world with the sensitive instruments at the disposal of anti-doping laboratories. The WADA laboratory statistics show that the percentage of positive results for clostebol (2% within drug class) places it at number 12 on the list of compounds found (32).

In terms of the veterinary aspect, the use of hormones and thyrostats in the fattening of slaughtered animals for consumption has been strictly forbidden in EU countries since 1981 following Directive 81/602/EEC, repealed by Council Directive 96/22/EC and amended successively by Directive 2003/74/EC and Directive 2008/97/EC (2, 12, 13). Under Regulation (EU) 2017/625 of the European Parliament and of the Council, EU member states are obliged to systematically carry out official controls and other official activities to ensure the application of food and feed law, with the aim of confirming the safety for consumers of their plant- and animal-based foods (14). Annex I of the relevant Commission Delegated Regulation (EU) 2022/1644, supplementing the overarching Regulation 2017/625, classifies substances with hormonal action in Group A 1(a–d) of prohibited or non-authorised pharmacologically active substances in food-producing animals (8, 10). Clostebol belongs to the steroid subgroup (A 1c). Because of the effects they can cause, hormones should not be present in biological material or tissues from animals. For this reason, maximum residue limits (MRLs) have not been established for these compounds. The EU Reference Laboratories (EURLs) proposed only the analytical limit of the minimum method performance requirement (MMPR) for monitoring of specific pharmacologically active substances in specific animal matrices in a document issued in 2022 (7). This limit provides a guideline for the appropriate design of the method validation process in order to obtain the decision parameters necessary to evaluate the test result. An MMPR analytical limit is one than which the decision limit values (CCα) for the confirmatory method should be lower, and in accordance with the guidelines of Commission Implementing Regulation (EU) 2021/808, any compound concentration result above the designated CCα value qualifies the sample as non-compliant with the established criterion (9). For clostebol, for which urine is the recommended matrix for testing for monitoring purposes, the MMPR value was set at 0.50 ug/L, similarly to other steroid hormones dedicated to determination in urine.

The required low analytical limit and the need to adjust decision-making parameters to it require the test method to allow detection and identification at the lowest possible concentration level (well below the MMPR) and a level appropriate for the current legislative criterion.

Different instrumental techniques were used for metabolism studies and for the determination of clostebol in urine and other biological matrices such as liver, muscle, hair or faeces: an ELISA (3, 5), gas chromatography-mass spectrometry (GC-MS) (3, 4, 15, 18, 19, 31) and liquid chromatography-mass spectrometry (LC-MS/MS) (1, 15, 16, 17, 25, 26). A confirmatory method operable under the conditions of the National Veterinary Research Institute laboratory and suited to the determination of clostebol in urine based on

LC-MS/MS was developed, validated and verified in international proficiency testing (PT). This was pursuant to the 2023 extension of the scope of the National Control Plan for veterinary medicinal products residues in Poland to include clostebol tests in animal urine.

## Material and Methods

**Reagents and chemicals.** Acetic acid, sodium hydrogen carbonate and sodium anhydrous were of analytical grade and obtained from POCH (Gliwice, Poland). Sodium carbonate was obtained from Sigma Aldrich (Steinheim, Germany). Methanol of analytical, HPLC and residue extraction grade and acetone of residue extraction grade were obtained from J.T. Baker (Deventer, the Netherlands). Type I water with the highest purity was obtained with a Milli-Q apparatus (MilliporeSigma, Burlington, MA, USA). β-Glucuronidase (23 U $mL^{-1}$)/aryl sulfatase (68 U $mL^{-1}$) *Helix pomatia* (AS HP) and Tris(hydroxymethyl)aminomethane (analytical grade) were purchased from Merck (Darmstadt, Germany). *n*-Pentane (Picograde) was provided by LGC Standards (Wesel, Germany). SPE C18 (500 mg/3 mL) and $NH_2$ (500 mg/3 mL) columns were obtained from Mallinckrodt Baker (Deventer, the Netherlands). Acetate buffer (2.0 M, pH 5.2) was prepared by dissolving acetic acid (225.2 g) and sodium acetate (129.5 g) in water (800 mL), adjusting the pH value to 5.2 and diluting with water to a final volume of 1,000 mL. Tris buffer (20 mM), pH 8.5 was prepared by dissolving solid tris (4.8 g) in water (500 mL), mixing 50 mL of prepared solution with 9 mL of hydrochloric acid (0.1 M), diluting it with water to a volume of 200 mL and adjusting the pH to 8.5. A 10% sodium hydrogen carbonate solution was prepared by dissolving the solid substance (100 g) in distilled water (900 mL). A carbonate buffer was prepared by mixing 10% sodium hydrogen carbonate solution (100 mL) with 10% sodium carbonate solution (500 mL) and adjusting the pH value to 10.25. The injection solution solvent consisted of methanol and water.

Standards of 17β-clostebol (17β-CLOS, $C_{19}H_{27}ClO_2$, molecular weight (m.w.) 322.87 Da, CAS 1093-58-9) was ordered from Dr Ehrenstorfer (Augsburg, Germany), 17α-clostebol (17α-CLOS, $C_{19}H_{27}ClO_2$, m.w. 322.87 Da, CAS 155021-07-1) and chlorandrostenedione (CLAD, $C_{19}H_{25}ClO_2$, m.w. 320.85 Da, CAS 6765-84-0) as well as 17β-clostebol-D3 (17β-CLOS-D3, $C_{19}H_{24}ClO_2D_3$, m.w. 325.89 Da) used as internal standard (IS) were purchased from Wageningen Food Safety Research (WFSR – Wageningen, the Netherlands). All standards were kept at room temperature or at 2–8°C, and labelled standards were stored at 2–8°C according to the recommendations of their accompanying certificates. Primary standard stock solutions were prepared in methanol at concentrations of 1 mg $mL^{-1}$, 100 µg $mL^{-1}$ and 10 µg $mL^{-1}$ and were stored in the freezer for not longer than one year. Working solutions were obtained by tenfold dilution of primary standard solutions to the concentration of 1 µg $mL^{-1}$ in methanol and were stored at a temperature of 2–8°C for not longer than six months. The structural formulas of molecules of clostebol acetate and its metabolites 17β-CLOS, 17α-CLOS and CLAD are presented in Fig.1.

**Sample preparation.** The method of isolation of 17β-CLOS, 17α-CLOS and CLAD from the matrix was selected based on the outcomes of testing samples spiked with hormones, so that the method made correct recovery possible without indications of matrix effect. As a result of these preliminary activities, the following procedure was used to further handle the urine samples. The samples of urine were stored frozen until the start of the test and were thawed at room temperature prior to testing. The urine was centrifuged and passed through filters for biological material to remove macroscopic contamination (ø25 mm, 0.45 µm, Millex-HA, Millipore Sigma, Bedford, MA, USA). The extraction of clostebol and marker residue metabolites from urine and purification of the sample was optimised and as a result of actions taken pursuing optimisation, the procedure was applied which is described next. Five mL of urine was measured, the pH was adjusted to 5.2 by adding 5 mL of acetate buffer and few droplets of glacial acetic acid or acetate buffer, if needed; the internal standard 17β-CLOS-D3 was added to the sample in an amount of 5 ng, which corresponded to a concentration of 1 µg $L^{-1}$ in the sample. In sequence, 50 µL of AS HP glucuronidase was added and the sample was thoroughly mixed and subjected to enzymatic hydrolysis at 37°C (± 2°C) for 16–20 h. The digested sample was cooled at room temperature. The isolation of free hormones from urine was carried out on a $C_{18}$ SPE column which had previously been conditioned with 3 mL of methanol and 3 mL of water. The column was washed with 3 mL of acetone/water mixture (45:55, v/v) and stored under vacuum. Steroid hormones were eluted with 3 mL of acetone in a 10 mL tube, and next the solvent was evaporated to dryness at 60°C under a nitrogen stream. The residues were dissolved with 200 µL of methanol by mixing on a laboratory shaker and next 2 mL of tris buffer at pH 8.5 was added. The tube's content was extracted with 6 mL of *n*-pentane twice. The organic layers were collected and evaporated under a gentle stream of nitrogen at 60°C (± 2°C). The dry residue was dissolved in 3 mL of acetone and loaded into an $NH_2$ SPE column which had previously been conditioned with 5 mL of methanol/water mixture (80:20, v/v). After passing through the column, the extract was collected and evaporated at 60°C (± 2°C) to dryness. The extract was reconstituted in 200 µL of a mobile phase consisting of a methanol and water mixture (70:30, v/v), mixed thoroughly and dispensed into the LC-MS/MS system.

**LC-MS/MS analysis.** Chromatographic separation was performed on a Shimadzu Nexera X2 (Kyoto, Japan) system equipped with a Poroshell 120 EC-C18 (150 mm × 2.1 mm × 2.7 µm) (Agilent Technologies, Santa Clara, CA, USA) column that was coupled with a C18 pre-column (4 mm × 2 mm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of a mixture of methanol (eluent A) and water (eluent B). The separation of

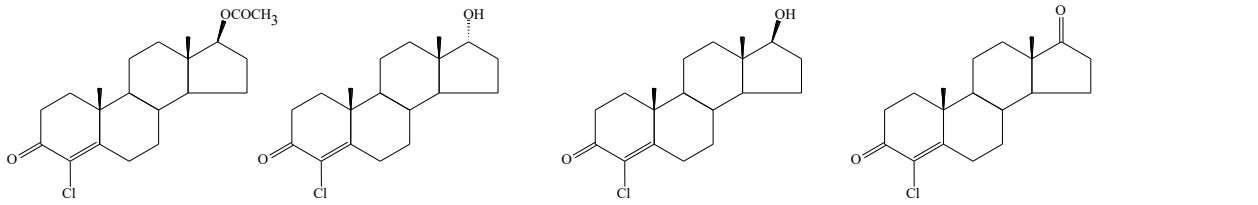
analytes was performed under an isocratic elution condition (A:B = 70:30, v/v) at a flow rate of 0.15 mL $min^{-1}$. The column temperature was kept at 40°C and an injection volume of 25 µL was used.

Mass spectrometry analysis was carried out with the AB SCIEX 5500 triple quadrupole QTRAP instrument (Applied Biosystems, Foster City, CA, USA) equipped with an ESI source operating in positive mode. The following detection condition were set: 5300 V ion spray voltage, 500°C temperature of ion source, 30 psi curtain gas ($N_2$) pressure, 40 psi nebulising gas (air) pressure, 40 psi drying gas (air) pressure and medium position collision gas ($N_2$), 40 ms multiple-reaction monitoring (MRM) dwell time and a 5 ms pause between mass ranges. Data acquisition and quantification were performed using Analyst software version 1.6.3 (AB Sciex). The LC-MS/MS acquisition parameters used for the identification and confirmation of 17α/β-CLOS, CLAD and relevant IS are presented in Table 1.

**Samples.** During validation of the test method, the reference material was cattle and pig urine pooled in the laboratory from available monitoring samples, which had previously been tested for residues of clostebol and its metabolites found free of them. In studies conducted officially in Poland, the test material consisted of urine samples collected from slaughter animals by authorised veterinary inspectors, according to the specifications of the 2023-and-onwards National Control Plan (NCP) for the presence of prohibited substances and residues of chemical and biological medicinal products in live animals and animal products. Samples were taken on farms and at slaughterhouses in accordance with regulations on sampling frequency requirements. Until July 19, 2024, 53 urine samples from slaughter animals had been collected and analysed, including 27 from cattle, 21 from pigs, 2 from sheep and 3 from horses. Of these, 17 came from farms and the remaining 36 from slaughterhouses.

**Validation study.** The LC-MS/MS method developed in this research uniquely for confirmation purposes was validated in accordance with the guidelines for confirmatory methods in Commission Implementing Regulation (EU) 2021/808, which replaced Commission Decision 2002/657/EC as of June 10, 2022 (9). The validation required method performance parameters such as instrumental linearity, linearity of the matrix-matched calibration curve, specificity, trueness (apparent recovery), precision (repeatability, reproducibility), ruggedness, matrix effect and individual decision limits to be determined (9). For the calculation of the CCα parameter, determining the use of the method for confirmation purposes, one of the methods recommended in Commission Implementing Regulation (EU) 2021/808 was adopted.

As a technical tool to analyse the numerical data, ResVal software version 4.0 provided by the WFSR EURL and validated Excel spreadsheets were used. The instrumental linearity of the method was evaluated using calibration curves on 17α/β-CLOS and CLAD standard solutions, prepared at seven points each. In the calibration curves, adjusted at each point for the enrichment level of the urine samples, the analyte concentrations corresponded to 0, 0.20, 0.50, 1.00, 2.00, 4.00 and 6.00 µg $L^{-1}$, and the amount of internal standard (IS) used corresponded to 1.00 µg $L^{-1}$ in the sample.




Clostebol acetate | Epi-clostebol (17α-clostebol) | Clostebol (17β-clostebol) | 4-chloro-4-androsten-3,17-dione (CLAD)

**Fig. 1.** Chemical structures of clostebol acetate and its metabolites

**Table 1.** LC-MS/MS ion acquisition parameters used for the identification of 17β-clostebol (17β-CLOS), 17α-clostebol (17α-CLOS) and 4-chloroandrostenedione (CLAD)

| Compound | MRM transition (m/z) | Collision energy(V) | Declustering potential(V) | Entrance potential(V) | Collision cell exit potential (V) | Ion ratio average ± standard deviation | Samples fulfilling the confirmation criteria (%) |
|---|---|---|---|---|---|---|---|
| | | | | | | | CCα – 2.50 µg $L^{-1}$ |
| 17β-CLOS | **323.2 > 143.1**[a] | 32 | 70 | 10 | 24 | - | - |
| | 323.2 > 131.0 | 33 | | | | 0.532 ± 0.028 | 100.0 |
| | 323.2 > 95.2 | 52 | | | | 0.048 ± 0.009 | 99.1 |
| 17α-CLOS | **323.3 > 143.0**[a] | 40 | 70 | 11 | 20 | - | - |
| | 323.3 > 131.0 | 31 | | | | 0.381 ± 0.021 | 100.0 |
| | 323.3 > 95.0 | 53 | | | | 0.141 ± 0.018 | 97.2 |
| CLAD | **321.0 > 143.0**[a] | 31 | 70 | 10 | 20 | - | - |
| | 321.0 > 131.0 | 32 | | | | 0.551 ± 0.015 | 100.0 |
| | 321.0 > 95.2 | 53 | | | | 0.056 ± 0.008 | 99.1 |
| 17β-CLOS-D3 | **326.4 > 143.0**[a] | 31 | 75 | 9 | 22 | - | - - |

[a] – transitions shown in bold were used for quantification

As a preliminary step, an experiment to estimate the relative matrix effect (ME) and check if it affected the signal response was designed. The extent of the ME was evaluated by comparing the mass spectrometric response for 17β-CLOS, 17α-CLOS and CLAD in the urine samples spiked after extraction and in a solvent at the same concentration of 0.50 μg $L^{-1}$ according to the following formula:

MF (standard normalised for IS) = MF(standard)/ MF(IS)

where, MF is the matrix factor, MF(standard) = peak area of matrix-matched standard/peak area of solution standard and MF(IS) = peak area of matrix-matched IS/peak area of solution IS (9). The ME numerical values were evaluated based on the coefficient of variations for the MF (standard normalised for IS).

Subsequently, the principle validation step was carried out. A validation level (VL) of 0.25 μg $L^{-1}$ recognised as the lowest calibrated level (LCL) – the lowest concentration at which the measurement system was calibrated) was applied to LC-MS/MS. Three series of spiked samples were prepared at concentrations levels of were 0.00, 0.25, 0.50, 0.75, 1.00 and 2.50 μg $L^{-1}$. For the 0.00, 0.25, 0.50 and 0.75 μg $L^{-1}$ concentration levels, seven samples were assayed in each validation run, while for concentration levels of 1.00 and 2.50 μg $L^{-1}$, three samples were assayed. Based on these acquisition data, matrix-matched calibration curves were plotted. Regression parameters of both standard and matrix-matched curves were calculated. Analyte concentration calculations were based on the matrix-matched calibration curves prepared with the internal standard 17β-CLOS-D3. Based on blank samples of the matrix from each validation run, the occurrence or non-occurrence of interfering peaks in the range of retention times of the tested compounds was evaluated. Also apparent recovery, precision and expanded uncertainty (as the sum of variances of reproducibility multiplied by the coverage factor of 2) were also determined from the above three validation experiments at each level of sample enrichment.

Calculations were made of the CCα (the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant, where the α error is the probability of a false non-compliant decision). They came from the application of the mathematical formula assuming for banned and unauthorised compounds the calculation of CCα values based on the LCL and a k-factor of 2.33 assuming a Gaussian distribution and taking into account reproducibility at the LCL level. The averaged values of these parameters were chosen from the three experiments used. After determining the CCα for each compound, an additional series of validations including ten urine samples spiked at the designated concentration level was performed.

The ruggedness test was conducted using Youden's J statistic. It involved selecting seven different factors in the format of A/a–G/g (X/x – uppercase letter/lowercase letter pairs for each factor) in which minor changes could influence the measurement results and preparing eight samples spiked to a concentration of 0.50 μg $L^{-1}$, each in a combination of individual factors. The group of selected factors included urine species (A/a: A – porcine urine; a – bovine urine), pH of acetate buffer (B/b: B – 5.6; b – 4.8), the series of SPE C18 columns (C/c: C – serial number 1828101862; c – serial number 2028201876), the composition of the mixture of acetone and water used for SPE C18 column washing (D/d: D – 43:57, v/v; d – 37:63, v/v), pH of tris buffer (E/e: E – 8.8; e – 8.2), the temperature of extract evaporation (F/f: F – 63°C; f – 57°C) and time to perform this stage of the analysis (G/g: G – half an hour longer than until dryness; g – until dryness). The robustness of the method was estimated based on the calculated standard deviations for the differences between the two levels of each factor. These were compared with standard deviations determined under laboratory conditions during the validation process to assess the influence of all factors on the result. In addition, the effect of each factor individually on the results was checked using the Student's *t*-test statistic (9). As a final step, the criteria for identifying compounds (pertaining to retention time and relative ion ratios) required for methods classified, like the one above, as confirmatory were checked for all spiked samples made during the validation process (9).

The expanded measurement uncertainty for relevant hormones was calculated automatically by the ResVal software, and determined for each level of spiking individually, as reproducibility variance multiplied by the coverage factor of k = 2.

**Evaluation of the competence and method performance in proficiency test.** The method for determining CLOS in the urine of animals was assessed in the PT organised by the WFSR EURL in 2022. In that test 17β-CLOS and CLAD were included in the field of analytes, and the test results were statistically evaluated (30). The material for the study was three samples: two of them were incurred bovine and porcine urine containing ethinylestradiol, 17β-19-nortestosterone and 17β-trenbolone appropriately and the other one was bovine urine spiked with 17β-CLOS and CLAD. The results of the PT were statistically summarised and evaluated with the z-score parameters.

## Results

A summary of the validation results of the confirmatory method for the analysed steroid hormones is presented in Table 2. The linear regression parameters for the standard and matrix-matched calibration curves were correct for all three compounds tested over the entire range of the CCα – 2.50 μg $L^{-1}$.

The calculated regression coefficients for the plotted curves were greater than 0.98, as shown in the Table 2. The calibration parameters are given at the bottom part of that table.

**Table 2.** Validation parameters of the liquid chromatography–tandem mass spectrometry method for the determination of 17β-clostebol (17β-CLOS), 17α-clostebol (17α-CLOS) and 4-chloroandrostenedione (CLAD) in animal urine

| Parameter | Number of samples | Spiking level (μg $L^{-1}$) | Compound | | |
|---|---|---|---|---|---|
| | | | 17β-CLOS | 17α-CLOS | CLAD |
| Mean concentration (μg $L^{-1}$)/ Apparent recovery (%) | n = 21 | 0.25 | 0.24/ 95.1 | 0.24/ 96.8 | 0.25/ 100.8 |
| | | 0.50 | 0.54/ 107.4 | 0.52/ 103.2 | 0.53/ 106.4 |
| | | 0.75 | 0.77/ 102.9 | 0.75/ 100.5 | 0.78/ 104.4 |
| | n = 9 | 1.00 | 0.97/ 96.9 | 0.99/ 98.5 | 0.96/ 96.3 |
| | | 2.50 | 2.49/ 99.6 | 2.50/ 100.1 | 2.50/ 100.0 |
| | n = 10 | CCα | 0.31/ 112.4 | 0.27/ 94.5 | 0.32/ 99.1 |
| Repeatability ($s_r$, μg $L^{-1}$/ RSD, %) | n = 21 | 0.25 | 0.01/ 4.2 | 0.01/ 5.3 | 0.02/ 8.0 |
| | | 0.50 | 0.03/ 6.4 | 0.03/ 5.8 | 0.02/ 4.3 |
| | | 0.75 | 0.06/ 7.3 | 0.04/ 5.4 | 0.05/ 6.1 |
| | n = 9 | 1.00 | 0.05/ 5.0 | 0.05/ 4.8 | 0.03/ 3.5 |
| | | 2.50 | 0.04/ 1.5 | 0.11/ 4.3 | 0.10/ 3.9 |
| | n = 10 | CCα | 0.03/ 9.1 | 0.03/ 10.0 | 0.03/ 10.3 |
| Within-lab reproducibility ($s_R$, μg $L^{-1}$/ RSD, %) | n = 21 | 0.25 | 0.02/ 6.3 | 0.02/ 8.0 | 0.03/ 12.3 |
| | | 0.50 | 0.05/ 9.7 | 0.05/ 8.8 | 0.03/ 6.4 |
| | | 0.75 | 0.08/ 10.9 | 0.06/ 8.0 | 0.07/ 9.2 |
| | n = 9 | 1.00 | 0.07/ 7.6 | 0.07/ 7.2 | 0.05/ 5.2 |
| | | 2.50 | 0.06/ 2.3 | 0.16/ 6.5 | 0.15/ 5.9 |
| Decision limit (CCα, μg $L^{-1}$) | | | 0.28 | 0.29 | 0.32 |
| Expanded measurement uncertainty (U, k = 2, %) | | 0.25 | 13 | 16 | 24 |
| | | 0.50 | 18 | 17 | 13 |
| | | 0.75 | 21 | 16 | 17 |
| | | 1.00 | 15 | 15 | 11 |
| | | 2.50 | 9 | 13 | 12 |
| Matrix effect (%) | | | 14 | 14 | 19 |
| Ruggedness | A/a factors | $t_{A/a}$ | 0.0600 | 0.5250 | 1.8583 |
| | B/b factors | $t_{B/b}$ | 0.3650 | 0.0550 | 1.1417 |
| | C/c factors | $t_{C/c}$ | 0.3950 | 0.2400 | 0.3000 |
| | D/d factors | $t_{D/d}$ | 0.0850 | 0.0250 | 0.3833 |
| | E/e factors | $t_{E/e}$ | 0.3950 | 0.0200 | 0.1917 |
| | F/f factors | $t_{F/f}$ | 0.3200 | 0.2200 | 0.7750 |
| | G/g factors | $t_{G/g}$ | 0.1200 | 0.1600 | 0.5500 |
| | All factors | $S_{I/i}$ | 0.01 | 0.01 | 0.02 |
| Standard calibration curve | Slope ± $s_b$ | | 1.0014 ± 0.2316 | 0.8378 ± 0.3434 | 0.8479 ± 0.2464 |
| | y-intercept ± $s_a$ | | −0.0808 ± 0.0658 | −0.1093 ± 0.0530 | −0.0767 ± 0.0390 |
| | Correlation coefficient | | 0.9975 | 0.9935 | 0.9969 |
| | Standard error | | 0.1819 | 0.2462 | 0.1714 |
| Matrix-matched calibration curve | Slope ± $s_b$ | | 0.8586 ± 0.2401 | 0.7704 ± 1.2009 | 0.7997 ± 0.2704 |
| | y-intercept ± $s_a$ | | 0.0293 ± 0.0263 | −0.0007 ± 0.0444 | 0.0131 ± 0.0208 |
| | Correlation coefficient | | 0.9992 | 0.9999 | 0.9997 |
| | Standard error | | 0.0324 | 0.0121 | 0.0188 |

RSD – relative standard deviation; $s_r$ – standard deviation under repeatability conditions; $s_R$ – standard deviation under reproducibility conditions; $s_a$ – standard deviation of slope; $s_b$ – standard deviation of intercept; $S_{I/i}$ – standard deviation of the differences between all factors; $t_{A–G/a–g}$ – index of the Student's *t*-test statistic; $t_{ctit.}$ = 2.0198 (spiking level 0.50 μg $L^{-1}$; number of sample, n = 18 in three series of validation)

Chromatographic analysis of blank urine samples showed no associated peaks in the retention time ranges of compounds, which confirmed the specificity of measurement of 17α/β-CLOS and CLAD.

Overall apparent recovery of compounds tested from urine at all validation spiking levels ranged from 95.1% for 17β-CLOS to 107.4% for 17β-CLOS, with the relative standard deviation (RSD) not exceeding 10% (1.5–8.0%) and the RSD under reproducibility conditions being less than 15% (2.3–12.3%). The calculated CCα values as presented in Table 2 were below 0.50 μg $L^{-1}$ of the MMPR level and in line with

the guidelines in the legislation. The apparent recoveries of 17α/β-CLOS and CLAD from the urine samples spiked to estimated values of the CCα were correct and in the range of 94.5–112.4%.

The determined values of expanded measurement uncertainty expressed as percentages ranged from 9% for 17β-CLOS for a spiking level of 2.50 μg $L^{-1}$ to 24% for CLAD for a spiking level of 0.25 μg $L^{-1}$.

The numerical values of ME expressed as a percentage indicated matrix enhancement (positive values) for all compounds tested, but were less than the 20% limit.

In the evaluation of the method's ruggedness, it was shown that calculated standard deviations of the differences between the two levels of each factor (A/a–G/g) for samples spiked to 0.50 μg $L^{-1}$ were smaller than the standard deviations carried out under within-laboratory reproducible conditions during validation ($S_{I/i}<S_R$). It was also demonstrated that no selected factor crucial for the method individually affected the analytical performance significantly ($t_{A–G/a–g}<t_{crit.}$).

Regarding the criteria for the confirmatory method, the percentage of samples meeting the criteria required for this purpose ranged from 97.2% to 100% depending on the compounds and specific MRM transition (Table 1).

In the WFSR "Proficiency test for A3 steroids in porcine and bovine urine" assessed in terms of the compounds tested, two satisfactory z-scores amounting to −1.76 for 17β-CLOS and −1.22 for CLAD and meeting the required evaluation criterion $|z| \leq 2$ were obtained (30).

The presence of 17α/β-CLOS and CLAD was not detected or confirmed above the CCα level under the rules for assessing the sample result set out in the legislation in any of the 53 urine samples taken as a part of the official monitoring studies conducted from 2023 in Poland (7). Representative LC-MS/MS MRM chromatograms are presented in Fig. 2.

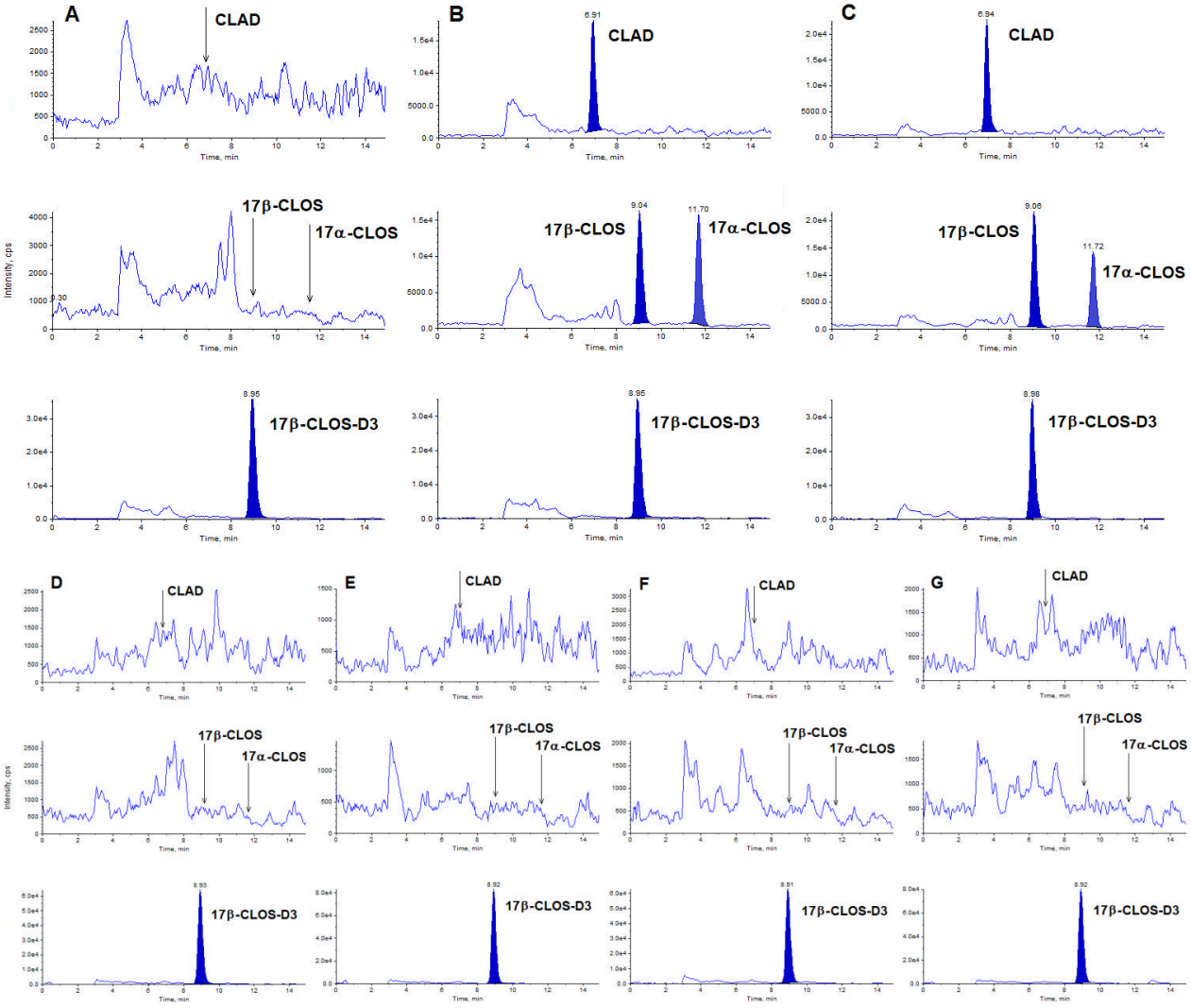



**Fig. 2.** Liquid chromatography–tandem mass spectrometry chromatograms of A – a blank animal urine sample (mixed bovine and porcine urine); B – a blank urine sample spiked with 17α/β-CLOS and CLAD at the individual CCα level; C – a blank urine sample spiked with 17α/β-CLOS and CLAD at MMPR=0.50 μg $L^{-1}$; D – a compliant routine bovine urine sample; E – a compliant routine porcine urine sample; F – a compliant routine ovine urine sample; G – a compliant routine equine urine sample. Note: chromatograms show transitions used for quantification for each analyte according to Table 1

## Discussion

The initial phase of the analytical procedure development was the optimisation of detection conditions of 17α/β-CLOS and CLAD and the relevant IS. The analysis conditions were determined by individually infusing the compound's standards. During the instrument tuning step, the positive and negative ionisation modes were evaluated, but finally, as expected trusting experience working with the vast majority of hormones determined in biological material, the positive one proved to be suitable and was selected for further proceedings (1, 15, 16, 17). Three MRM transitions were obtained for 17α/β-CLOS and also CLAD as indicated by the data in Table 1. The most intensive MRM transitions for particular hormones were designated quantitative and the others qualitative, and were selected as the transitions on which to carry out the identification of analytes according to the required legislative criteria. For all transitions, optimal technical parameters of the collision energy, declustering potential, entrance potential and collision cell exit potential physical quantities were selected. The established optimal values of the technical parameters of the MS instrument were verified by examining the signal intensity of the analytes on a chromatography column.

The Poroshell column, which is currently used for the determination of other hormones in the steroid group, was chosen for the study of 17α/β-CLOS and CLAD because it had proved to be the most suitable for this group of hormones during earlier testing. Also for 17α/β-CLOS and CLAD, the optimal compromise between ionisation, hormone peak geometry and peak intensity was achieved on that kind of column.

Other authors used different chromatographic columns from multiple manufacturers, namely Hypersil Gold, Kinetex, Zorbax and Ascentis Express, with different diameters and lengths, dissimilar specified packing materials and a range of film thicknesses (1, 15, 16, 17). The next stage of developing the procedure was the selection of the best of three methods for isolating 17α/β-CLOS and CLAD from the urine matrix. The first one involved the extraction of analytes from the sample with diethyl ether and further purification on an SPE C18 and $NH_2$ column. It is used in the laboratory to determine mainly stilbenes and resorcylic acid lactones and only a few steroids. The second method was quick, easy, cheap, effective, rugged and safe (QuEChERS) and based on dispersive SPE using C18, primary secondary amine (PSA) and anhydrous magnesium sulphate sorbents. The third method was based on solid-phase SPE followed by liquid–liquid extraction using n-pentane and an $NH_2$ column. The parameters tested during validation, namely recovery and repeatability, were used as criteria for assessing and selecting the most appropriate method. For the first method, low hormone recovery was achieved of on average 65%, with a good coefficient of variation for repeatability around 30%.

For the second method, the recovery was correct and ranged from 75% to 89%; however, the method probably would not be suitable for the intended confirmation purposes because obtaining sufficiently intense MRM transitions is difficult. For the third method, correct recovery of 75–105% with a repeatability variation coefficient not exceeding 20% and a satisfactory chromatogram appearance were achieved. The third method having previously been validated with positive results for a wide range of steroid hormones and being proven to operate well in the laboratory, and above all taking into account its provision of the best evaluation indicators, it was chosen for 17α/β-CLOS and CLAD as optimal for their determination in urine.

Our actions intended to use the developed analytical method for confirmatory purposes. Following the guidelines for quantitative confirmatory procedures, the required validation technical parameters were determined (9). The results proved that method had sufficient selectivity and specificity, which was justified by chromatograms of a blank animal urine sample not containing signals of compounds interfering with 17α/β-CLOS or CLAD as in Fig. 2A.

The correlation coefficients for the standard and matrix-matched calibration curves, which for all compounds tested exceeded 0.98, showed good curve fit according to statistical modelling theory and provided a linear regression response within the adopted concentration range.

The trueness of the method was satisfactory. All apparent recovery values were within the reference range defined in Commission Implementing Regulation (EU) 2021/808 as the minimum trueness of quantitative methods and ranged from −50% to +20% under the provisions for concentrations less than or equal to 1 μg $L^{-1}$ ($kg^{-1}$) and from −30% to +20% for concentrations between greater than 1 and 10 μg $L^{-1}$ ($kg^{-1}$) (9). For all levels of urine spiking with analytes, good precision was obtained and shown in the RSD under repeatability not exceeding 11%, which is in line with the assumptions of Regulation 2021/808. According to that regulation, for concentrations below 10 μg $L^{-1}$ ($kg^{-1}$), it should be as low as possible and equal to or below two thirds of the set maximum value of 30% (9); also the within-lab reproducibility RSD of less than 15% is in line with the requirements of the legislation in force. The values of the apparent recovery and RSD of repeatability and reproducibility parameters obtained in our validation study are consistent with the those obtained by other authors (2, 15, 32).

Furthermore, apparent recoveries of 17α/β-CLOS and CLAD in urine samples spiked at estimated CCα concentrations were in the required range of 50–120% stipulated by Commission Implementing Regulation (EU) 2021/808 for concentrations below 1 μg $L^{-1}$ ($kg^{-1}$) (9). The CCα calculated values for all compounds tested were below the MMPR level of 0.50 μg $L^{-1}$, which was in line with the EURL guidance on MMPRs, and also served the principle that the detection parameters values for banned and unauthorised compounds should be as

low as reasonably achievable (ALARA) (7). Other authors reported validation data for clostebol determination in matrices other than urine, namely meat and plasma, therefore it is difficult to compare validation parameters. The lack of published results in urine is due to the inclusion of clostebol in the list of mandatory compounds for monitoring as recently as in 2022. Pertinent data are also not available in the PT report, as during the testing, most of the participants (from National Reference Laboratories) subjected methods to validation under the then-emerging legislation.

The uncertainty values not exceeding 25% determined at each spiking level in the overall assessment were correct for all compounds tested. The uncertainty calculations took into account the two factors that have the greatest impact on the uncertainty value, namely reproducibility and apparent recovery. Since the values of these parameters were consistent with the criteria, the uncertainty values appear realistic.

Some components of biological matrices may cause the phenomenon of matrix effect, which is most often encountered in LC techniques in the form of enhancement or suppression of the analytical signal for tested compounds. The method did not confirm any significant influence of the matrix on the test result. The low numerical values describing ME indicated no interference from the site of endogenous matrix components that could interfere with analytes, influencing the analytical signal. The ME can be quantified in several ways: based on the principle of spiking after extraction, variation of the calibration curve slopes or comparison of the slope of the matrix-matched calibration curve to that of the standard calibration curve; however, for a long time there were no official criteria for assessing and accepting ME as value data. According to the content of Commission Implementing Regulation 2021/808/EU, it actually should be assumed that the existence of any ME has not been detected if the estimated coefficient of variation is not greater than ±20% (9). Moreover, it is justified to perform calculations from the calibration curve on the matrix in each series of analyses, which is one of the tools to eliminate this undesirable phenomenon.

The experiment on the robustness of the analytical method did not show any irregularities and confirmed that the parameters finally selected as crucial for sample preparation and chromatographic separation are optimal for obtaining satisfactory method performance.

For the reason that the method is used as confirmatory, work was also undertaken to address the requirements for identifying compounds regarding identification points (IPs), determined on the basis of relative intensities of particular characteristic ions and relative retention time (9). For prohibited compounds such as hormones, 5 IPs are required, one of which may be related to the type of chromatographic separation. One precursor and three daughter ions allow a total of 6.5 IPs to be observed in the method used for 17α/β-CLOS and CLAD. For all spiked samples tested in this series, the criterion for the retention time of 17α/β-CLOS and CLAD to be within 1% of the retention time of the internal standard was met. Regarding the relative ion intensities in 97.2–100% of samples in the CCα-2.50 μg $L^{-1}$ concentration range depending on the MRM transition, the criteria for specified ion ratios were met considering ±40% RSD, which is suitable for a confirmatory method.

The assumption is that for prohibited compound α-error, the probability that a non-compliant tested sample is indicated as compliant is 1%, which means that 99% of non-compliant samples are indicated correctly as non-compliant. Considering that, it can be concluded that the LC-MS/MS based method is sufficient for confirmatory purposes.

The LC-MS/MS method developed was verified in PT which was passed by the laboratory using it. This confirms its suitability for the intended purposes. From 2023 to the time of writing, all samples tested for 17α/β-CLOS and CLAD residues as part of the NCP in Poland were classified as compliant with the applicable criteria. Similarly on the basis of the European Food Safety Authority published reports summarising the monitoring results in EU member states, it can be concluded that no member state reported non-compliant results regarding 17α/β-CLOS and CLAD for the years 2010–2022 (11).

## Conclusion

In order to ensure the safety of food of animal origin and the health of its consumers, it is reasonable for laboratories supervising the testing for residues of prohibited compounds in biological material of animal origin to expand the spectrum of obligatory analytes.

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