ANALYSIS OF CORTICOSTEROIDS IN SAMPLES OF ANIMAL ORIGIN USING QuEChERS AND ULTRA HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO HIGH RESOLUTION MASS SPECTROMETRY

Iñaki Beguiristain[1], Angelina Alongi[2,3], Antoni Rúbies[2], Mercè Granados[1]

[1] Department d'Enginyeria Química i Química Analítica, Universitat de Barcelona, Martí Franquès 1-11, 08028 Barcelona, Spain
[2] Laboratori de l'Agència de Salut Pública de Barcelona, Avinguda Drassanes 13-15, 08001 Barcelona, Spain
[3] Istituto Zooprofilattico Sperimentale della Sicilia, Via G.Marinuzzi 3, 90129 Palermo, Italy

Corresponding author:
Mercè Granados
e-mail address: mgranados@ub.edu
telephone number: +34 934034875

ORCID:
Mercè Granados: 0000 0003 2032 3352

Abstract

A rapid and sensitive method for the confirmatory analysis of 8 synthetic corticosteroids (betamethasone, dexamethasone, prednisolone, 6 methyl prednisolone, triamcinolone, flumethasone, beclomethasone, fluocinolone acetonide) is proposed. The method is useful for detecting illegal treatments in different animal species. It consists of an extraction and clean-up using the Quick Easy Cheap Effective Rugged Safe (QuEChERS) strategy. Quantitative determination is achieved by ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry with heated electrospray ionization in negative mode. Quantification is performed using surrogate matrix matched standard calibration curve with dexamethasone-D_4 as the internal standard. The method was validated for analyzing liver samples according to the criteria established by Decision 2002/657/EC. Linearity was assessed in the 1-10 μg kg^{-1} range and linear correlation coefficients were over 0.99 for all the analytes. CCα ranged from 0.04 to 0.16 μg kg^{-1} for substances without maximum residue limit. The method allows confident quantification and confirmation of corticosteroids in liver samples, and its simplicity makes it suitable for analyzing large numbers of samples.

Keywords: *Synthetic Corticosteroids, Liquid Chromatography, High Resolution Mass Spectrometry, Orbitrap, Food analysis, Liver*

Introduction

Natural corticosteroids are a family of steroidal hormones derived from cholesterol and produced in the external portion of the adrenal cortex. These compounds have anti-inflammatory properties with analgesic and antipyretic effects. Pharmacological doses are used for their palliative anti-inflammatory or immunosuppressant effects [1]. Large numbers of synthetic steroids with these specific characteristics are produced [2, 3]. Some of these corticosteroids are used for therapeutic purposes in human or veterinary medicine. For instance, dexamethasone (DEX) is twenty times more powerful than cortisol and has a longer period of action [4], and it is used in human and veterinary medicine.

In veterinary practice, when used in low doses for long periods of time corticosteroids are considered to be growth promoters, since they act on lipid metabolism [1,5]. The association between corticosteroids and other growth promoters give them synergic effect and depletes the urinary concentration of molecules administered for illicit purposes [6-8]. Moreover, corticosteroids negatively affect the organoleptic properties of meat and cause adverse effect on human health [9].

In the European Union, corticosteroids are strictly regulated and classified in Group B2f, as "*Other substances that exert a pharmacological activity"* [10]. European Regulation 37/2010 [11] only sets maximum residue limits (MRL) in liver matrix for betamethasone (BET) (2 μg kg^{-1} in bovine and pig), DEX (2 μg kg^{-1} in ovine and pig), prednisolone (PLN) (10 μg kg^{-1} in bovine) and 6-methylprednisolone (MPLN) (10 μg kg^{-1} in bovine). However, these corticosteroids may only be used for therapeutic purpose, with regular prescription by the veterinary [12]. To detect the use of prohibited substances or improper administration of regulated ones, and with the ultimate aim of protecting consumer health from unwary intake of residues, the European authorities have established national residue control plans [10]. These plans require analytical methods with high sensitivity, specificity and accuracy.

In food testing laboratories, veterinary drug residues are normally analyzed using liquid chromatography (LC) coupled to mass spectrometry (MS), mainly using triple quadrupole (QqQ) instruments. However, the resolution of QqQ instruments is not always sufficient for resolving coeluting isobaric interferences in complex matrices, thus affecting the reliability of the results [13-15]. In this sense, it is advantageous to use high-resolution mass spectrometry (HRMS) to analyze this type of compounds [15-17]. HRMS is a powerful tool because of its resolving power and mass accuracy, which allow to discriminate between interference and target ions, thus providing high sensitivity for analyzing low concentrations of analytes [18].

LC/HRMS has greatly improved laboratories' ability to characterize and confirm detected substances, and official control laboratories are increasingly using HRMS to detect and confirm food contaminants.

Moreover, control laboratories need high throughput analysis methods. In this sense, sample treatment is the bottleneck for analyzing corticosteroids in food samples. Regarding the extraction of

corticosteroids, relatively polar solvents, such as ethyl acetate, acetonitrile, methanol or hydro-organic mixtures have been proposed, in most cases using conventional shaking, or ultrasonic bath. Extracts are further cleaned up, mostly by solid phase extraction (SPE), especially in the reverse mode. Thus, some authors describe the use of C_{18} [17, 19] or polymeric cartridges [20,21], and less frequently the combination of two SPE steps, with C_{18} and SiO_2 cartridges [22], or the use of mixed mode strong cation exchange cartridges [23].

The QuEChERS methodology [24] allows to markedly speed up the sample treatment and is a very attractive approach for control laboratories dealing with large numbers of samples. Recent reports describe a QuEChERS based method for analyzing flumethasone (FLU) in beef muscle [25], and multifamily residue methods that include some corticosteroids [16].

The aim of this work is to develop a high throughput and sensitive method for simultaneously determining 8 synthetic corticoids in liver samples, namely DEX, BET, PLN, MPLN, FLU, triamcinolone (TRI), beclomethasone (BCL) and fluocinolone acetonide (FCN). The QuEChERS strategy is applied for the extraction and clean up steps, and the quantitative determination is performed by ultra-high performance liquid chromatography (UHPLC) coupled to high-resolution mass spectrometry, using a hybrid quadrupole Orbitrap mass spectrometer, with heated electrospray ionization (HESI).

Materials and Methods

Chemicals and Reagents

BCL, BET, DEX, FLU, FCN, MPLN, PLN and TRI with a minimum purity of 95% each, were purchased from Sigma-Aldrich (Seelze, Germany); Dexamethasone-D_4 (DEX D_4) 95%, was purchased from CDN Isotopes Inc. (Canada).

Purified Water Type I was obtained from a Milli-Q System (Millipore, Billerica, MA, USA); methanol HPLC was obtained from Fisher Scientific (UK); acetonitrile HPLC hypergrade (ACN) was obtained

from LiChrosolv (Darmstadt, Germany); formic acid and anhydrous sodium acetate were obtained from Sigma-Aldrich (Seelze, Germany); chloroform, hexane, sodium hydroxide and glacial acetic acid 99% were obtained from Panreac (Barcelona, Spain); QuEChERS salts (6 g of magnesium sulfate and 1.5 g of sodium acetate) and dispersive tubes (0.4 g of Primary Secondary Amines (PSA), 0.4 g of C18 and 1.2 g of magnesium sulfate) were obtained from Agilent Technologies (New Castle, DE, USA). All other chemicals were analytical reagent grade.

Individual stock solutions (1000 mg L^{-1}) of BCL, BET, DEX, FLU and TRI, were prepared by weighing 25 mg separately, and dissolving it into 25 mL of methanol in dark volumetric flasks. Individual stock solutions (1000 mg L^{-1}) of FCN, MPLN and PLN were prepared by weighing 25 mg separately and dissolving it into 25 mL of ACN:chloroform (50:50) mixture in dark volumetric flasks. These solutions were stable at least for 2 years at -20±3°C. Intermediate solutions (20 mg L^{-1}) were prepared by taking 0.5 mL of each stock solution with automatic pipette, bringing it to a 25 mL dark volumetric flask and wiping with ACN. These solutions were stable at least for 1 year at -20±3°C. Working solutions (0.4 mg L^{-1}) were prepared by taking 0.5 mL of 20 mg L^{-1} solution and diluting to 25 mL with mobile phase. These solutions were stable for 3 months at + 4±1°C.

Internal standard (DEX-D_4) stock solution (1000 mg L^{-1}) was prepared by weighing 10 mg of commercial standard and dissolving it in a dark flask with 10 mL of methanol. This solution was stable at least for 2 years at -20±3°C. Internal standard intermediate solution (20 mg L^{-1}) was prepared by taking 0.5 mL of DEX-D_4 1000 mg L^{-1} solution and diluting to 25 mL with ACN. This solution was stable for 1 year at -20±3°C. Internal standard working solution (0.4 mg L^{-1}) was prepared taking 0.5 mL of intermediate solution of DEX-D_4, putting it to a 25 mL volumetric dark flask and mixing with re-suspension solution (water:ACN (80:20) mixture). This solution was stable for 3 months at + 4±1°C.

Formic acid 0.1% (mobile phase A) was prepared daily by measuring 1 mL of formic acid with an automatic pipette and transferring it into a one liter flask with water type I, to obtain 1 liter of solution, and filtering through 0.22 µm polyvinylidene fluoride (PVDV) filter.

Instrumentation

A Vanquish UHPLC Ultimate 3000 binary pump, with a CORTECS column (Waters) C18 (50 x 2.1) mm, 1.6 µm, was used. Oven temperature was +40ºC and flow rate was 400 µL min^{-1}. The mobile phases were aqueous 0.1% formic acid (A) and ACN Hypergrade (B), and the gradient program was (time min, % B): (0, 20); (6, 30); (6.1, 100); (7, 100); (7.5, 20); (9.0, 20). Total run time was 9 minutes.

The chromatographic system was coupled to a hybrid quadrupole-Orbitrap HRMS instrument (Q-Exactive, Thermo Scientific, Bremen, Germany), with a heated electrospray ionization (HESI) in negative ionization mode. The Product Reaction Monitoring (PRM) acquisition mode was applied. In the PRM mode the precursor ions are isolated by the quadrupole and fragmented at the high energy collisional dissociation (HCD) cell. The product ions are analyzed at the Orbitrap mass analyzer, and the traces for the selected quantification and confirmation ions are acquired, using time windows, with high resolution.

The following conditions were used: heater temperature 310°C, capillary temperature 350°C, sheat flow gas (N_2) 40 L h^{-1}, auxiliary gas 10 L h^{-1} and spray voltage 3.5 kV. The mass spectrometer was calibrated daily to ensure a mass accuracy better than 5 ppm, using Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions (Thermo Fisher Scientific, Rockford, IL, USA). The isolation window in the quadrupole was 2 Da. We set a collision energy (NCE) of 10% for all compounds, except for FCN (15%), a resolution of 70,000 (m/z 200, FWHM), scanning rate 2 Hz and automatic gain control of $5e^6$ with a maximum injection time of 100 ms. The information about precursor, quantification and confirmation ions for each analyte is shown in Table 1.

To control the UHPLC-HRMS and data processing, TraceFinder 4.1 software (Thermo Fisher Scientific, San Jose, CA, USA) was used.

Nitrogen was obtained from a Zephyr generator (Clantecnologica, Seville, Spain).

A Retsch GM200 blender (Haan, Germany) was used for sample homogenization. A vertical agitation system (AGYTAX SR1 CP57, Spain), a refrigerated centrifuge (Hettich Rotanta 460R, Germany), a nitrogen evaporation system with controlled temperature (Turbo-VAP LV, Biotage, Sweden), an

ultrasonic bath (Selecta, Barcelona, Spain), a multiple vortex agitator (Genius 3, Germany) were used in the extraction steps, Whatman 2 mL vials with 0.45 µm PVDF filter and class A volumetric glass material were used.

Samples

Liver samples from various animal species (bovine, porcine, ovine, aviary and rabbit) were stored immediately on reception at -20°C in a properly numbered plastic jar until analysis. Liver samples were properly homogenized before analysis using a laboratory blender.

All blank samples used to validate the procedure were analyzed to confirm the absence of corticosteroids.

Extraction and clean up procedure

For extraction, 50 mL tubes to weigh 5g of liver sample were used, and 100 µL of internal standard 0.4 mgL^{-1} was added. After shaking for 30 seconds, 10 mL of water type I was added. Then 15 ml of ACN was added, and this mixture was shaken in a vortex. QUECHERS salts (6 g $MgSO_4$, 1.5 g CH_3COONa) were added, and the sample was shaken using vertical shaker, sonicated, and centrifuged at 3500 rpm for 5 minutes at + 5°C. One 10 ml aliquot was transferred to a dispersive tube containing 0.4 g PSA, 0.4 g C18 and 2 g $MgSO_4$, shaken using a multivortex and a vertical shaker, sonicated, and centrifuged at 3500 rpm for 5 minutes at + 5°C. The supernatant was evaporated to dryness at +45°C for 45 minutes. The dry residue was re-dissolved with 500 µL of re-suspension solution, shaken in vortex, sonicated and filtered using vials with an incorporated filter. Finally, 5µL of the extract was injected into UHPLC-HRMS system.

UHPLC/HMRS conditions for confirmatory analysis

Compounds were identified by retention time (RT) and accurate mass. Maximum permitted deviation of the retention time was set at 1%, related to the RT of the analytes in surrogate matrix matched standards (SMMS) extracts. Ions selected in the quadrupole were the formate adducts for all compounds, due to the ability of corticosteroids to form adducts, except for TRI. These ions were partially fragmented in the collision cell and afterwards analyzed using HRMS.

We used as quantification ion a fragment of the molecule in the majority of cases, except for BCL and FCN (Table 1): in these cases we used the formate adduct of the molecule. In addition to the quantification ion, two confirming ions were monitored (when possible) in the same experiment using a mass accuracy better than 5 ppm. Table 1 shows the confirmation ions for each analyte. The ion ratio (ratio between the quantification ion and the confirmation ion) was used as an additional criterion for confirming positive results. The ion ratio of the compounds in the sample should be in agreement with that of the surrogate matrix matched standards (SMMS) with a maximum tolerance of ± 20%.

Calibration

Calibration was performed using SMMSs in the range 1-10 µg kg^{-1} and adding Dexamethasone-D_4 as internal standard according to 657/2002/CE Decision and its interpretation [26, 27]. SMMSs were prepared as follows: blank samples were spiked with a mixture of analytes and internal standard, and were left during 30 min before carrying out the analytical procedure. The SMMSs were then submitted to the complete analytical procedure (*i.e*. QuEChERS+UHPLC-MS/HRMS).

The calibration curve for each analyte was obtained by linear regression analysis of the ratio between the peak area of the analyte and that of the internal standard plotted against the analyte concentration. This type of calibration provides results already corrected for matrix effect and recovery.

Linearity was assessed by calculating the correlation coefficient (r^2) and point-to-point deviation. The value of r^2 was > 0.999 for all analytes except DEXA, for which r^2 was 0.998. Residuals requirements were set as follows: < 25% at the first point of the calibration curve and < 15% for higher calibration concentrations.

Results and Discussion

Optimization of UHPLC/HRMS

The chromatographic separation was performed at +40ºC with a CORTECS C18 core shell column using a mobile phase based on water with 0.1% formic acid and ACN. The chromatographic conditions, in terms of gradient and flow rate, are described in section 2.2. Separation of the 8 compounds was achieved within 6 minutes, and DEX and BET stereoisomers were completely resolved (Figure 1). Total chromatographic run time, including re-equilibration, was 9 minutes.

MS detection is generally based on corticosteroids' ability for adduct formation. Addition of low concentration (0.1%) of acetic or formic acid in the mobile phase induces the formation of acetate or formate adducts [18, 28-30]. For HRMS detection with the Q-Orbitrap spectrometer, negative ionization and the PRM mode were used; PRM is similar to Product Ion Scan in triple quadrupole instruments, but generates a HRMS product ion spectrum. Thus, in PRM, the precursor ion is selected in the quadrupole (isolation window 2 Da), then fragmented in the HCD cell, and the product ions are analyzed at the Orbitrap mass analyzer. For all the analytes the formate adduct was selected as precursor ion, except for TRI, for which the $[M-H]^-$ ion was chosen (Table 1). Fragmentation experiments, varying the collision energy, were performed to select suitable quantification and confirmation ions. It was observed that formate adducts for BCL and FCN underwent lower fragmentation, with $[M+HCOO]^-$ ions showing intense signals in the HRMS product ions spectra, and thus they were selected as quantification ions. The formate adduct was selected as confirmation ion for all analytes except for TRI, BCL and FCN. Information about quantification and confirmation ions for all the analytes is given in Table 1.

Optimization of the extraction method

Liver samples were used to optimize various parameters of the extraction method.

The QuEChERS approach was first proposed for the analysis of pesticides in fruits [24], but it is currently applied in different fields of analysis [31, 32]. Commercial QuEChERS Kits for fatty

samples were assayed. ACN was used as extraction solvent, sodium acetate and magnesium sulphate as a salting-out medium and a mixture of PSA and C18 as a dispersive media for clean-up.

The standard QuEChERS procedure uses 10 g of sample and 15 mL of ACN. During the assays, it became necessary to add water to the samples to prevent them from becoming too viscous and to allow proper extraction. Thus, in the preliminary tests, the ratio of sample to the amount of water added was evaluated, and proportions of 2, 1, 0.5 and 0.3 were tested using samples spiked at 1 μg kg^{-1} concentration. The ratio 0.5 gave the extracted sample an appropriate density and an effective separation of the mixture, with a clean upper layer after centrifugation, suitable for chromatographic analysis.

In order to avoid matrix overload in the chromatographic system, the sample weight was investigated (3, 5 and 10 g). 5 g was enough to detect and confirm concentrations of at least 1 μg kg^{-1}, and thus it was not necessary to analyze a larger amount of sample. Therefore, for the extraction step the established conditions were: 5 g of sample, 10 mL of water, 15 mL of ACN, and 6 g of magnesium sulfate and 1.5 g of sodium acetate. Afterwards, the organic layer was cleaned-up by dispersive solid phase extraction with PSA, C18 and magnesium sulfate, the extract was evaporated to dryness, and the solid residue dissolved in water:ACN (80:20).Finally, a commercial QuEChERS kit for drug residues in meat was also tested. This kit uses sodium chloride in the extraction step and C18 as the dispersive medium for clean up; the results were not successful, since the clean-up step was not effective enough and the extracts were too fatty, and thus this kit was discarded.

The efficiency of the QuEChERS extraction method using the fatty samples kit was evaluated by recovery studies. Extraction recoveries were determined using spiked blank porcine liver samples, previously analyzed and confirmed to be free of analytes. We followed this approach, that can led to some overestimation of recoveries, because there were no certified reference materials available. Thus, aliquots of a blank liver sample were fortified at 1, 2 and 10 μg kg^{-1}. For each concentration level, two sets of samples (n=6 each) were prepared, the first one spiked before the extraction step and the second one spiked after extraction and clean-up, and the results of both sets were compared. The procedure was repeated on three different days, for a total of 18 replicates at each concentration level. Absolute recoveries ranged from 35 to 49%, with RSD values below 15%. Although absolute

recoveries lie in the low range, values were reproducible. In addition, calibration by SMMS corrects for extraction recoveries.

The proposed method is very convenient for routine analysis of corticosteroids in food samples, in laboratories with high workload. Compared to methods based on a conventional extraction followed by a clean-up by SPE, the new method reduces the time spent in sample treatment in about 50%.

Method validation

The method was validated according to 657/2002/EC Decision [26], and the results are collected in Table 2. The validation study was performed using porcine liver as the basic matrix.

Blank studies

The analytical method was tested in blank liver samples from different species (bovine, ovine, swine, avian and rabbit) with satisfactory results. No signal in the retention time of the analytes could be measured, demonstrating the method's selectivity. The results were also evaluated in terms of internal standard response: RSD% among different species was 7.8%, proving that the matrix effect was very limited. These results evidenced that the different liver matrixes were equivalent in terms of the method performance.

Linearity

The criteria for evaluating linearity are described in calibration section. We found satisfactory results for all analytes in the validation range (1-10 μg kg^{-1}), with correlation coefficients > 0.99. Response factor as a second linearity criterion was also determined. Response factor was calculated as the ratio between the response (peak area) of each calibration point and its theoretical value of concentration. A good linearity implies the parameter to be equal for all calibration points. The parameter was evaluated in terms of RSD between all calibration points. In all cases was below 20%, demonstrating the good linearity of the method.

Precision

Precision was determined, in terms of reproducibility, from experiments conducted on three different days and extracting 6 spiked samples per day. Samples were spiked at 3 different concentrations,

namely 1, 2 and 10 μg kg^{-1}, and were extracted using different reagent batches and by different analysts. The method's precision, expressed as the RSD, ranged from 2.9 to 13.5%.

Trueness

Since no certified reference material was available, trueness was calculated in terms of recovery of spiked samples. Results were satisfactory, in the 78-101% range, in agreement with 657/2002/EU recommendations. Slopes obtained in regression analysis of spiked and calculated concentration data were close to 1, with regression coefficients higher than 0.99.

CC*α* and CC*β*

On one hand, for compounds with a published MRL (namely DEX, BET, PLN, MPLN in liver of some particular species) [11], *CCα* and *CCβ* were calculated from blank samples spiked at the MRL level, according to the guidelines in Decision 657/2002/CE. On the other hand, for compounds with no MRL, these parameters were calculated using the ISO 11843 extrapolation approach [33]. *CCα* ranged from 0.04 to 0.16 μg kg^{-1}, depending on the analyte. *CCβ* results ranged from 0.08 to 0.35 μg kg^{-1}. These values point out that the method is highly sensitive. Results are shown in Table 2.

The laboratory has participated in proficiency tests using the new method and very satisfactory results have been achieved; Fapas 02339 Food Chemistry exercise consisted of a bovine liver containing BET (5 μg kg^{-1}). Results were excellent, with a Z-score of 0.0, proving the suitability of the proposed method. In the 4^{th} run of Progetto Trieste interlaboratory exercises, the method was applied to the analysis of a lyophilized bovine liver sample. DEX was detected and quantified (2.49 μg kg^{-1}), and the obtained z-score was 0.41.

The new method is currently used at the laboratory for the analysis of liver samples from various species from different slaughterhouses in Catalonia, as a routine method of analysis. Figure 2 shows the chromatogram of a porcine liver sample that contained 1.5 μg kg^{-1} of DEX. In addition to the DEX peak, some small peaks can be observed at the trace chromatograms of PLN, BCL and FCN, at their corresponding retention time windows. However, these peaks are below the limit of quantification and do not fulfill the ion ratio criteria.

Conclusions

The new method combines the QuEChERS strategy for sample treatment with the confirmatory analysis by UHPLC-MS/HRMS of 8 synthetic corticosteroids. On the one hand the QuEChERS approach allows to notably increasing the method throughput, since time for sample treatment is reduced by about 50% in comparison with methods using conventional extraction and SPE clean up. In addition, there is a remarkable waste reduction. On the other hand, the use of HRMS provides excellent performance in terms of selectivity and also sensitivity, which are critical issues in the analysis of low concentration levels in complex samples. The method is very simple and rapid, and up to 30 samples can be daily processed at the laboratory by a single operator. These characteristics are essential for an effective and reliable food safety control. In addition, they facilitate the market withdrawal of non-compliant products, containing illicit synthetic compounds used as growth promoters in livestock farming.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest

References

1. Martindale W. The Extra Pharmacopoeia. J.E.F. Reynolds, editors. 31st ed. London: Royal Pharmaceutical Society; 1996.
2. Miller W L, Auchus RJ. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. Endocr Rev. 2016; 32(1):81-151.

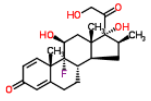
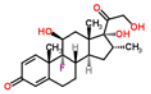
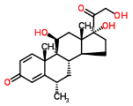
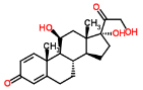
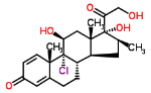
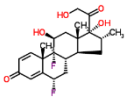
3. Quanson JL, Stander MA, Pretorius E, Jenkinson C, Taylor AE, Storbeck KH. High-throughput analysis of 19 endogenous androgenic steroids by ultra-performance convergence chromatography tandem masss spectrometry. J Chromatogr B. 2016;1031: 131-138.
4. Soma LR, Uboh CE, Liu Y, Li X, Robinson MA, Boston RC, Colahan PT. Pharmacokinetics of dexamethasone following intra-articular, intravenous, intramuscular and oral administration in horses and its effects on endogenous hydrocortisone. J Vet Pharm Therap. 2013;36:181-191.
5. Richelmi GB, Pezzolato RC, Botta M, Varello K, Pitardi D, Baioni E, Bellino C, D'Angelo A, Caramelli M, Bozzetta E. Thymus atrophy is an efficient marker of illicit treatment with dexamethasone in veal calves: Results from a triennial experimental study. Res Vet Sci. 2017;113: 67-72.
6. Mitra, R. Adverse effects of corticosteroids on bone metabolim: a review. A A J Phys Med Rehab. 2011;3: 466-471.
7. Vicenti M, Girolami F, Capra P, Pazza M, Carletti M, Gardini G, Nebbia C. Study of dexamethasone urinary excretion profile in cattle by LC-MS/MS: comparison between therapeutic and growth-promoting administration. J Agric Food Chem. 2009;57: 1299-1306.
8. Deceuninck Y, Bichon E, Monteau F, Antignac JP, Le Bizec B. Determination of MRL regulated corticosteroids in liver from various species using ultra high performance liquid chromatography tandem mass spectrometry (UHPLC). Anal Chim Acta. 2011;632: 137-143.
9. Schmidt KS, Stachel CS. In-house validation and factorial effect analysis of a liquid chromatography-tandem mass spectrometry method for the determination of corticosteroids in bovine and porcine muscle tissue. Anal Bioanal Chem. 2013;405: 6287-6297.
10. Council of the European Union. Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. Off J Eur Comm. 1996;L125: 10-32.
11. European Commission. Commission Regulation (EU) Nr. 37/2010, on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. Off J Eur Union. 2010;L15:1-72.

12. Council of the European Union. Regulation (EU) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin. Off J Eur Union. 2009;L152: 11-22.
13. Herrero P, Cortés-Francisco N, Borrull F, Caixach J, Pocurull E, Marcé RM. Comparison of triple quadrupole mass spectrometry and orbitrap high-resolution mass spectrometry in ultra-high performance liquid chromatography for the determination of veterinary drugs in sewage: benefits and drawbacks. J Mass Spectrom. 2014;49: 585-96.
14. Kumar P, Rúbies A, Centrich F, Granados M, Cortés-Francisco N, Caixach J, Companyó R. Targeted analysis with benchtop quadrupole-orbitrap hybrid mass spectrometer: Application to determination of synthetic hormones in animal urine. Anal Chim Acta. 2013;780: 65-73.
15. Kaufmann A , Butcher P , Maden K , Walker S, Widmer M. Reliability of veterinary drug residue confirmation: high resolution mass spectrometry versus tandem mass spectrometry. Anal Chim Acta. 2015; 856: 54-67.
16. León N, Roca M, Igualada C, Martins CPB, Pastor A, Yusá V. Wide-range screening of banned veterinary drugs in urine by ultra-high liquid chromatography coupled to high-resolution mass spectrometry. J Chromatogr A. 2012;1258: 55– 65.
17. De Clercq N, Van den Bussche J, Croubel S, Delahaut P, Vanhaecke L. Development and validation of a high-resolution mass-spectrometry based method to study the long-term stability of natural and synthetic corticosteroids in faeces. J Chromatogr A. 2014;1336: 76-86.
18. Fidani M, Pompa G, Mungiguerra F, Casati A, Fracchiolla ML, Arioli F. Investigation of the presence of endogenous prednisolone in equine urine by high-performance liquid chromatography mass spectrometry and high resolution mass spectrometry. Rapid Commun Mass Spectrom. 2012;26: 879-886.
19. Delahaut P, Demoulin L, Gillard N, Fichant E, Courtheyn D. Preliminary study on the presence of prednisolone in porcine urine and liver - How to distinguish endogenous from therapeutically administered prednisolone. Drug Test Anal. 2014; 6: 325–335.

20. Dusi G, Gasparini M, Curatolo M , Assini W, Bozzoni E , Tognoli N, Ferretti E. Development and validation of a liquid chromatography tandem mass spectrometry method for the simultaneous determination of nine corticosteroid residues in bovine liver samples. Anal Chim Acta. 2011;700: 49-57.
21. Tölgyesi A , Sharma V K , Fekete J. Development and validation of a method for determination of corticosteroids in pig fat using liquid chromatography-tandem mass spectrometry. J Chromatogr B. 2011;879: 403-10.
22. Deceuninck Y, Bichon E, Monteau F, Dervilly-Pinel G, Antignac JP, Le Bizec B. Fast and multiresidue determination of twenty glucocorticoids in bovine milk using ultra high performance liquid chromatography–tandem mass spectrometry. J Chromatogr A. 2013;1294: 76– 86.
23. Tölgyesi A, Tölgyesi L, Sharma VK, Sohn M, Fekete J. Quantitative determination of corticosteroids in bovine milk using mixed-mode polymeric strong cation exchange solid-phase extraction and liquid chromatography-tandem mass spectrometry. J Pharm Biomed Anal. 2010;53: 919-28.
24. Anastassiades M, Lehotay SJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. J AOAC Int. 2003;86: 412-443.
25. Park K H, Choi J H, Abd El-Aty AM, Cho SK, Park JK, Kwon KS, Park HR, Kim HS, Shin HC, Kim MR, Shim JH. Development of QuEChERS-based extraction and liquid chromatography–tandem mass spectrometry method for quantifying flumethasone residues in beef muscle. Meat Sci. 2012;92: 749-753.
26. European Commission. Commission Decision (EU) 657 of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off J Eur Comm. 2002;L221: 8-36.
27. SANCO. Conclusion of the meeting on the interpretation of the implementation of decision 2002/657/EC. Standing Committee of the food Chain and Animal Health, 16 November 2004. Available from: https://ec.europa.eu/food/sites/food/files/safety/docs/cs_vet-med-residues_cons_2004-2726rev4_en.pdf. Accessed 10 July 2018.

28. Chen D, Tao Y, Liu Z, Wang Y, Huang L, Yuan Z. Development of a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of glucocorticoid residues in edible tissues of swine, cattle, sheep, and chicken. Food Add Contam. 2010;27 (10): 1363-1371.
29. Noh E, Yoon CY, Lee JH, Baek SY, Oh HB, Do JA. A liquid chromatography-quadrupole-time of flight mass spectrometry (LC-Q-TOF-MS) study for analyzing 35 corticosteroid compounds: Elucidation of MS/MS fragmentation pathways. Bull Korean Chem Soc. 2016;37: 1029–1038.
30. Chiesa L, Panseri S, Pavlovic R, Cannizzo FT, Biolatti BB, Divari S, Villa R, Arioli F. HPLC-ESI-MS/MS assessment of the tetrahydrometabolites of cortisol and cortisone in bovine urine: promising markers of dexamethasone and prednisolone treatment. Food Add & Contam A. 2016;33: 1175-1189.
31. Rúbies A, Antkowiak S, Granados M, Companyó R, Centrich F. Determination of avermectins: A QuEChERS approach to the analysis of food samples. Food Chem. 2015;181: 57–63.
32. Rúbies A, Guo L, Centrich F, Granados M. Analysis of non-steroidal anti-inflammatory drugs in milk using QuEChERS and liquid chromatography coupled to mass spectrometry: triple quadrupole versus Q-Orbitrap mass analyzers. Anal Bioanal Chem. 2016;408: 5769-5778.
33. International Organization for Standardization. ISO 11843-2. Capability of detection. Part 2: Methodology in the linear calibration case. 2000

Table 1. Compounds information. Precursor, quantification and confirmation ions

Compound	Acronym	Structure	Molecular formula	Molecular mass	RT (min)	Precursor ion [m/z]	Quantification ion [m/z]	Confirmation ions [m/z]
Betamethasone	**BET**		$C_{22}H_{29}FO_5$	392.46	3.80	437.2	359.1666	437.1993 361.1824
Dexamethasone	**DEX**		$C_{22}H_{29}FO_5$	392.46	3.94	437.2	359.1666	437.1993 361.1824
Methylprednisolone	**MPLN**		$C_{22}H_{30}O_5$	374.47	3.65	419.22	341.1758	419.2019 313.1812
Prednisolone	**PLN**		$C_{21}H_{28}O_5$	360.44	2.22	405.2	327.1604	405.1931 299.1653
Beclomethasone	**BCL**		$C_{22}H_{29}ClO_5$	408.92	4.40	453.2	453.1689	407.1632 377.1527
Flumethasone	**FLU**		$C_{22}H_{28}F_2O_5$	410.45	3.93	455.2	379.1726	455.1893 409.1828

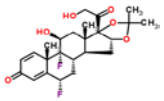
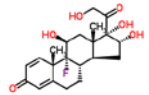
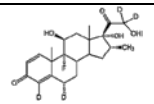
Fluocinolone acetonide	**FCN**		$C_{24}H_{30}F_2O_6$	452.48	5.26	497.2	497.2007	431.1875 ----
Triamcinolone	**TRI**		$C_{21}H_{27}FO_6$	394.43	1.07	393.2	345.1727	325.1444 ----
Dexamethasone-D4	**DEX-D4**		$C_{22}H_{25}D_4FO_5$	396.46	3.89	441. 2	363.1948	----

Table 2. Results of the validation study in porcine liver matrix

Compound	MRL (µg kg^{-1})	Concentration level (µg kg^{-1})	Trueness (%)	Precision (RSD%)	CCα (µg kg^{-1})		CCβ (µg kg^{-1})	
					MRL	No MRL	MRL	No MRL
BET	2[a]	1	80.1	8.3	2.3	0.16	2.6	0.27
		2	78.5	11.0				
		10	87.0	9.4				
DEX	2[b]	1	84.6	13.9	2.4	0.11	2.7	0.21
		2	78.4	13.5				
		10	85.9	10.0				
MPLN	10[c]	1	94.0	4.4	11.1	0.04	12.2	0.08
		2	89.2	4.6				
		10	91.9	7.4				
PLN	10[c]	1	87.2	7.2	11.2	0.04	12.4	0.13
		2	83.1	9.3				
		10	88.9	8.4				
BCL	---	1	98.6	5.5	---	0.15	---	0.35
		2	94.0	2.9				
		10	94.5	8.1				
FLU	---	1	92.6	4.6	---	0.06	---	0.13
		2	92.2	4.6				
		10	92.6	7.1				
FCN	---	1	93.1	5.0	---	0.10	---	0.18
		2	89.5	6.4				
		10	92.0	7.4				
TRI	---	1	101.2	4.8	---	0.05	---	0.13
		2	90.9	6.2				
		10	89.9	6.8				

a: bovine and porcine liver; b: ovine and porcine liver; c: bovine liver

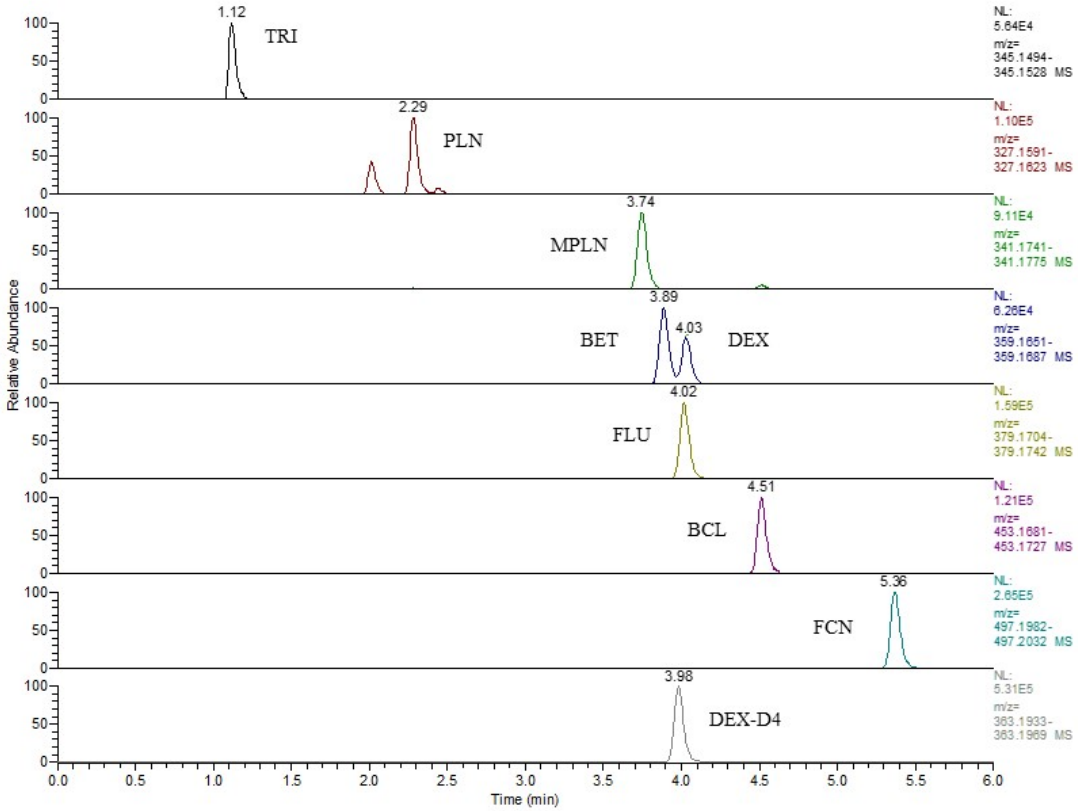


Fig. 1 LC-MS/HRMS ion chromatograms of an extract from a porcine liver sample spiked with corticosteroids at 1 µg kg^{-1} level

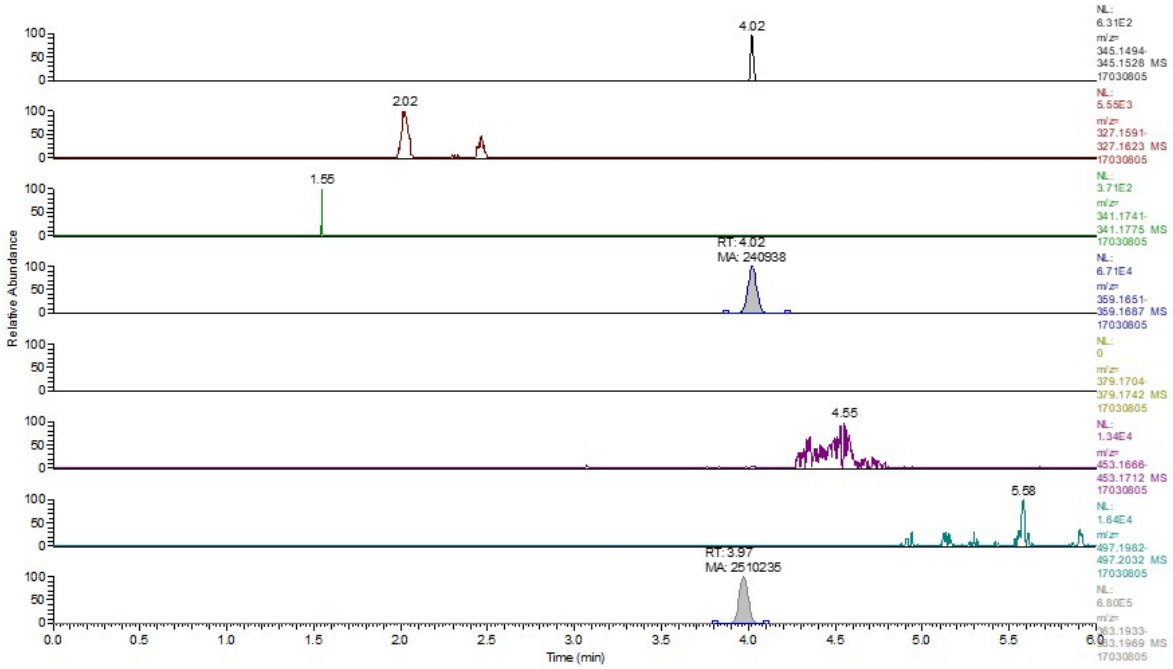


Fig. 2 LC-MS/HRMS ion chromatograms of a porcine liver sample containing DEX (1.5 µg kg^{-1})