Detection of boldenone, its conjugates and androstadienedione, as well as five corticosteroids in bovine bile through a unique immunoaffinity column clean-up and two validated liquid chromatography–tandem mass spectrometry analyses.

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Abstract

The presence of β-boldenone II phase metabolites and prednisolone in urine samples, owing to endogenous or natural origin or illicit treatment, is under debate within the European Union. The detection of β-boldenone conjugates, α-boldenone conjugates at a concentrations higher than 2 ng mL^{-1} and prednisolone above the cut-off level of 5 ng mL^{-1} in urine have been, until now, critical in deciding if illegal drug use has occurred. The use of urine as a matrix for the control of illegal drug treatment is not, however, entirely satisfactory. In this study, we have developed and validated a simple and unique immunoaffinity clean-up procedure, which was applied to bovine bile samples, followed by two different analytical liquid chromatography, ion electrospray, tandem mass spectrometry methods. The first of these methods tests for androstadienedione, α- and β-boldenone sulphate, glucuronate and free forms, and the other method tests for prednisolone, prednisone, dexamethasone, cortisone and cortisol. The methods were validated according to the European Commission Decision 2002/657/EC. The evaluated parameters were linearity, specificity, precision (repeatability and intra-laboratory

reproducibility), recovery, decision limit and detection capability. The decision limits (CCα) were between 0.38 and 0.45 ng mL^{-1} and 0.13 and 0.15 ng mL^{-1} for anabolic steroids and corticosteroids, respectively. Intra- and inter-day repeatability was below 15.8 and 19.9% for all analytes, respectively. The methods were applied to the analysis of some bile samples collected from untreated young bulls in order to investigate the presence of the studied steroids in this matrix.

Keywords: boldenone sulphate, boldenone glucuronide, prednisolone, prednisone, dexamethasone, bovine bile.

1. Introduction

The use of growth promoters in food-producing animals allows animal performances to be improved, such as a better transformation rate, a higher meat yield at slaughter, an increase in milk production or a decrease in muscle fat. The use of growth promoters is prohibited, as detailed in Council Directives 96/22/EC and 96/23/EC [1, 2], which contain guidelines for controlling veterinary drug residues in animals and their products, with all the necessary information to set up national monitoring plans [3].The ban of any growth-promoter was accomplished on 1 January 2006 with the prohibition of the last four antimicrobial agents [4].

Regulations on substance residues with hormonal activity in food of animal origin is essential to safeguard animal welfare, to avoid consumer health risks derived from the exposure and to ascertain commercial frauds.

Nevertheless, the simple detection of some steroids in bovine urine is currently considered to provide insufficient evidence of illicit treatment. Parameters such as cut-off levels, presence of metabolites, or both, must be accounted for. As an example, the α-epimer of boldenone was proposed, in 2003, as a naturally occurring steroid in bovine animals by experts within the EU, who set the "natural threshold" for the α-boldenone conjugates in urine at 2 ng mL^{-1}; a concentration above this could come from illicit treatment [5]. The authorities responsible for the control of residues in food must, therefore, consider either the possible endogenous production of these molecules or the existence of natural feed ingredients, such as phytosterols, as possible precursors to boldenone [6]. The faecal contamination of urine can also generate false positives for boldenone presence [7, 8]. An analogous explanation considers the *in vitro* formation of prednisolone from cortisol in bovine [9] and human urine [10]. Moreover, cattle that are under stress conditions [11] could produce prednisolone. Based on recent findings and on a study that has been carried out on 100 bovine urine samples, de Rijke et al. have suggested a threshold level

of 5 ng mL^{-1} for regulatory purposes [12], which was based on the following calculation: average level in non-treated animals + (3 x the standard deviation). From all considerations, it emerges that no ultimate answer is available on the topics of boldenone and prednisolone in bovine urine. We, therefore, suggest a different biological matrix, such as bile, which represents a fairly complex matrix containing a lot of information. Many substances undergo, through the biliary tract, entero-hepatic recycling. Until now, scientific reports have indicated urine, liver, faeces and hair as the major biological matrices for the detection of such important analytes, whereas data are scarce for bile.

Particular research in this area includes the study of trenbolone in bovine bile and faeces [13], the analysis of hormonal steroids in fish plasma and bile [14], and an automated multi-immunoaffinity chromatography screening to detect anabolic agents, including boldenone, in bile and urine [15]. However, these methods do not allow for the simultaneous determination of corticosteroids and anabolic steroids, and the developed clean-up method relied on a number of steps, resulting in complicated and expensive procedures.

In this paper, we describe two methods based on a unique immunoaffinity column (IAC) clean-up and two liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses of bile, which are validated according the technical guidelines on the analytical performance criteria for confirmatory and validation procedures, as described in the Commission Decision (2002/657/EC) [16].

Anabolic steroids 17α- and 17β-boldenone, their glucuronate and sulphate conjugates, and their precursor androstadienedione (ADD) (Figure 1) are the analytes that were investigated in the first method. Corticosteroids prednisolone, prednisone, dexamethasone, cortisone and cortisol are the analytes used for the second method (Figure 2).

The methods both demonstrated a good performance, allowing for the detection and identification of the analytes at levels lower than 0.5 ng mL^{-1}. These validated methods were ultimately applied to the analysis of bile samples collected from untreated young bulls in order to investigate the presence of the studied steroids in this matrix.

2. Materials and methods

2.1. Sample collection

Bile samples were collected after slaughtering untreated young Charolaise bulls (14–17 months old); following collection they were immediately frozen, taken to the laboratory and stored at −40°C until the analysis was performed.

2.2. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA). Formic acid 98–100% was obtained from Riedel-de Haën (Sigma-Aldrich, St.Louis, MO, USA). Water was purified by a Milli-Q System. The IAC was provided by Randox (DM 2185, Randox Laboratories, Antrim, UK). Concentrated wash and storage buffers, which were diluted following the manufacturer's instructions before use, were supplied with the columns. ADD and β-boldenone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA); β-boldenone sulphate (triethylamine salt), β-boldenone glucuronide, and α-boldenone were obtained from LGC Standards (Teddington, UK). The internal standards were β-boldenone sulphate-d3 for the sulphate forms, β-boldenone-d3 for the free forms (LGC Standards, Teddington, UK) and epitestosterone (EpiT) glucuronide-d3 for the glucuronate forms (National Measurement Institute, Pymble, NSW, Australia). The sulphate and glucuronate forms of α-boldenone, provided by research partners, were prepared by a two-step synthesis procedure, in which β-

boldenone (Steroid SpA, Cologno Monzese, Milan, Italy) was epimerised using a modified Mitsunobo protocol, according to Dodge and Lugar [17, 18], which was followed by sulphation, according to Sanaullah and Bowers [19], or glucuronation, according to Casati et al. [20]. Cortisone, cortisol, prednisone, prednisolone and dexamethasone were purchased from Fluka (Sigma-Aldrich, St.Louis, MO, USA) and their internal standard, prednisolone-d6, was obtained from C/D/N Isotopes Inc (Pointe-Claire, Quebec, Canada).

2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at -40°C. Working solutions, containing each of the studied analytes at the concentrations of 10 and 100 ng mL^{-1}, were prepared daily. Each working solution was maintained at 4°C during the method validation procedures.

2.4. Sample extraction

Samples of bovine bile (5 mL) were centrifuged, spiked with the internal standards to the final concentration of 2 ng mL^{-1} and then purified by using the IAC. The column was previously washed with 5 mL ethanol:water (70:30 v/v) and equilibrated with 3 × 5 mL wash buffer (flow rate ≤ 3 mL min^{-1}, i.e. about one drop per second). The pH value of the bile samples was measured resulting within the operative range (7.5–8.5) of the column. The samples were loaded by gravity flow. Wash buffer (2 x 5 mL) and water (1 x 5 mL) were used to wash the column. The elution of the bound analytes was then performed by the application of 4 mL ethanol:water (70:30 v/v) (flow rate ≤ 3 mL min^{-1}), which was collected in a 15 mL polypropylene tube. The eluate was evaporated in a rotary vacuum evaporator. The dried extract was reconstituted in 200 µL of methanol:water (50:50 v/v) and transferred in an auto-sampler vial. The injection volume was 10µL. The IAC could be

used again, starting from the equilibration described above, after a wash step with 2 x 5 mL ethanol:water (70:30 v/v).

2.5. LC-MS/MS analyses

LC analysis was carried out with an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA), constituted by a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with 20 µL loop. Chromatographic separation was achieved using a Synergi Hydro RP reverse-phase HPLC column (150 x 2.0 mm, 4 µm internal diameter), with a C18 (4 x 3.0 mm) guard column (Phenomenex, Torrance, CA, USA), which was kept at 30°C. The mobile phase consisted of methanol (solvent A) and 0.1% aqueous formic acid (solvent B). The gradient program for boldenone and its conjugates began at 60% A for 1 min, changing to 95% A in 11 min, which was then held for 2 min. Then, it returned to 60% A in 2 min and equilibrated for another 7 min. The flow rate was 200 µL min^{-1} and the overall run time was 22 min.

The gradient profile for corticosteroids began at 75% B, changing to 30% B in 18 min and then to 5% B in 1 min, which was held for 2 min. Finally, it returned to 75% B in 2 min and equilibrated for another 6 min. The flow rate was 250 µL min^{-1} and the overall run time was 29 min.

The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher, San Jose, CA, USA) equipped with an electrospray interface (ESI) set both in the positive (ESI+) and in the negative (ESI-) electrospray ionisation modes. Acquisition parameters were optimised in the ion-spray mode by direct continuous pump-syringe infusion of the standard solutions of analytes at a concentration of 1 µg mL^{-1}, a flow rate of 20 µL min^{-1} and a MS pump rate of 100 µL min^{-1}. The following conditions were used: capillary voltage 3.5 kV, ion-transfer capillary temperature 340°C; nitrogen as sheath and auxiliary gases at 30 and 10 arbitrary units, respectively, argon as the collision gas at 1.5 mTorr and peak

resolution 0.70 Da at full width half maximum (FWHM). The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. Three diagnostic product ions were chosen for each analyte and internal standard. The acquisition was made in multiple reaction monitoring (MRM). The selected diagnostic ions, one of which was chosen for the quantification, and the collision energies are reported in Table 1 for boldenone and its conjugates; those results for the corticosteroids are reported in Table 2. The reconstructed LC–MS/MS chromatograms for the anabolic steroids and corticosteroids in a bile sample spiked with each analyte at the lowest concentration level of the validation are shown, together with the ion spectra, in Figure 3 and Figure 4, respectively; in addition, on the right-hand side of each figure, are the relative internal standards (2 ng mL^{-1}). Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

2.6. Method validation

The validation was performed according to the criteria and recommendations of the European Commission Decision 2002/657/EC [16]. Some bile samples that were previously tested contained residues of α-boldenone glucuronide, cortisone and cortisol at concentrations higher than 3 ng mL^{-1}. We could, therefore, use pooled-bile blank samples from untreated young bulls for the validation of all steroids, except for the three mentioned. The method for these last analytes was validated in water adjusted to pH 8 with NaOH 0.1 N as a surrogate matrix of the bile, following the directions of van de Merbel [21].

For each analyte, the method performance was assessed through its qualitative parameters, such as the analyte specificity, molecular identification in terms of retention time (RT) and transition ion ratios, through its quantitative parameters, such as the linearity, recovery, accuracy in term of trueness and of precision expressed as the intra- and inter-day repeatability, and through the analytical limits [decision limit (CCα) and detection capability (CCβ)].

Specificity identification includes detecting any extra peaks in the blank matrix chromatograms as well as checking the matching of the relative retention time observed for the spiked analytes, compared to standard analytes in methanol, with a tolerance of ±2.5%. No evaluation of the specificity could be made for the validated analysis of the three chemicals in the surrogate matrix.

The instrumental linearity was evaluated by drawing five-point calibration curves in the solvent containing a fixed amount of the internal standards (2 ng mL^{-1} each), with analyte concentrations corresponding to 0.3, 1.0, 2.0, 3.0 and 5.0 ng mL^{-1} for ADD and the different forms of boldenone, and to 0.1, 0.5, 1.0, 3.0 and 5.0 ng mL^{-1} for the five corticosteroids.

Matrix calibration curves were obtained by spiking bile samples with each of the analytes (except the three validated in water), resulting in three analytical series; each series had three concentration levels (0.1, 0.2 and 0.3 ng mL^{-1} for corticosteroids and 0.3, 0.6 and 0.9 ng mL^{-1} for boldenone and its conjugate) in six replicates. Analogue curves, in water adjusted to pH 8, were obtained for α-boldenone glucuronide, cortisone and cortisol.

The trueness was assessed through recovery and was evaluated using the matrix curve results from the three analytical series, expressed in terms of a percentage of the measured concentration with respect to the spiked concentration.

The precision in terms of intra- and inter-day repeatability was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at three concentration levels of the three analytical series. The data from the matrix calibration curve were used to calculate the decision limit (CCα) and the detection capability (CCβ), according to the matrix calibration curve procedure described in the Commission Decision 2002/657/EC [16], as clarified in the document SANCO/2004/2726 revision 4 [22].

3. Results and discussion

The pseudo-endogenous nature of boldenone and prednisolone has so far hampered the control of residues of such substances in conventional matrices, such as urine, and resulted in the investigation of possible indications of treatment, such as biomarkers or cut-off levels, as already stated in Section 1.

A procedure that uses bovine bile as the biological matrix to detect and distinguish the boldenone epimers, their phase II metabolites, ADD and five corticosteroids is described herein and two methods are validated with the aim to be used as a tool to carry out research on the origin of these steroids and their conjugated forms.

The two developed methods use a unique IAC clean-up step in bovine bile, which is suitable for both anabolic steroids and corticosteroids, and one of two LC-MS/MS analyses steps. One of the LC-MS/MS analyses can detect ADD, α- and β-boldenone sulphate, glucuronate and free forms, and, in the other LC-MS/MS method, prednisolone, prednisone, dexamethasone, cortisone and cortisol, at concentration levels suitable for research and control purposes. The two LC-MS/MS methods were developed to provide confirmatory data for the analysis of bovine bile. After preliminary trials, in full-scan mode from 50 to 500 *m/z,* the three product ions with the higher signal-to-noise ratio (s/n), for each analyte and internal standard were chosen for identification. The collision energy (CE) and the de-clustering potential (DP) were adjusted in the MRM mode for each transition monitored, in order to reach the highest sensitivity for all analytes.

For a method to be deemed confirmatory under Commission Decision 2002/657/CE [16], it must yield four identification points (IPs). Each one of the three product ions is equal to 1.5 IPs, making a total of 4.5 IPs. The three diagnostic product ions, among which is the ion for the quantification, and the CEs are reported in Table 1 (for anabolic steroids) and Table 2 (for corticosteroids).

3.1. Performance characteristics of the methods

242 The instrumental linearity was evaluated over a concentration range of 0.3– 5.0 ng mL^{-1} for
243 the anabolic steroids and 0.1–5.0 ng mL^{-1} for the five corticosteroids, using solutions of the
244 analytes in methanol:water (50:50 v/v), containing a fixed amount of the internal standards
245 (2.0 ng mL^{-1} each). Correlation coefficients of the curves were higher than 0.9970 for all
246 compounds, indicating a good fit.
247 The matrix calibration curves built for the validation of each analyte were demonstrated to
248 be linear in the range 0.3–0.9 ng mL^{-1} for the anabolic steroids and 0.1–0.3 ng mL^{-1} for the
249 corticosteroids. The regression lines, obtained using the least-square method,
250 demonstrated a good fit for all analytes, with correlation coefficients always higher than
251 0.9860.
252 Specificity and matrix effect were evaluated for all analytes except the three validated in
253 the surrogate matrix. Blank and spiked samples were analysed and did not show any
254 interferences (signals, peaks, ion traces) in the region of interest, where the target
255 analytes were expected [16]. The matrix effect was less than 4% for all compounds.
256 Specificity and matrix effect were not evaluated for α-boldenone glucuronide, cortisone
257 and cortisol, as the validation of the method for these three steroids was made in water
258 adjusted to pH 8, as stated in Section 2.6.
259 The precision, calculated by applying the one-way analysis of variance (ANOVA), was
260 expressed as coefficient of variability (CV), in terms of intra- and inter-day repeatability.
261 The reported results show that the intra- and inter-day repeatability for all analytes was
262 below 15.8 and 19.9%, respectively. These CVs were lower than 22%, as proposed by
263 Thompson [23]. The high values were probably due to the low concentrations used for the
264 method validation. The levels chosen were, however, addressed to subsequent research
265 on the natural or endogenous origin of conjugated boldenone in bovine bile. The use of
266 these methods for control purposes could consider higher concentration ranges for
267 validation.

The mean recoveries ranged between 94 and 106% for α-boldenone sulphate, 91 and 109% for β-boldenone sulphate, 96 and 104% for α-boldenone glucuronide, 99 and 101% for β-boldenone glucuronide, 94 and 106% for ADD, 98 and 103 % for α-boldenone and 99 and 101% for β-boldenone. The mean recoveries for the corticosteroids ranged between 98 and 102% for prednisolone, 98 and 102% for prednisone, 94 and 106% for dexamethasone, 93 and 107% for cortisone and were about 100% for cortisol.

The data for the anabolic steroids are reported in Table 3 and Table 4 for the corticosteroids. CCα was calculated, as described in SANCO/2004/2726 revision 4 [17], using parallel extrapolation to the *x*-axis at the lowest experimental concentration. CCα and CCβ values are reported in Table 5 and Table 6.

3.2. Application of the methods

In order to verify the developed methods in actual conditions, 20 bile samples, randomly collected from untreated young bulls (14–17 months old) under veterinary control, were subjected to the analysis for the detection of the studied molecules.

All bile samples showed α-boldenone glucuronide residues at a concentration interval from 8.3 to 258.2 ng mL^{-1} (average concentration 68.9 ng mL^{-1}) and evidenced traces of β-boldenone sulphate (concentration < CCα); α-boldenone was found in seven samples at a concentration interval from 0.6 to 1.3 ng mL^{-1} (average concentration 0.9 ng mL^{-1}). Only three samples showed ADD at a concentration interval from 0.7 to 2.3 ng mL^{-1} (average concentration 1.3 ng mL^{-1}). β- boldenone glucuronate, α-boldenone sulphate and β-boldenone were not detected.

Our findings evidenced the presence of boldenone and some phase II metabolites in a matrix without faecal contamination.

Regarding corticosteroids, the concentration values found in all samples ranged from 0.3 to 13.5 ng mL^{-1} for cortisone (average concentration 5.0 ng mL^{-1}) and from 0.3 to 6.8 ng

294 mL^{-1} for cortisol (average concentration 2.3 ng mL^{-1}). Eight samples showed prednisolone
295 at a concentration interval from 0.2 to 0.4 ng mL^{-1} (average concentration 0.3 ng mL^{-1})
296 and six samples evidenced prednisone from 0.2 to 0.3 ng mL^{-1} (average concentration 0.2
297 ng mL^{-1}). Dexamethasone was not detected.
298
299 **4. Conclusion**
300 We presented two LC-MS/MS methods for the analysis of bile samples. The first included
301 17α and 17β-boldenone, their precursor androstadienedione (ADD) as well as their
302 glucuronides and sulphates, whereas the second one reported the detection of
303 prednisolone, prednisone, dexamethasone, cortisone and cortisol. All analytes were
304 extracted with a common and simple immunoaffinity chromatographic procedure. The
305 performance characteristics of the two methods were evaluated in accordance with the
306 criteria of the Commission Decision 2002/657/CE [16].
307 We found α-boldenone glucuronate at high concentrations as well as traces of β-
308 boldenone sulphate, ADD, α-boldenone, prednisolone, prednisone, cortisone and cortisol
309 in bile samples of untreated young bulls.
310 Further studies are ongoing in order to verify whether or not bile is an effective matrix for
311 investigating the endogenous nature of boldenone phase II metabolites to unambiguously
312 discriminate illicit treatments from their natural presence.
313
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317
318 **References**

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383

384 Figure captions

385 Figure 1. Chemical structures of α-boldenone and β-boldenone free and conjugated forms
386 and ADD.

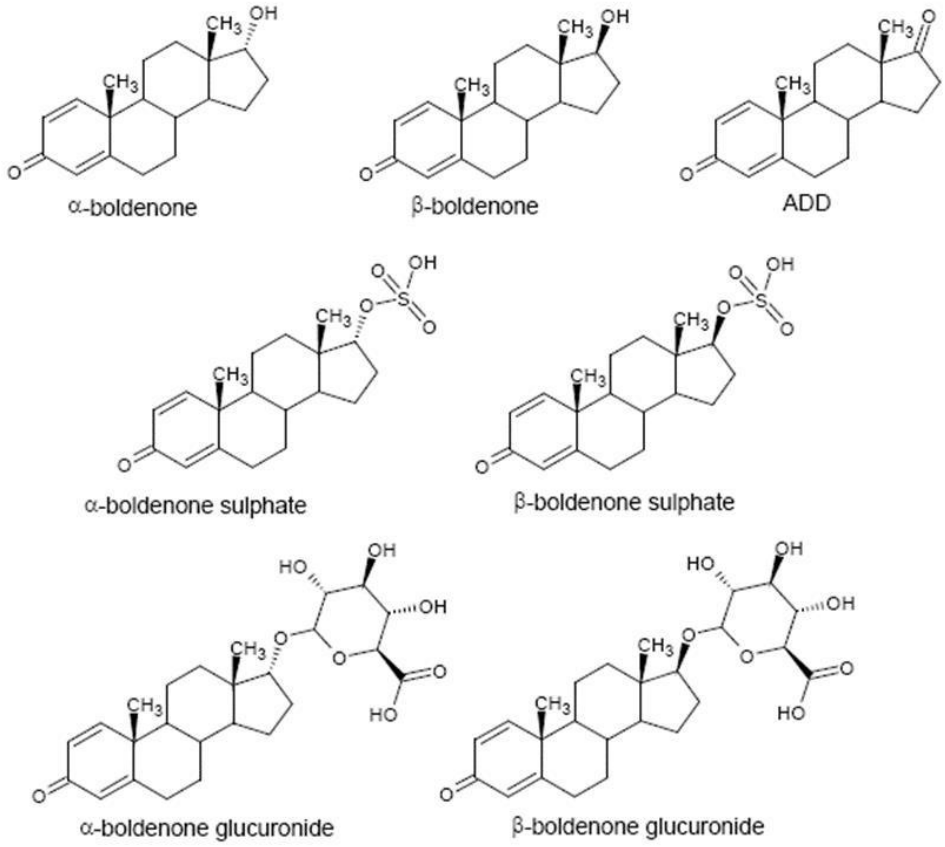
387 Figure 2. Chemical structures of the five studied corticosteroids.

388 Figure 3. Reconstructed LC–MS/MS chromatograms and relative ion spectra of α-
389 boldenone and β-boldenone free and conjugated forms and ADD in a bile sample spiked
390 at the validation lowest concentration level (0.3 ng mL^{-1}). Right-hand side: relative internal
391 standards (concentration = 2 ng mL^{-1}).

392 Figure 4. Reconstructed LC–MS/MS chromatograms and respective ion spectra of the five
393 corticosteroids in a bile sample spiked at the validation lowest concentration level (0.1 ng
394 mL^{-1}). Right-hand side: relative internal standard (concentration = 2 ng mL^{-1}).

395

396 Figure 1



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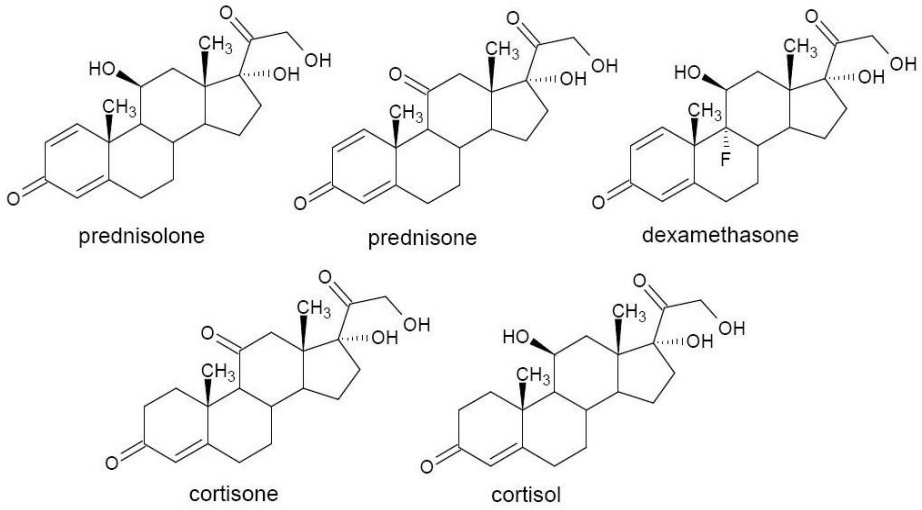
399 Figure 2



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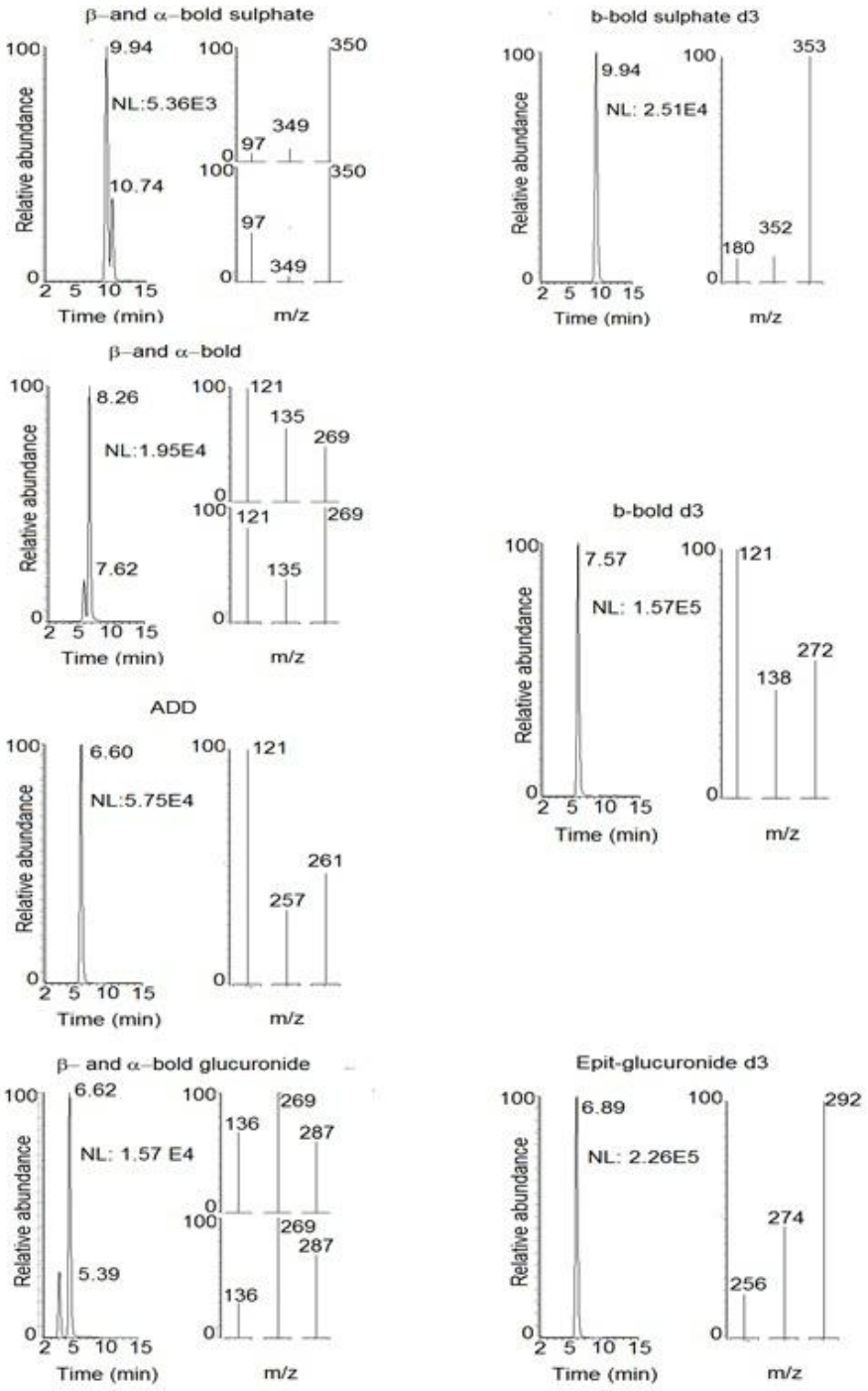
402 Figure 3.



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405 Figure 4.

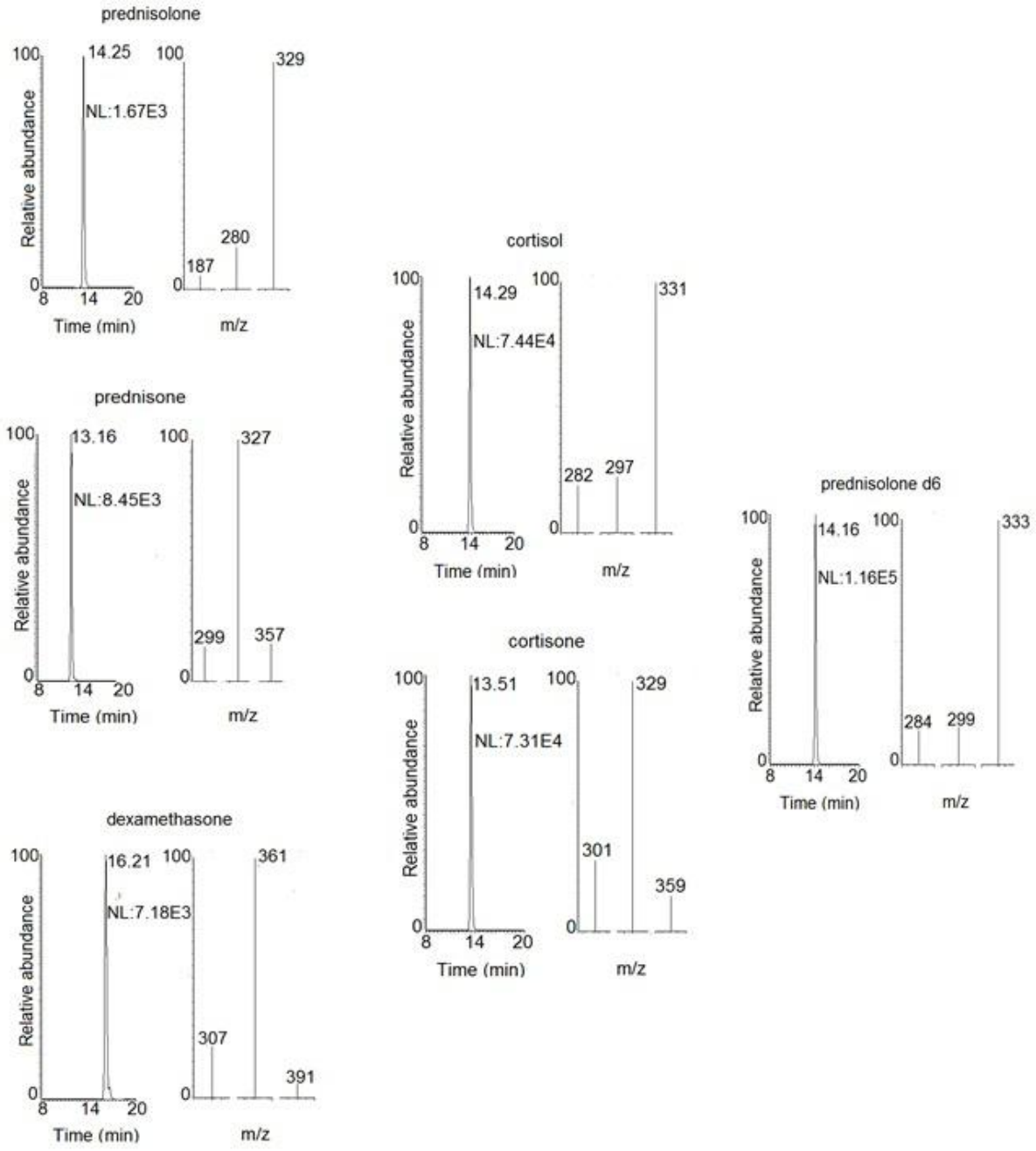

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Table 1. MS/MS conditions for the MRM acquisitions of α-boldenone and β-boldenone free and conjugated forms and ADD, as well as the relative internal standards. Ions for quantification are in boldenone. CE: collision energy, expressed in Volts.

Analyte	Precursor ion $[M-H]^-$ or $[M-H]^+$ (*m/z*)	Product ions$_{CE}$ (*m/z*)	ESI
α-boldenone sulphate	365	177_{39}, 349_{40}, **350_{30}**	(-)
β-boldenone sulphate	365	177_{39}, 349_{40}, **350_{30}**	(-)
β-boldenone sulphate-d3	368	180_{41}, 352_{40}, **353_{31}**	(-)
α-boldenone glucuronide	463	135_{21}, **269_{13}**, 287_{12}	(+)
β-boldenone glucuronide	463	135_{21}, **269_{13}**, 287_{12}	(+)
epitestosterone glucuronide-d3	468	256_{23}, 274_{16}, **292_{11}**	(+)
ADD	285	**121_{22}**, 151_{14}, 267_{11}	(+)
α-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone	287	**121_{23}**, 135_{14}, 269_{10}	(+)
β-boldenone d3	290	**121_{27}**, 138_{14}, 272_{10}	(+)

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415 **Table 2.** MS/MS conditions for the MRM acquisitions of the corticosteroids and internal
416 standards. Ions for quantification are in bold. CE: collision energy, expressed in Volts.

Analyte	Precursor ion $[M-H]^-$ or $[M-H]^+$ (*m/z*)	Product ions$_{CE}$ (*m/z*)	ESI
prednisolone	405	187_{30}, **280_{35}**, 329_{19}	(-)
prednisone	403	**299_{21}**, 327_{19}, 357_{12}	(-)
dexamethasone	437	307_{33}, **361_{20}**, 391_{14}	(-)
cortisone	405	**301_{21}**, 329_{20}, 359_{12}	(-)
cortisol	407	282_{37}, **297_{33}**, 331_{20}	(-)
prednisolone-d6	411	284_{37}, 299_{32}, **333_{19}**	(-)

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Table 3. Method precision for α-boldenone and β-boldenone free and conjugated forms and ADD.

Analyte	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV; *n*=18)
α-boldenone sulphate	0.3	105.8	15.6	17.5
	0.6	94.2	15.8	19.5
	0.9	101.9	14.6	19.8
β-boldenone sulphate	0.3	109.4	15.2	19.9
	0.6	90.5	12.9	16.5
	0.9	103.1	5.0	6.1
α-boldenone glucuronide	0.3	96.3	15.2	9.7
	0.6	103.7	10.1	6.8
	0.9	98.8	11.0	8.8
β-boldenone glucuronide	0.3	99.2	14.4	19.3
	0.6	100.8	15.8	19.2
	0.9	99.7	15.8	16.5
ADD	0.3	106.4	11.2	14.7
	0.6	93.6	10.3	13.9
	0.9	102.1	9.0	9.5
α-boldenonone	0.3	101.9	15.8	18.0
	0.6	98.1	10.5	12.2
	0.9	103.1	15.3	15.5
β-boldenone	0.3	99.1	15.0	19.0
	0.6	100.9	8.1	15.4
	0.9	99.6	10.3	11.2

Table 4. Method precision for the five corticosteroids.

Analyte	Concentration level (ng mL^{-1})	Recovery % (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV; *n*=18)
prednisolone	0.1	102.0	14.7	14.9
	0.2	98.0	12.2	12.4
	0.3	100.1	9.0	9.0
prednisone	0.1	101.9	15.7	19.8
	0.2	98.1	15.0	19.6
	0.3	101.	12.6	13.3
dexamethasone	0.1	93.8	11.0	19.4
	0.2	106.2	12.8	19.4
	0.3	97.0	14.0	15.0
cortisone	0.1	93.0	15.2	11.2
	0.2	107.0	10.7	18.3
	0.3	97.7	7.5	9.0
cortisol	0.1	99.9	15.4	19.4
	0.2	100.1	10.9	15.0
	0.3	100.0	15.0	19.3

Table 5. CCα and CCβ for α-boldenone and β-boldenone free and conjugated forms and ADD.

Analyte	CCα (ng mL^{-1})	CCβ (ng mL^{-1})
α-boldenone sulphate	0.42	0.55
β-boldenone sulphate	0.45	0.58
α-boldenone glucuronide	0.38	0.45
β-boldenone glucuronide	0.44	0.59
ADD	0.40	0.50
α-boldenone	0.43	0.69
β-boldenone	0.44	0.57

433 **Table 6.** CCα and CCβ for the five corticosteroids.

Analyte	CCα (ng mL^{-1})	CCβ (ng mL^{-1})
prednisolone	0.13	0.16
prednisone	0.15	0.21
dexamethasone	0.14	0.19
cortisone	0.15	0.20
cortisol	0.14	0.19

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Figure 1

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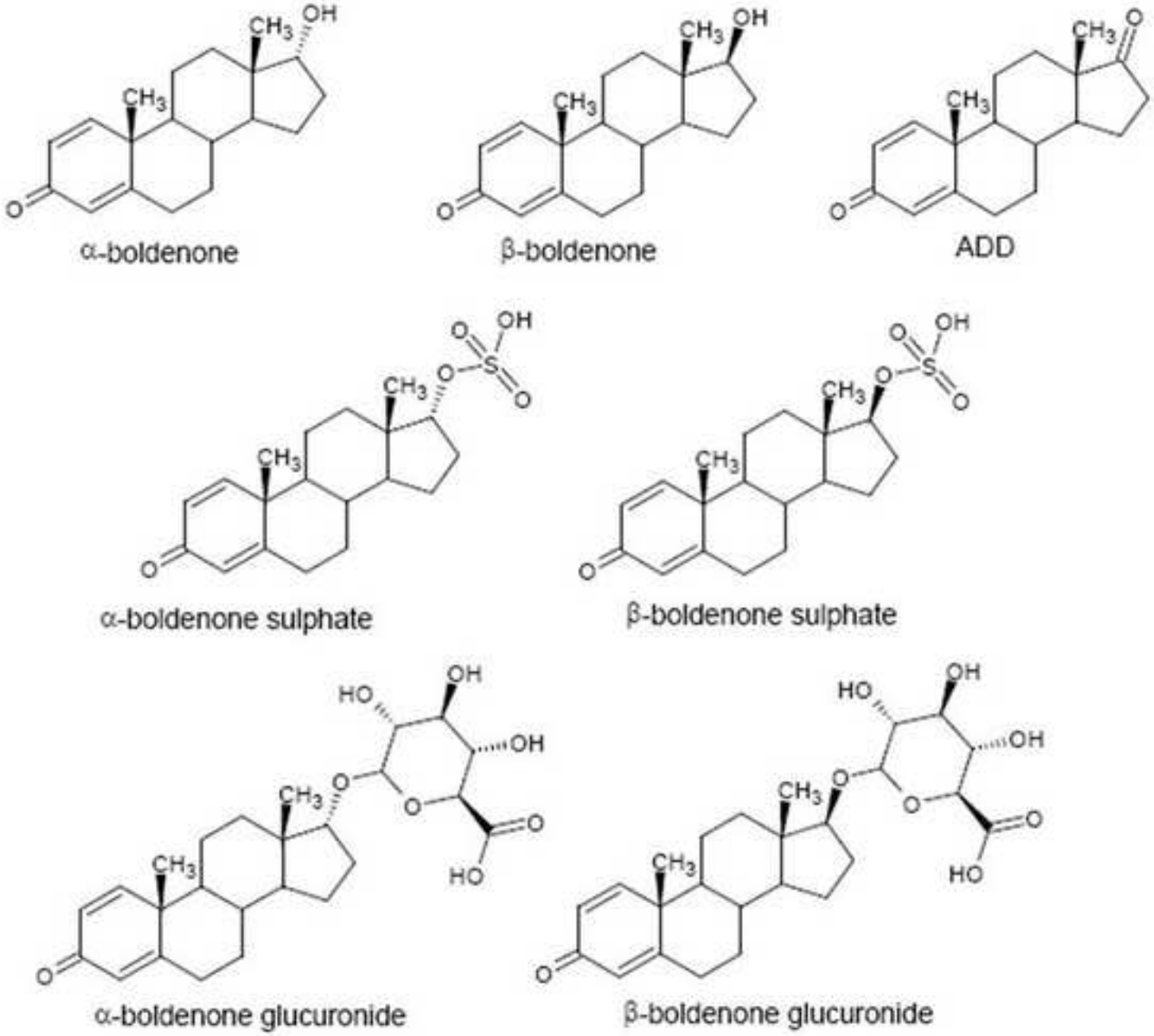

Figure 2

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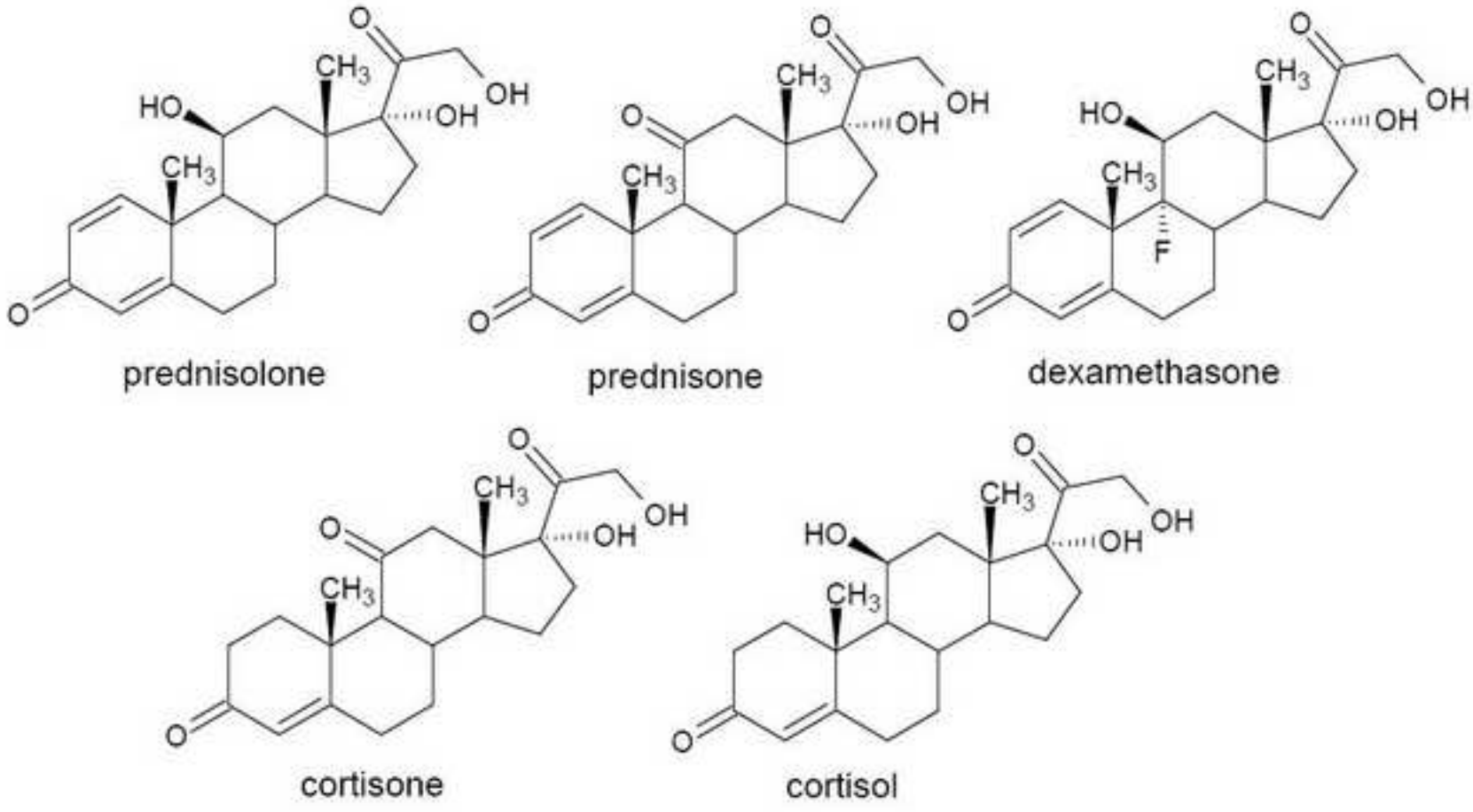

Figure 3

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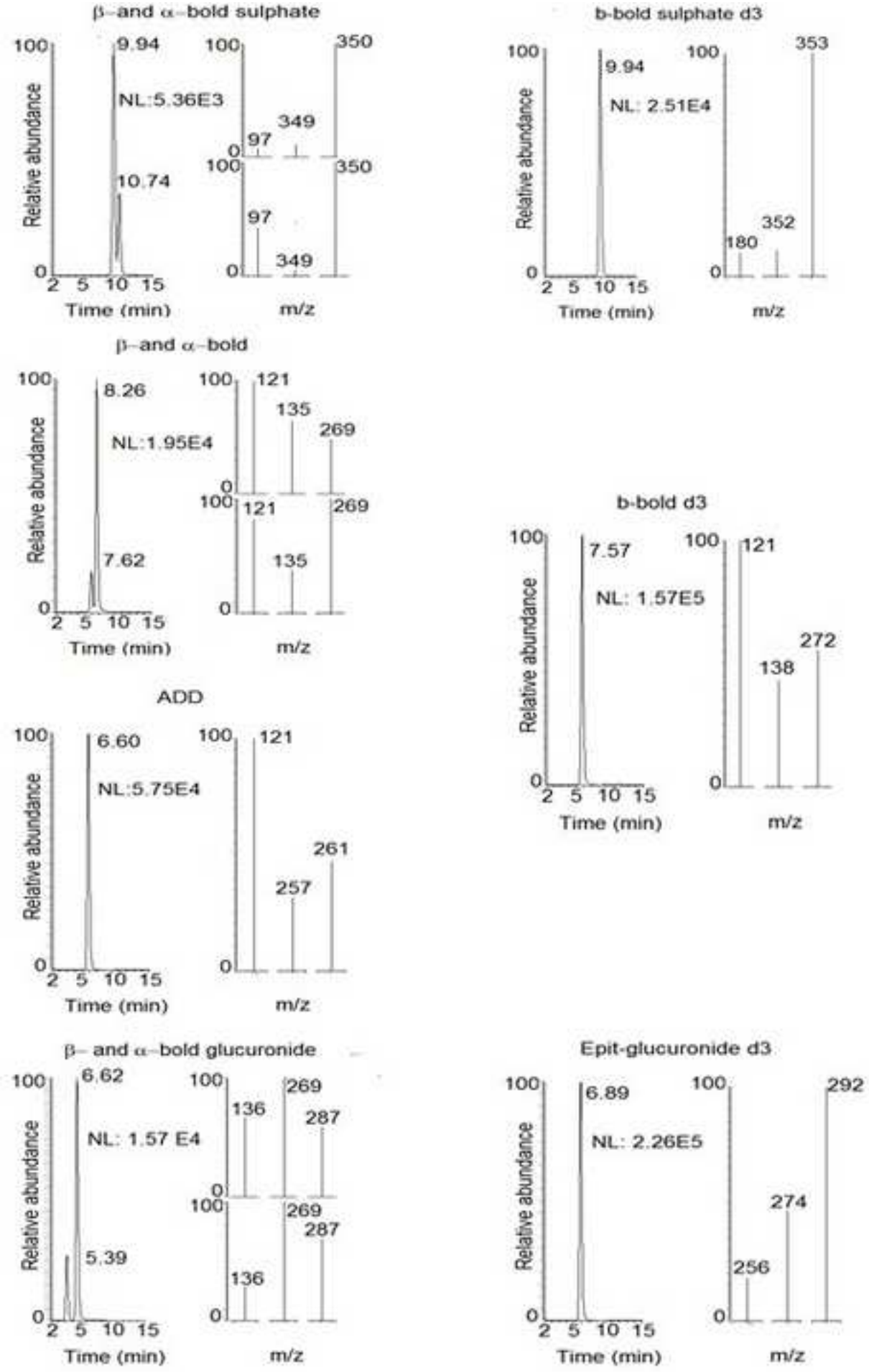

Figure 4

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