Journal of Chromatography B, 877 (2009) 635–644

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Optimization and validation of a liquid chromatography tandem mass spectrometry (LC/MS^n) method for analysis of corticosteroids in bovine liver: Evaluation of Keyhole Limpet β-glucuronidase/sulfatase enzyme extract

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ARTICLE INFO

Article history:
Received 5 September 2008
Accepted 23 January 2009
Available online 29 January 2009

Keywords:
Corticosteroids
Liquid chromatography tandem mass spectrometry
Spectrometry
Bovine liver
Keyhole Limpet
Validation

ABSTRACT

A liquid chromatography tandem mass spectrometry (LC/MS^n) method for the determination of 12 corticosteroids in bovine liver has been optimized and validated in accordance with the European Commission Decision 2002/657/EC. A bovine liver sample was deconjugated with β-glucuronidase/sulfatase enzyme, extracted with diethyl ether and further cleaned up with Solid Phase Extraction (SPE) before analysis with LC/MS^n. Two different enzyme extracts (originating from Helix Pomatia and Keyhole Limpet) and three SPE elution solvents (ethyl acetate, acetonitrile and methanol) were compared during the optimization. Helix Pomatia is generally known as the enzyme most being used for enzymatic hydrolysis purposes. Nevertheless, when detecting corticosteroids in the low μg kg^{-1} concentration range, the Helix Pomatia extract may lead to interferences in the final LC/MS^n chromatogram. When using the Keyhole Limpet enzyme extract, no interferences were observed and therefore, this extract was the best choice for enzymatic hydrolysis tested in this case. Ethyl acetate was used as elution solvent during the validation procedure since SPE elution with acetonitrile resulted in higher chromatographic backgrounds, while elution with methanol showed less reproducible results. Validation of the optimized method was carried out for 10 of the 12 corticosteroids, giving mean recoveries between 91 and 109%, and repeatability and reproducibility coefficients of respectively maximum 13.7 and 18.0%. The working ranges for the linear calibration curves were 5–20 μg kg^{-1} for prednisolone, methylprednisolone and prednisone and 0.5–4 μg kg^{-1} for the other compounds (coefficients of determination $R^2 \geq 0.97$). Specificity, decision limit (CCα) and detection capability (CCβ) were for all compounds within the EC specified limits.

1. Introduction

Corticosteroids belong to the class of the steroid hormones and can be divided into glucocorticoids (e.g. cortisone), which regulate protein and carbohydrate metabolism, and mineralocorticoids (e.g. aldosterone) which control the electrolyte and water balance [1,2] (Fig. 1). Besides naturally occurring corticosteroids, which are secreted by the adrenal cortex, a whole range of chemical synthetic analogs (e.g. dexamethasone and prednisolone) have been developed [3]. These corticosteroids, and in particular the glucocorticoids, are often used in human and veterinary medicine because of their anti-inflammatory and immunosuppressive properties [2,4–6].

In the European Union, the legal utilization of corticosteroids (and other substances having hormonal, thyreostatic or beta-agonistic action) in veterinary medicine is strictly regulated to protect consumers' health [7,8]. To prevent the presence of potentially harmful residues in animal derived products, withdrawal periods between treatment and slaughtering and maximal residue limits (MRLs) have been established for several compounds [9]. For bovine liver, MRLs have been set only for dexamethasone (dxm) (2 μg kg^{-1}), betamethasone (btm) (2 μg kg^{-1}), prednisolone (prolon) (10 μg kg^{-1}) and methylprednisolone (mprolon) (10 μg kg^{-1}) [10]. Unfortunately, corticosteroids are also used illegally as growth promoters increasing weight gain and water retention, improving feed conversion and having synergetic effects with other molecules like beta-agonists or anabolic steroids [2,3,9,11–13]. This (ab)use leads to the production of juicy and lean meat, which is commercially attractive for the cattle farmer [12]. Therefore, analytical methods have been developed to investigate the presence of corticosteroid residues in different edible tissues and in

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doi:10.1016/j.jchromb.2009.01.028

urine using GC/MS [14–17] and more recently LC/MS [12,15,18–24]. The aim of this study was to optimize and validate a liquid chromatography–negative electrospray ionization-tandem mass spectrometry (LC/ESI(-)/MS^n) method for the screening and confirmation of 12 corticosteroids (4 MRL substances and 8 banned substances) in bovine liver samples (Fig. 1). Since corticosteroids are metabolised in vivo to hydrophilic conjugates of glucuronic acid and sulfuric acid (phase II metabolites) [9], the first step in the analytical method was an enzymatic hydrolysis. After deconjugation with a β-glucuronidase/sulfatase enzyme, the sample was extracted with diethyl ether and further cleaned up with Solid Phase Extraction (SPE) before analysis with LC/MS^n. In order to harmonize the performances of the laboratories for prohibited substances in the EU, a minimum required performance limit (MRPL), which is the minimum concentration of an analyte in a sample that has to be detected and confirmed, has been introduced [25]. Since European legislation has not yet set MRPLs for prohibited corticosteroids [26,27] recommended concentrations[2] for the analytical methods have been suggested on a national level for triamcinolone acetonide (trmat) and flumethasone (flm) in liver (both 2 μg kg^{-1}) by the Federal Agency for the Safety of the Food Chain (FAVV-AFSCA). Due to the absence of recommended concentrations and MRPLs the following working levels have been chosen for the other investigated corticosteroids: 2 μg kg^{-1} for triamcinolone (trm), fluorometholone (fml), beclomethasone (bcm), fluocinolone acetonide (fnat) and fluocinolone acetonide acetate (fnatac), and 10 μg kg^{-1} for prednisone (pron) (a compound structurally similar to prednisolone and methylprednisolone). These working levels and recommended concentrations will be called 'MRPL' further in the text, but note that these are not official MRPLs.

2. Experimental

2.1. Reagents and materials

Betamethasone, dexamethasone, flumethasone, prednisone, prednisolone, methylprednisolone, triamcinolone, triamcinolone acetonide, fluorometholone, fluocinolone acetonide acetate, fluocinolone acetonide, beclomethasone and the external standard isoflupredone were purchased from Sigma (St. Louis, MO, USA) and Steraloids (Newport, USA). The internal standard, dexamethasone-d4, was obtained from CDN Isotopes (Quebec, Canada). All solvents and reagents were of HPLC grade. Diethyl ether was purchased from BDH Chemicals LTD (Pool, Dorset, UK), all other solvents were obtained from Merck (Darmstadt, Germany). The enzymatic hydrolysis was performed with β-glucuronidase/sulfatase extracts from Helix Pomatia (Roche, Penzberg, Germany) and Keyhole Limpet (Sigma, St. Louis, MO, USA). The SPE C18 cartridges (500 mg, 6 mL, Bond Elut) were purchased from Varian (Lake Forest, CA, USA).

2.2. Preparation of reagents

Acetate buffer (0.4 M, pH 5.2) was prepared from a 0.4 M sodium acetate solution and a 0.4 M acetic acid solution. Sodium acetate (32 g dry chemical powder) was dissolved in 1 L of ultrapure water. After adding 150 mL of the 0.4 M acetic acid solution, the pH was adjusted to 5.2 ± 0.1 with the 0.4 M acetic acid solution.

Carbonate buffer solution (pH 10.2) was prepared from a 28% (w/v) sodium carbonate decahydrate solution and a 10% (w/v) sodium hydrogen carbonate solution. From the sodium hydrogen carbonate solution, 120 mL was added to 850 mL sodium carbonate decahydrate solution and brought to pH 10.2 ± 0.1 with the 10% sodium hydrogen carbonate solution.

Ortho-phosphoric acid solution (1 M) was prepared by adding 29 mL of ortho-phosphoric acid (85%, w/w) to 500 mL of ultrapure water and stored below 8 °C. All solutions could be used for 3 months.

2.3. Preparation of stock and working standard solutions

Stock (1 g L^{-1}) and working solutions (100 mg L^{-1}) in methanol were prepared for each corticosteroid compound and stored at −18 °C. From the individual working solutions, a standard mixture – containing 9 corticosteroids in a concentration of 1 mg L^{-1} and the 3 other standards (prednisolone, methylprednisolone and prednisone) in a concentration of 5 mg L^{-1} – was prepared in methanol and stored in a refrigerator (<8 °C). A 10-fold dilution of this standard mixture in methanol was used for spiking control liver samples and preparing the LC/MS^n reference standard solution. For the internal and external standards, stock and working solutions of respectively 100 and 1 mg L^{-1} were prepared in methanol and stored below 8 °C.

2.4. Extraction, clean up and optimization

A bovine liver sample (20 g), spiked with 80 μL of a 1 mg L^{-1} dexamethasone-d4 internal standard solution, was buffered with 5 mL acetate buffer pH 5.2 and 45 mL ultrapure water, and homogenized for 1 min. Then, enzymatic hydrolysis was carried out by incubation for 2 h at 60 °C using either Helix Pomatia (10,000 units) or Keyhole Limpet (10,000 units, powder dissolved in 1 mL distilled water) extract. After hydrolysis and cooling down at room temperature, 5 mL distilled water, 100 mL methanol and 10 mL ortho-phosphoric acid (1 M) were added and the sample was shaken (10 min) and centrifuged (15 min, 10,000 rpm, 4 °C). The liquid phase, obtained after centrifugation, was then extracted two times with respectively 100 and 50 mL diethyl ether. The different ether phases were collected, washed with 5 mL carbonate buffer pH 10.2 and 10 mL distilled water, and evaporated until dryness with a rotavapor (40 °C). The residue was redissolved in 800 μL ethanol and 10 mL ultrapure water and applied onto a C18 SPE cartridge, previously activated with 6 mL methanol and 6 mL ultrapure water. After washing with 2 × 1.5 mL ultrapure water and 2 × 1.5 mL methanol/water (20/80, v/v) the cartridge was dried for 2 min, washed with 3 mL hexane, dried again for 2 min before the corticosteroids were eluted with 2 × 3 mL of one of the selected elution solvents (ethyl acetate, methanol or acetonitrile). The extract was further evaporated until dryness under oxygen-free nitrogen at 40 °C, the remaining residue was redissolved in 920 μL mobile phase (ultrapure water/acetonitrile (20/80, v/v) with 0.2% acetic acid) and 80 μL isoflupredone (1 mg L^{-1}) for analysis with LC/MS^n.

2.5. Liquid chromatography

High Performance Liquid Chromatography (HPLC) analyses were carried out on a Surveyor LC pump with an on-line degasser and automatic injector (Thermo Electron Corporation, San Jose, CA, USA). Separations were obtained with a Hypercarb column (2.1 mm × 100 mm, 5 μm, Thermo) with guard column (2.1 mm × 1 mm, 5 μm drop-in guard, Thermo). Elution was performed under isocratic conditions with ultrapure water/acetonitrile (20/80, v/v), acidified with 0.2% acetic acid, at a flow rate of 300 μL min^{-1}. The temperature of the autosampler tray and the column oven were respectively set at 15 and 35 °C. The injection volume was 3 μL and the total runtime 60 min. Due to

[2] Recommended concentrations are the levels that should be detected and confirmed by the current analytical methods in residue control. The CCβ for screening methods or CCα for confirmatory methods should be lower than this level (CRL guidance paper: CRLs view on state of the art analytical methods for national residue control plans, 7 December 2007).

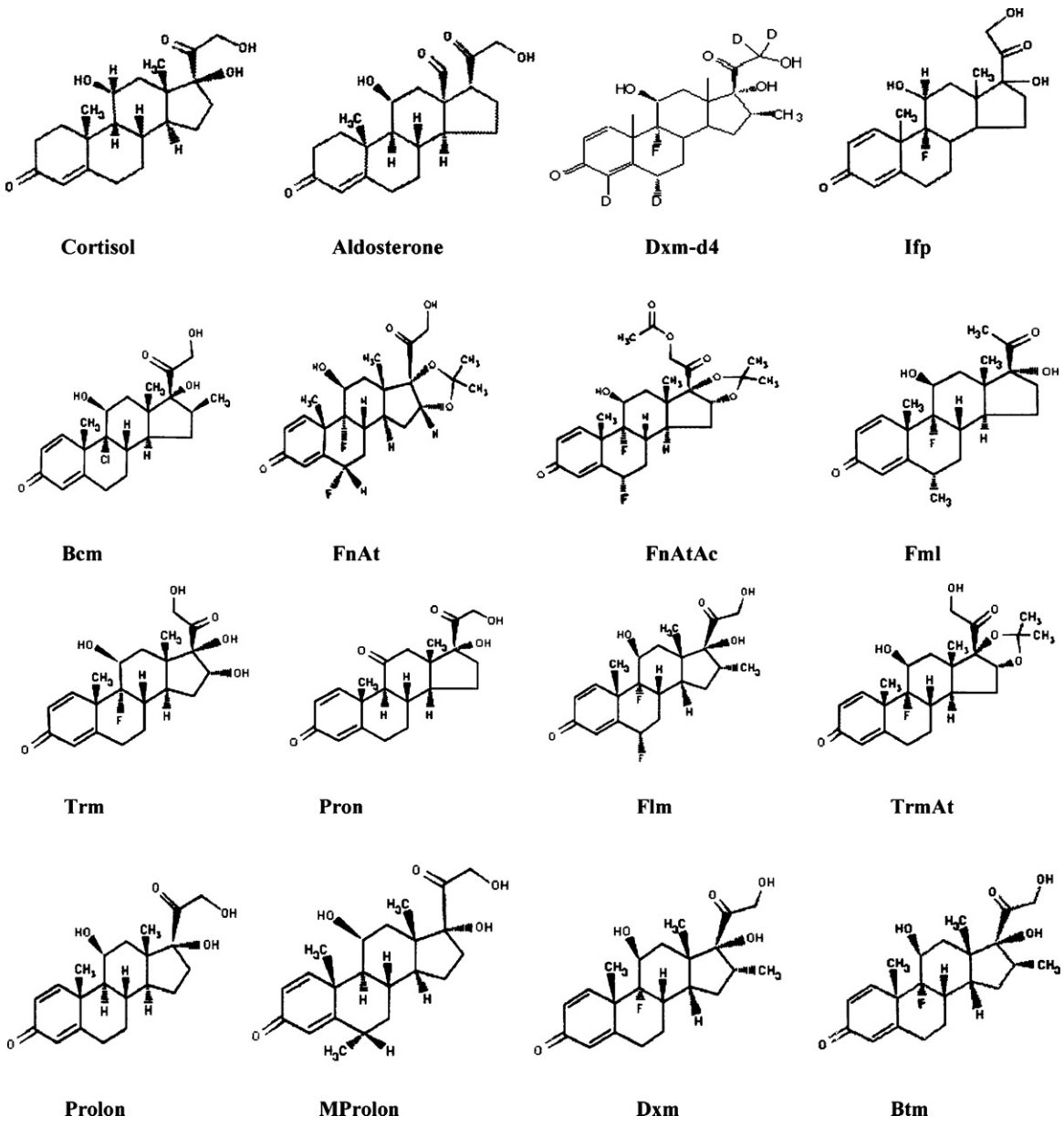

Fig. 1. Structures of cortisol, aldosterone, the internal standards (ifp and dxm-d4) and the twelve analyzed corticosteroids.

Table 1
Retention times, precursor ions, fragment ions and collision energies.

Analyte	Retention time (min)	Precursor ion (*m*/*z*)	Fragment ions (*m*/*z*)	Collision energy (%)
Beclomethasone	32.04	467	377–407	23.0
Fluorometholone	7.75	435	355–375	22.7
Triamcinolone*	8.26	453 and 393 (MS^3)	345–363	18.5 and 18.0
Fluocinolone Acetonide Acetate*	14.35	553	375–493	22.2
Fluocinolone Acetonide	4.02	511	373–393–431–451	23.3
Prednisone	4.79	417	327–357	18.6
Dexamethasone	8.83	451	361–391	21.5
Betamethasone	12.28	451	361–391	21.5
Flumethasone	6.79	469	379–409	22.2
Triamcinolone Acetonide	5.10	493	337–357–375–413–433	24.5
Prednisolone	4.27	419	329–359 (SRM)	22.6
Methylprednisolone	6.90	433	343–373	22.7
Dexamethasone-d4	8.58	455	363–395	21.8
Isoflupredone	6.09	437	347–377	22.7

* Could not be detected in bovine liver samples.

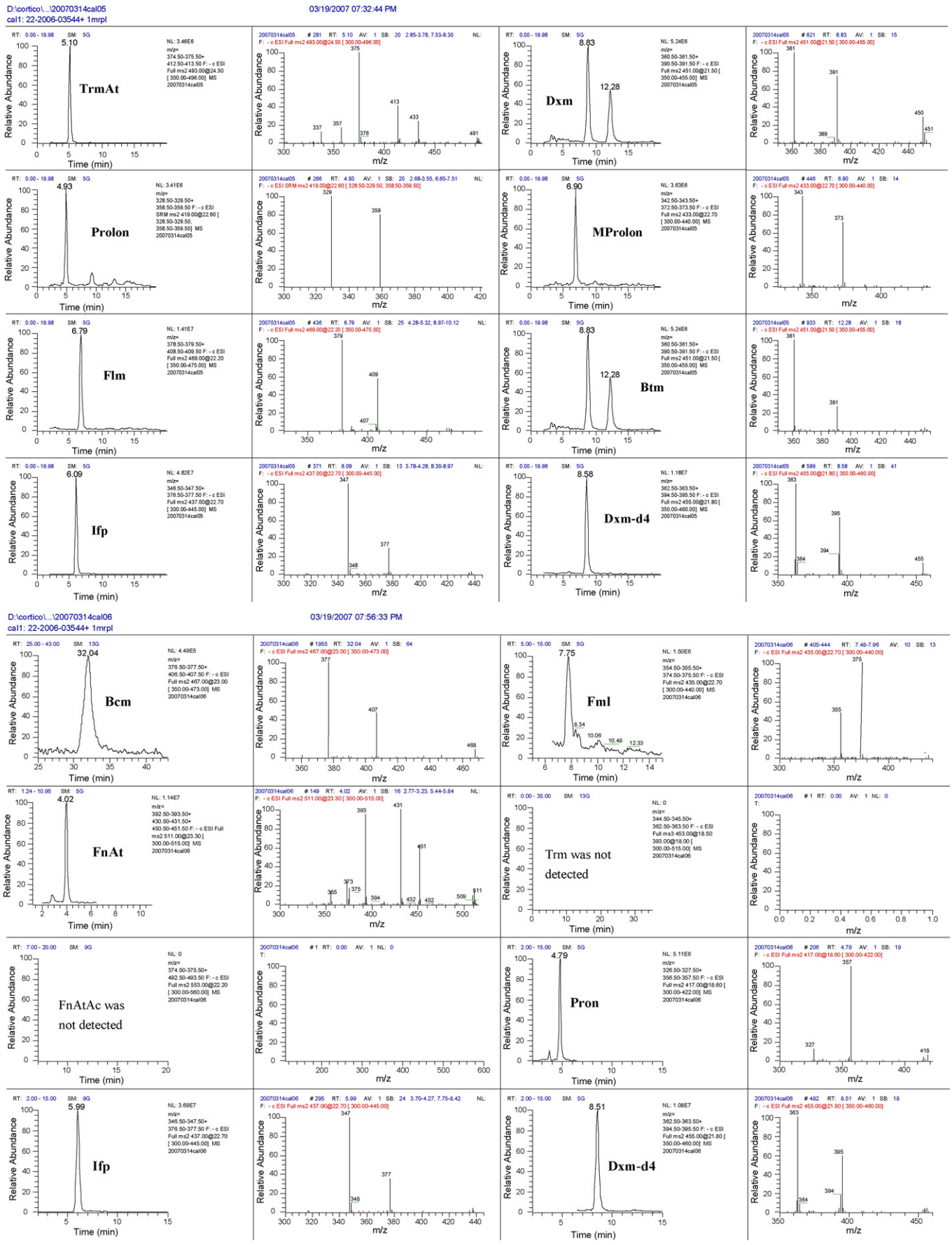

Fig. 2. Chromatograms and mass spectra of a bovine liver sample spiked at 1 MRL or MRPL for (a) compounds analyzed during the first run and (b) compounds analyzed during the second run.

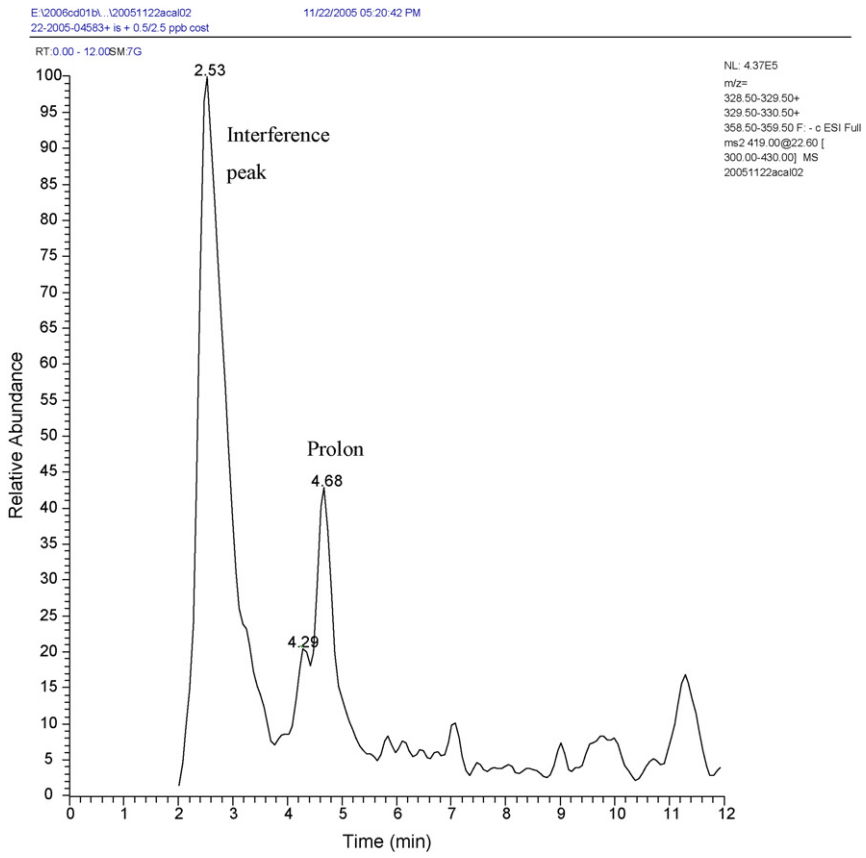

Fig. 3. Extracted ion chromatogram of a liver sample spiked with a low concentration (0.25 MRL) prednisolone; hydrolysis was performed with Helix Pomatia.

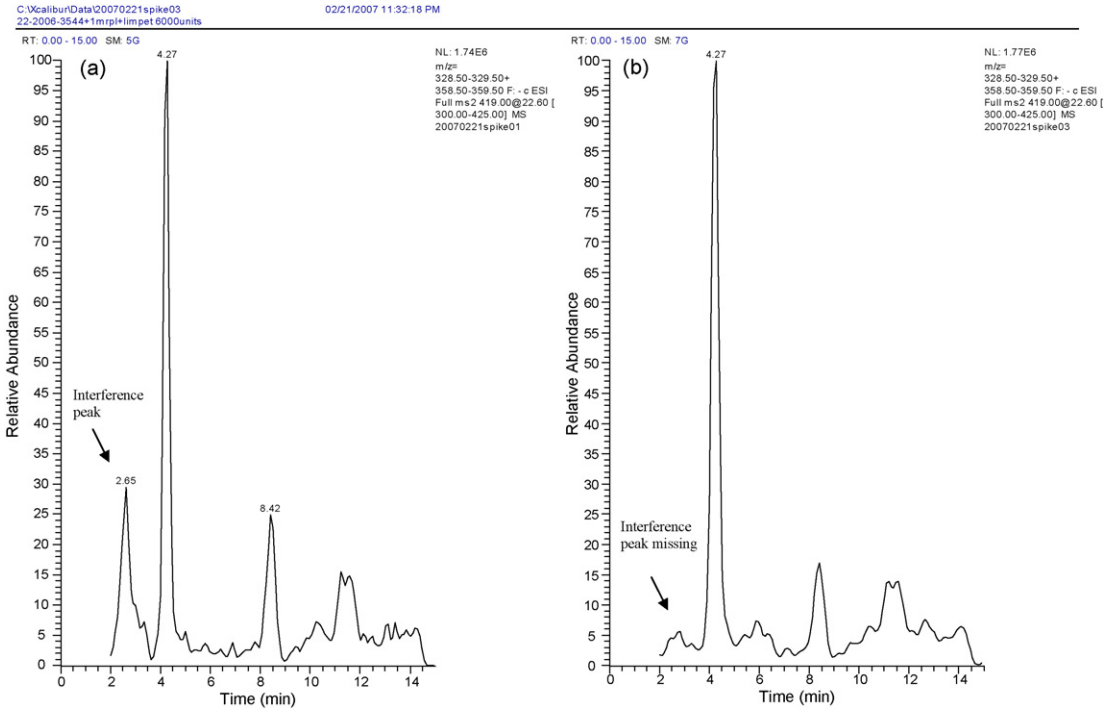

Fig. 4. Comparison of extracted ion chromatograms of prednisolone after hydrolysis of a spiked liver sample (1 MRL) with (a) 10,000 units Helix Pomatia and (b) 12,000 units Keyhole Limpet extract.

overlapping peaks in the chromatograms, the mixture of 12 corticosteroids was analyzed in 2 different runs. Therefore, the sample extract was injected twice and in each run 6 corticosteroids were analyzed. During the first run (23 min) dexamethasone, betamethasone, prednisolone, methylprednisolone, triamcinolone acetonide and flumethasone were analyzed. Triamcinolone, beclomethasone, fluorometholone, fluocinolone acetonide acetate, fluocinolone acetonide and prednisone were detected during a second run (47 min). The internal and external standard, dexamethasone-d4 (dxm-d4) and isoflupredone (ifp), were analyzed in both runs (Fig. 2a and b). Retention times of all compounds are shown in Table 1.

2.6. Mass spectrometry

Data were acquired using a Thermofinnigan LCQ Deca XP ion trap mass spectrometer (San José, CA, USA) operating in negative electrospray ionization mode (ESI). Nitrogen was used as the nebulization and desolvation gas at flow rates of 63 and 720 L h^{-1}, respectively. The capillary temperature was maintained at 350 °C, the potential applied on the capillary was 4.5 kV and the capillary voltage was −8 V. All corticosteroids were analyzed in full scan MS/MS except for triamcinolone and prednisolone. For triamcinolone (full scan) MS^3 was necessary to obtain two fragment ions and for prednisolone selected reaction monitoring (SRM) was used instead of full scan to suppress the high background ion intensities (m/z 327 and 357) from the liver extract. Helium was used as the collision gas at a pressure of 3 bar and the collision energy was adapted for each compound. In Table 1, precursor and fragment ions and collision energies (for MS^2 or MS^3) are shown for all corticosteroids. The isolation width was set at 3.0 Th and an activation Q of 0.250 was applied.

2.7. Validation

The validation was carried out in accordance with the performance criteria described in Commission Decision 2002/657/EC [25]. Recovery, repeatability and reproducibility were determined only for the MRL compounds (dexamethasone, betamethaosone, prednisolone and methylprednisolone); specificity, linearity, decision limit (CCα) and detection capability (CCβ) were determined for all compounds. For the qualitative interpretation, relative retention times (i.e. the ratios of the chromatographic retention time of the analyte and that of the internal standard) and identification points (IP) were calculated for all substances. For substances listed in Group A (substances having anabolic effects and unauthorized substances) of Annex I to Directive 96/23/EC [28], minimum 4 IPs were required, while in case of Group B substances (veterinary drugs and contaminants, i.e. the MRL corticosteroids) a minimum of 3 IPs had to be obtained [28,29].

Calibration curves were obtained by plotting the (area) ratios of the analyte peak areas and the internal standard peak areas against the analyte concentrations, with the results being analyzed by linear regression. Four spiked samples (concentrations 0.5, 1, 1.5 and 2 MRL or MRPL) and one blank liver sample were analyzed within each run. With these calibration curves, the decision limits (CCα) and detection capabilities (CCβ) were calculated (ISO 11843). For banned substances α is set at 1% and β at 5% while for MRL substances both the α and β error are 5%. The calculated CCα was then verified by analysis of a liver sample fortified at CCα level. The signal to noise ratio (S/N) should be equal to 3 for all compounds. Since no certified reference material was available, the trueness was determined by recovery. The recovery for each corticosteroid was calculated by analysis of blank liver samples spiked with a mixture of the above mentioned corticosteroids at 0.5, 1 and 1.5 MRL or MRLP. At least 6 spiked samples at each concentration level were analyzed and the mean recovery was calculated for each concentration. Repeatability and intra-laboratory reproducibility were tested by analysis of 3× 18 spiked blank samples at concentrations of 0.5, 1 and 1.5 MRL or MRPL on three different days. Results were expressed as coefficients of variation (CV). The measurement uncertainty was calculated from the bias and 2 times the intra-laboratory reproducibility. Specificity was checked by analysis of 20 blank liver samples.

2.8. Stability

The stability of the standard mixtures in methanol and in LC/MS^n mobile phase (ultrapure water/acetonitrile (20/80, v/v) with 0.2% acetic acid) containing the 12 corticosteroids was investigated at 2 concentration levels (the standard mixture and a 10-fold dilution). All solutions were kept below 8 °C before analysis. LC/MS^n reference standards (0.5 MRL or MRPL) were prepared and the area ratios, averaged from triplicates, were calculated for each compound. Short and long-term stability was investigated by reanalyzing the mixtures after 1, 4, 6 and 8 weeks. Changes in stability of the analytes were calculated by means of a two-tailed t-test ($\alpha = 0.05$).

The effect of the temperature of the autosampler tray (4 or 15 °C) on the stability of the target analytes was investigated by analyzing the same reference standard at the beginning (first injection) and at the end of the sequence (last injection). At both temperatures, the difference in peak area ratio between the first and the last injection was calculated by means of a two-tailed t-test ($\alpha = 0.05$).

3. Results and discussion

3.1. Hydrolysis optimization

Most routine methods use the β-glucuronidase/arylsulfatase Helix Pomatia enzyme extract for hydrolysis, as it leads to total deconjugation of the steroids [9,12,18,19,22,23,30]. A disadvantage of Helix Pomatia juice for the analysis of corticosteroids in bovine liver is that a significant peak just in front of the prednisolone peak is observed (Figs. 3 and 4a). This peak, which elutes around 2.6 min, can suppress the signal of prednisolone and make identification and quantification of the compound difficult, especially at low concentrations, as can be seen in Fig. 3. In this study, an alternative hydrolysis enzyme, the Keyhole Limpet enzyme extract [30,31], was evaluated on 3 liver and 3 solvent blank samples (both spiked at 1 MRL or MRPL). One liver sample and one solvent blank were spiked with 10,000 units Helix Pomatia (sample and blank 1), while the other samples were deconjugated respectively with 12,000 (sample and blank 2) and 6000 units (sample and blank 3) Keyhole Limpet extract. All deconjugation experiments were performed at pH 5.2 and an incubation temperature of 60 °C for 2 h, since these were the most optimal hydrolysis conditions for steroids [9,12,18,30].

Quantitative interpretation of the results gave no significant difference in absolute area of the chromatogram peaks for the different corticosteroids. However, by deconjugation with Keyhole Limpet enzyme extract no interfering peaks were detected in the chromatogram of prednisolone, both at low and high enzymatic concentration (Fig. 4b). The chromatograms of the other corticosteroids gave similar qualitative results (chromatograms and mass spectra) using either Helix Pomatia or Keyhole Limpet. Since there was also no quantitative or qualitative difference using 6000 or 12,000 units of the enzyme extract, a concentration of 10,000 units – similar to Helix Pomatia – was chosen for further analysis and validation.

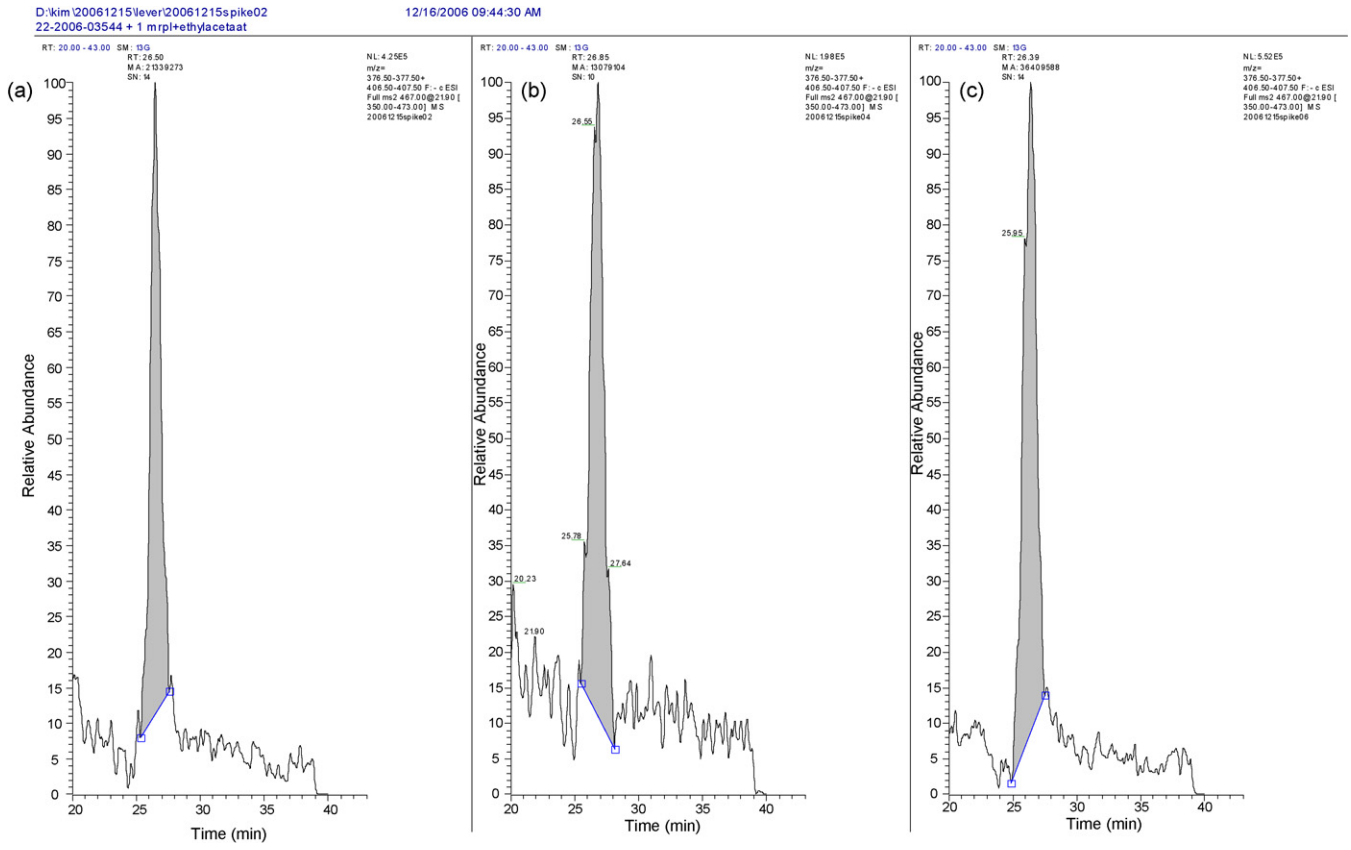

Fig. 5. Extracted ion chromatograms of beclomethasone: SPE elution with (a) ethyl acetate, (b) acetonitrile and (c) methanol.

Table 2
Repeatability for all corticosteroids using different elution solvents.

Compound	Absolute area					
	Ethyl acetate		Acetonitrile		Methanol	
	Spike01	Spike01b	Spike02	Spike02b	Spike03	Spike03b
Dexamethasone-d4	21.7 10^7	23.6 10^7	12.2 10^7	12.3 10^7	23.6 10^7	26.4 10^7
Triamcinolone acetonide	73.1 10^5	84.1 10^5	32.7 10^5	42.9 10^5	77.0 10^5	95.7 10^5
Prednisolone	39.5 10^6	42.7 10^6	22.9 10^6	29.2 10^6	46.2 10^6	64.9 10^6
Betamethasone	62.1 10^6	64.4 10^6	37.8 10^6	35.6 10^6	65.7 10^6	58.2 10^6
Dexamethasone	83.2 10^6	92.8 10^6	46.7 10^6	55.2 10^6	92.6 10^6	91.0 10^6
Methylprednisolone	41.4 10^6	39.8 10^6	22.5 10^6	25.3 10^6	49.3 10^6	47.3 10^6
Flumethasone	17.4 10^7	20.8 10^7	10.4 10^7	13.9 10^7	19.9 10^7	24.0 10^7
Fluocinolone acetonide	21.3 10^7	22.3 10^7	94.9 10^6	22.2 10^7	23.2 10^7	19.6 10^7
Fluorometholone	26.9 10^6	23.2 10^6	16.4 10^6	24.9 10^6	30.9 10^6	21.0 10^6
Prednisone	76.2 10^6	81.6 10^6	35.0 10^6	64.7 10^6	96.9 10^6	63.7 10^6
Beclomethasone	21.5 10^6	20.7 10^6	13.1 10^6	13.9 10^6	35.5 10^6	19.0 10^6

3.2. Optimization of the SPE elution solvents

As a last step in the clean up procedure, the Solid Phase Extraction was applied. Three different elution solvents (acetonitrile, methanol and ethyl acetate) were evaluated on a qualitative and quantitative basis. Therefore, 3 standard solutions, 3 solvent blanks and 3 liver samples, were analyzed. All samples were spiked with 80 ng of the internal standard and a mix of the 12 corticosteroids at a concentration of 1 MRL or MRPL. The three standard solutions were only subjected to SPE, while the liver samples were first hydrolyzed with Keyhole Limpet enzyme and extracted following the procedure described above. Fig. 5 shows the chromatograms of beclomethasone, obtained from 3 spiked liver samples after a final clean up with the 3 different SPE elution solvents.

Elution with acetonitrile gave higher background noise (as illustrated for beclomethasone in Fig. 5b) and lower absolute areas compared to the 2 other elution solvents (Table 2). As a result, elution with acetonitrile gave higher detection limits and a more difficult integration of the chromatogram peaks. Elution with methanol and ethyl acetate resulted in comparable qualitative results, but the latter yielded more reproducible quanti-

Table 3
Maximum permitted tolerances for relative ion intensities (Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJ N° L 221, 17.8.2002).

Relative intensity (% of base peak)	LC–MS^n
>50%	±20%
>20–50%	±25%
>10–20%	±30%
≤10%	±50%

Table 4
Qualitative interpretation (RRT and IP) of bovine liver samples spiked with respectively 0.5 and 1.5 MRPL fluocinolone acetonide.

Reference standard	Precursor ion	Ion m/z 451(%)	Ion m/z 431(%)	Ion m/z 393(%)	Ion m/z 373(%)	RRT
FnAt	511	68.22	100.00	90.54	31.88	0.48
Ion ratio range (lower and upper limit)		54.58 81.86	80.00 120.00	72.43 108.65	23.91 39.85	0.47 0.49
I.P.	1	1.5	1.5	1.5	1.5	

Liver sample	Precursor ion	Ion m/z 451(%)	Ion m/z 431(%)	Ion m/z 393(%)	Ion m/z 373(%)	RRT	IP
FnAt (0.5 mrpl or 1 μg kg^{-1})	511	58.01	93.08	100.00	22.41	0.48	5.5
FnAt (1.5 mrpl or 3 μg kg^{-1})	511	72.31	87.87	100.00	29.67	0.47	7.0

tative LC/MS^n results. Especially for prednisolone, prednisone and beclomethasone less variation in absolute areas was observed using ethyl acetate (Table 2). Therefore, this solvent was selected as SPE elution solvent for the validation.

To detect losses during clean up, all SPE fractions from a standard solution were collected and analyzed by LC/MS^n. In none of the washing steps' fractions corticosteroids could be detected.

3.3. LC/MS^n analysis

LC/MS^n analyses were carried out on standard solutions, spiked solvent blanks and spiked liver samples. All corticosteroids could be detected in the standard solutions, but some compounds were not detected in the spiked solvent blanks or in the spiked liver samples (Fig. 2). Triamcinolone could only be detected in standard solutions and fluocinolone acetonide acetate only in standard solutions and spiked solvent blanks. Stability testing showed that triamcinolone is not stable in methanol and this compound is probably degraded during extraction and clean up. Fluocinolone acetonide acetate was stable in methanol for minimum 8 weeks, but could not be detected in the spiked liver samples. A possible explanation is the inability to extract the component from the liver matrix due to the presence of the acetate group.

For these reasons, validation was carried out only for 10 compounds: dexamethasone, betamethasone, prednisolone, methylprednisolone, flumethasone, triamcinolone acetonide, fluorometholone, beclomethasone, fluocinolone acetonide and prednisone.

3.4. Validation

For all 10 corticosteroids, the relative retention time was within the allowed 2.5% retention time window and a minimum of 4 IPs were obtained. One IP was given for the precursor ion and 1.5 IP for the fragment ions, when their relative ion intensities were within the permitted tolerance levels. These limits were calculated from the relative ion intensities of the reference standard, according to the criteria given in Table 3. The RRT, ion ratios and IPs for fluocinolone acetonide are shown in Table 4 as an example. The calibration curves were linear in the working range of 0.5–2 MRL or MRPL ($R^2 \geq 0.97$). The mean recovery of the MRL compounds was for all concentration levels within the EC specified limits (Table 5) [25], but for prednisolone a total of 5 outliers were found at the different concentration levels. A possible explanation was an interconversion between prednisolone and prednisone during extraction [15]. To investigate this assumption, two spiked liver samples (one spiked with 40 ng prednisolone and one with 40 ng prednisone) and two solvent blanks (similarly spiked) were analyzed. In both liver samples, prednisolone and prednisone were found, while in the solvent blanks only the added compound was detected. From these results it can be concluded that interconversion occurs between the compounds. The coefficients of variation for repeatability (CV_r) and reproducibility (CV_{Rw}) were maximum 13.7 and 18.0%, which is lower than acceptance limits based on the Horwitz equation in the European Decision 2002/657. The measurement uncertainty (MU) was between 12.4 and 43.4%. Table 6 gives an overview of the data obtained at 0.5, 1 and 1.5 MRL. Decision limits and detection capabilities, calculated from the calibration curves, are presented in Table 7.

The calculated decision limits for banned substances were verified by analysis of a spiked liver sample fortified at and below the calculated CCα. Table 8 gives an overview of the spiked concentrations and the signal to noise ratios (S/N) of the secondary

Table 5
Minimum trueness of quantitative methods (Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, OJ N° L 221, 17.8.2002).

Concentration	Range
≤1 μg kg^{-1}	−50% to +20%
>1 μg kg^{-1} to 10 μg kg^{-1}	−30% to +10%
≥10 μg kg^{-1}	−20% to +10%

Table 6
Recovery and precision at 0.5 MRL (a), 1 MRL (b) and 1.5 MRL (c).

Analyte	Mean recovery (%)	CV_r (%)	CV_{Rw} (%)	MU (%)
a				
Dexamethasone	107	6.32	7.59	22.1
Betamethasone	106	7.49	12.4	30.9
Prednisolone	109	11.7	13.0	40.2
Methylprednisolone	103	11.4	13.9	30.4
b				
Dexamethasone	97	7.69	7.69	18.2
Betamethasone	104	4.27	4.27	12.3
Prednisolone	107	13.7	15.9	43.4
Methylprednisolone	105	11.2	11.2	26.9
c				
Dexamethasone	91	5.85	6.10	21.5
Betamethasone	94	7.80	8.86	23.8
Prednisolone	97	7.65	12.5	28.0
Methylprednisolone	99	8.61	18.0	37.9

Table 7
Decision limits and detection capabilities calculated from the calibration curves.

Analyte	CCα (μg kg^{-1})	CCβ (μg kg^{-1})
Dexamethasone	2.32	2.64
Betamethasone	2.34	2.67
Prednisolone	12.6	15.2
Methylprednisolone	12.5	15.0
Triamcinolone Acetonide	0.97	1.65
Flumethasone	0.43	0.74
Beclomethasone	0.66	1.12
Fluocinolone Acetonide	0.47	0.82
Prednisone	8.41	14.5
Fluorometholone	0.74	1.28

Table 8
Signal to noise ratio (S/N) for banned substances.

Analyte	Concentration ($\mu g\,kg^{-1}$)	S/N
Triamcinolone Acetonide	0.25	41
Flumethasone	0.43 (CCα)	14
Beclomethasone	0.66 (CCα)	4
Fluocinolone Acetonide	0.47 (CCα)	12
Prednisone	2.50 (=0.25 MRPL)	33
Fluorometholone	0.74 (CCα)	5

(confirmatory) ions. All substances could be detected with a signal to noise ratio > 3, which implies that the decision limits will be lower than the values indicated in Table 7. The ion ratio criteria were also met for all the S/N values mentioned in Table 8.

The method is also specific since no co-eluting interfering compounds at the appropriate retention times were found in the chromatograms.

3.5. Stability

Stability testing showed that, due to the long analysis time, the temperature of the autosampler tray has a significant influence on the results. With the temperature of the autosampler tray at 15 °C significant differences in peak area ratios between standard solutions analyzed in the beginning and at the end of the run were found ($p = 0.036$), while with the temperature at 4 °C no significant differences were detected ($p = 0.416$). Therefore, it is recommended to change the temperature of the autosampler to 4 °C for longer runs (>24 h).

Stability of the corticosteroids in standard mixtures was dependent on both the solvent and the concentration. All compounds, except triamcinolone, were stable for at least 6 weeks. Most stable compounds were betamethasone, triamcinolone acetonide, fluocinolone acetonide, fluocinolone acetonide acetate and fluorometholone. These compounds were stable for minimum 8 weeks in both methanol and mobile phase, and at concentrations of 1 and 0.1 mg L^{-1}. Also stable for 8 weeks in both solvents were beclomethasone, flumethasone, dexamethasone (1 mg L^{-1}), prednisolone and methylprednisolone (5 mg L^{-1}). In mobile phase, concentrations of prednisolone, methylprednisolone (0.5 mg L^{-1}), prednisone (5 and 0.5 mg L^{-1}), dexamethasone, flumethasone and beclomethasone (0.1 mg L^{-1}) were not significantly altered after 6 weeks. In methanol, beclomethasone, flumethasone (0.1 mg L^{-1}) and prednisone (5 and 0.5 mg L^{-1}) were stable for 6 weeks, while dexamethasone (0.1 mg L^{-1}), prednisolone and methylprednisolone (0.5 mg L^{-1}) could be used for minimum 8 weeks. Triamcinolone working standard solution (100 mg L^{-1}) in methanol showed a decrease in area ratio of 44% after only 2 weeks. For lower concentrations (1 and 0.1 mg L^{-1}) in methanol already after 1 week, a significant alteration was found ($p = 0.0000342$). Standard solutions in mobile phase (1 and 0.1 mg L^{-1}) were stable for minimum 6 weeks. Therefore, it is recommended to prepare the stock, working and standard solutions of triamcinolone in mobile phase. For longer storage, the influence of microbiological activity should be investigated.

4. Conclusion

A multiresidue LC/MS^n method for the analysis of corticosteroids in bovine liver was optimized and validated in accordance with the European Commission Decision 2002/657/EC. Ten out of 12 target corticosteroids could be detected in spiked liver samples. Triamcinolone was not stable in methanol and presumably degraded during extraction, and fluocinolone acetonide acetate could not be extracted from the liver matrix.

Investigations on the enzymatic hydrolysis and the SPE elution showed that the Keyhole Limpet enzyme extract was the best choice for hydrolysis and that ethyl acetate was suitable as elution solvent for Solid Phase Extraction.

A complete validation was carried out for the following corticosteroids: dexamethasone, betamethasone, prednisolone and methylprednisolone (all MRL compounds), and flumethasone, triamcinolone acetonide, fluorometholone, beclomethasone, fluocinolone acetonide and prednisone (all banned glucocorticoids).

Recovery, repeatability and reproducibility (determined only for the MRL compounds), and specificity, linearity, decision limit and detection capability (determined for all compounds) were all within the limits specified in Commission Decision 2002/657/EC. For prednisolone, some outliers were observed during calculation of the recovery due to interconversion with prednisone.

Stability testing showed that the concentration of the corticosteroids, the solvent used and the temperature of the autosampler can have a significant influence on the results. Methanol was preferred as the solvent for storage since there was no risk for microbiological activity during the tested periods. Standard mixtures in methanol, containing all validated corticosteroids at concentrations of 1 and 5 mg L^{-1} or 0.1 and 0.5 mg L^{-1}, could be used for 6 weeks. For longer runs, a lower temperature of the autosampler tray (4 °C instead of 15 °C) was preferred.

Acknowledgements

The work was performed at the Scientific Institute of Public Health in the framework of a master thesis at the Free University of Brussels (VUB). The authors are grateful to the members of the Scientific Institute of Public Health for their technical support.

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