ELSEVIER

Journal of Chromatography B, 757 (2001) 11–19

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Multi-residue extraction–purification procedure for corticosteroids in biological samples for efficient control of their misuse in livestock production

Jean-Philippe Antignac*, Bruno Le Bizec, Fabrice Monteau, Frédéric Poulain, François Andre

Laboratoire National de Référence (LDH/LNR), Ecole Nationale Vétérinaire de Nantes, BP 50707, 44307 Nantes Cedex 3, France

Received 28 March 2000; received in revised form 14 December 2000; accepted 14 December 2000

Abstract

A fast and efficient multi-residue extraction–purification procedure was developed for 12 corticosteroids in biological matrices (hair, urine and meat), in order to control their illegal use as growth promoters in cattle. Detection and identification of the analytes were achieved using a previously described LC–MS–MS method based on negative electrospray ionisation and a triple quadrupole analyser. The presented procedures included acid (hair) or enzymatic (urine and meat) hydrolysis, C_{18} reversed-phase SPE, Na_2CO_3 liquid–liquid clean-up and SiOH normal-phase SPE. The detection limits of the developed methods were between 2.9 and 9.3 pg/mg (ppb) for hair samples and in the 40 – 70 pg/g (ppt) range for the urine or meat samples. The acid hydrolysis used for corticosteroid extraction in hair was optimised using an experimental design and response surface methodology. Achieved performances were linked to a physico–chemical approach based on the corticosteroids specific C_{17} side-chain. This original multi-residue and multi-matrices analytical methodology will be used for further metabolism studies.

Keywords: Corticosteroids; Growth promoter; Electrospray

1. Introduction

1.1. Illegal use of corticosteroids as growth promoters

Because of their anti-inflammatory properties, chemical synthesis of many corticosteroids was investigated, with applications in human and veterinary medicine. Besides this therapeutic use, some zootechnical research demonstrated these compounds' capability to increase weight gain and reduce feed conversion ratio, and they have a synergetic effect when combined with other molecules like β-agonists or anabolic steroids [1–3]. Thus, corticosteroids are illegally used as growth promoters in cattle, administered through livestock food or by injection [4]. Their use as growth promoters has never been allowed in Europe, and maximal residue levels (MRL) have only been established for dexamethasone (0.75 ppb in liver, 0.5 ppb in muscle and 0.3 ppb in milk). Consequently, controls were necessary to survey their

*Corresponding author. Tel.: +33-2-4068-7766; fax: +33-2-4068-7878.

E-mail address: ldhlnr@vet-nantes.fr (J.-P. Antignac).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00626-5

misuse in meat producing animals and to assure the food safety. The few analytical methods for this purpose are generally performed in urine or meat, and more recently in hair. If liver and muscle are important matrices due to the fixed MRL and their incidence for consumers, they can not be used before slaughtering. Urine is a more practicable test material, but drug urinary elimination occurs in the first days or weeks after their administration, preventing long term survey. Hair is currently used in forensic analysis and should be a matrix of choice for long term corticosteroid misuse determination.

1.2. Present analytical state

Because of the low residue levels of corticosteroids or their metabolites in the investigated biological matrices, only a very sensitive and specific detection technique can be used. The present study was based on a previously described liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS) method [5], based on negative electrospray (ESI) ionisation, triple quadrupole analyser and multiple reaction monitoring (MRM) acquisition mode. But before their detection, target analytes have to be isolated from original matrices in order to minimise interfering compounds, to increase signal-to-noise ratio and to enhance specificity. So the objective of this work was to develop a fast and efficient extraction–purification procedure for corticosteroids in bovine hair, urine and meat. Several authors have proposed various methods for the extraction and purification of corticosteroids in urine [6–8], meat [9,10] or plasma [11–13]. When it is employed (about 20% of the studies), the first step is an enzymatic hydrolysis of sulfate and glucuronide conjugated forms. Then, liquid–liquid extraction (LLE) with diethylether or methanol, C_{18} reversed solid-phase extraction (SPE) or liquid–liquid clean-up with NaOH or Na_2CO_3 are commonly used. All these methods present advantages in terms of rapidity but are not very specific. The direct consequence of this is the presence of numerous interfering compounds in the extract that disturb the ion chromatogram interpretation and reduce the signal-to-noise ratio. The generally observed detection limits are in the 0.1–1 ng/g (ppb) range. A HPLC purification stage is sometimes added [14], providing better specificity but is time consuming. Moreover, only few studies were available on specific extraction of corticosteroids in hair [15,16], for which the LOD are in the 30 – 100 pg/mg (ppb) range.

1.3. Present work objectives

The priority of the developed method was its specificity and its applicability to different matrices. Physico–chemical properties of the analytes were then considered, in particular their relative high polarity compared to other steroids due to the side chain in C_{17}. These properties were exploited through purification stages adapted to the analytes rather than to the matrix nature. In a second step, an optimisation of hair acid hydrolysis was conducted through an experimental design and response surface methodology. Finally, our purpose was to develop an homogenous methodology for corticosteroid detection and identification in different biological matrices at low residue levels compared to existing methods (i.e., under 50 ppb in hair and under 0.1 ppb in urine or meat).

2. Experimental

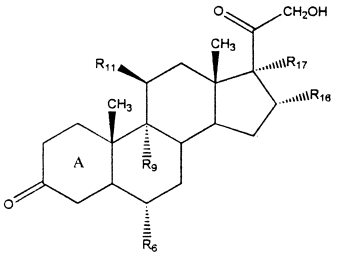
2.1. Reagents

Methanol (analytical and HPLC grade), cyclohexane, diethylether, ethyl acetate, glacial acetic acid and 1 *M* HCl were provided by Solvents Documentation Syntheses (SDS, Peypin, France); sodium acetate and sodium carbonate were purchased by Merck (Darmstadt, Germany) and standard reference corticosteroids (Table 1) were from Sigma (St Louis, MO, USA). Standard solutions were prepared at 1 mg/ml in methanol and working solutions were prepared monthly by ten-fold successive dilutions at concentrations from 100 ng/μl to 1 pg/μl, and were stored at −20°C. A fludrocortisone internal standard solution was prepared at 1 ng/μl in methanol.

2.2. Liquid chromatography

An Alliance 2690 HPLC pump with automatic injector was used (Waters, Milford, MA, USA). Reversed-phase liquid chromatography was per-

Table 1
Structures, molecular weights and diagnostic ions of the investigated corticosteroids



Compound	M_w	A	R_6	R_9	R_{11}	R_{16}	R_{17}	Precursor ion	Fragment ion 1	Fragment ion 2
1. Beclomethasone	408.9	$\Delta^{1,4}$	–	–Cl	–OH	$-CH_3$	–OH	467	377	407
2. Betamethasone	392.5	$\Delta^{1,4}$	–	–F	–OH	$-CH_3$	–OH	451	361	391
3. Cortisol	362.5	Δ^{4}	–	–	–OH	–	–OH	421	331	361
4. Cortisone	360.5	Δ^{4}	–	–	=O	–	–OH	419	329	359
5. Desoxycortisone	346.5	Δ^{4}	–	–	–OH	–	–OH	405	315	345
6. Dexamethasone	392.5	$\Delta^{1,4}$	–	–F	–OH	$-CH_3$	–OH	451	331	361
7. Fludrocortisone (I.S.)	380.5	$\Delta^{1,4}$	–	–F	–OH	–	–OH	439	349	379
8. Flumethasone	410.5	$\Delta^{1,4}$	–F	–F	–OH	$-CH_3$	–OH	469	379	409
9. Methylprednisolone	374.5	$\Delta^{1,4}$	$-CH_3$	–	–OH	–	–OH	433	343	373
10. Prednisolone	360.5	$\Delta^{1,4}$	–	–	–OH	–	–OH	419	329	359
11. Prednisone	358.4	$\Delta^{1,4}$	–	–	=O	–	–OH	417	327	357
12. Triamcinolone	394.4	$\Delta^{1,4}$	–	–F	–OH	–OH	–OH	453	345	393

formed on octadecyl grafted silica Nucleosil C_{18}AB (50×2 mm, 5 μm) stationary phase (Macherey-Nagel, Düren, Germany) with a guard column (Nucleosil C_{18}AB, 10×2 mm, 5 μm). Elution solvents were methanol (A) and 0.5% (v/v) acetic acid in water (B). The mobile phase composition (A:B; v/v) was 40:60 at 0 min, 90:10 at 10 min, and 40:60 from 20 to 30 min. The flow-rate was 220 μl/min and the injected volume was 10 μl.

2.3. Mass spectrometry

Data were acquired in the negative electrospray mode using a QuattroLC® triple quadrupole analyser (Micromass, Manchester, UK). Nitrogen was used as the nebulisation and desolvation gas, at flow-rates of 90 and 600 L/h, respectively. Source and desolvation temperatures were 130 and 400°C. Potentials applied on the capillary (from 3.0 to 4.0 kV) and on the cone (from 15 to 35 V) were optimised to each molecule. MS–MS experiments were performed using argon as the collision gas at a pressure of 4.0×10^{-4} mBar, and the collision energy varied from 2 to 30 V. For each molecule (M), the diagnostics ions were the precursor ion $[M+acetate]^-$ and the two fragments ions $[M-H]^-$ and $[M-H-CH_2O]^-$ (Table 1).

2.4. Extraction–purification

The developed procedure for the extraction–purification of corticosteroids in the three investigated matrices (hair, urine and meat) is represented in Fig. 1.

2.4.1. Hair samples

The different techniques used in the literature for the extraction of pharmaceutics in hair (methanolic sonication, enzymatic, alcaline or acid hydrolysis) were tested. The hair sample used for this study came from a cow treated with dexamethasone and methylprednisolone. The measured response was the signal intensity of the dexamethasone $[M+acetate]^-$>$[M-H-CH_2O]^-$ chromatographic trace

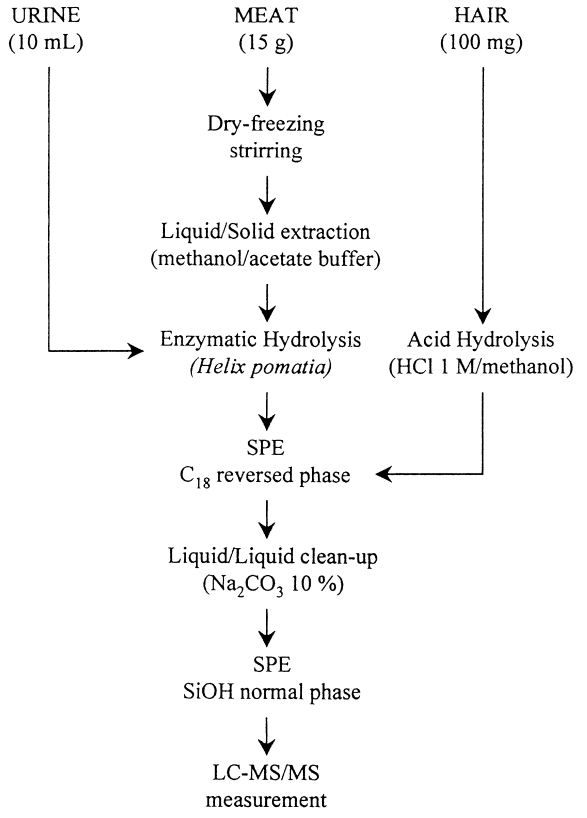


Fig. 1. Schematic representation of the developed extraction–purification procedure for corticosteroids in urine, meat and hair.

(i.e., 451>361). Acid hydrolysis giving the more satisfying results (data not shown), this technique was chosen and the acid hydrolysis conditions were optimised using a central composite experimental design and response surface methodology. Data analysis were realised with Statgraphic® software (Manugistic Inc., Rockville, USA). Fixed parameters were the sample weight (100 mg), the liquid phase nature (methanol–1 *M* HCl) and total volume (5 ml). Parameters to optimise were the volume of HCl (V), the incubation duration (D) and temperature (T). The experimental domain, determined after a rapid preliminary study, is presented in Table 2. For each sample, 50 ng of fludrocortisone are added before hydrolysis as the internal standard. After hydrolysis, supernatant was extracted by C_{18} SPE and purified with Na_2CO_3 liquid–liquid clean-up.

Table 2
Experimental domain of the central composite experimental design used for the hair acid hydrolysis optimisation[a]

Factor	Unit	Level				
		*	−1	0	1	*
V	ml	0.0	1.5	2.5	3.5	5.0
D	h	0.5	3.0	4.0	5.0	7.5
T	°C	30	40	45	50	60

[a] V=HCl volume; D=incubation duration; T=incubation temperature; −1/+1=low/high factor level; 0=central factor level; *=factor level for ''star points''.

2.4.2. Urine and meat samples

A 1-ml aliquot of acetate buffer pH 5.2 and 50 μl of *Helix pomatia* juice (Biosepra, Villeneuve-la-Garenne, France) were added to the bovine urine sample (10 ml). A sample of 50 ng of fludrocortisone was added as the internal standard and enzymatic hydrolysis was carried out for 15 h at 52°C. After centrifugation, supernatant was applied to C_{18} SPE cartridges (2 g of solid-phase, SDS, Peypin, France) previously activated with 5 ml of methanol and 5 ml of water. After washing with 5 ml of water and 5 ml of cyclohexane, analytes were eluted with 5 ml of diethylether. Then, 1 ml of Na_2CO_3 (10%) was added, and after stirring and centrifugation, the organic layer was transferred in a tube. The liquid–liquid cleanup was repeated once. The diethylether was evaporated and the extract was reconstituted in 0.5 ml of cyclohexane–ethylacetate (50:50, v/v), and applied to SiOH SPE cartridges (1 g of solid-phase, SDS, Peypin, France) previously activated with 15 ml of cyclohexane. After washing with 5 ml of cyclohexane–ethylacetate (50:50, v/v), corticosteroids were eluted with 10 ml of ethylacetate–cyclohexane–acetic acid (90:5:5, v/v/v). Purified extracts were evaporated and reconstituted in 50 μl of water–methanol–acetic acid (60:40:0.5, v/v/v).

For the meat samples, 15 g of bovine fresh meat were dry-frozen and pulverised. Aliquots of 12 ml of methanol and 15 ml of acetate buffer, pH 5.2, were added, as well as 50 ng of fludrocortisone as the internal standard, and stirring was performed during 30 min. After centrifugation, 80 μl of *Helix pomatia* juice were added to the supernatant and enzymatic hydrolysis was carried out for 15 h at 52°C. After

centrifugation, about 30% of the volume was evaporated and the extract is purified as with the urine samples, with C_{18} SPE, two Na_2CO_3 liquid–liquid clean-up and SiOH SPE.

3. Results and discussion

3.1. Hair samples

Optimisation of the acid hydrolysis conditions for corticosteroid extraction in hair concerned the volume of HCl (V), the incubation duration (D) and temperature (T). It was conducted using an experimental design and response surface methodology of which only the main results are presented here. More details can be found elsewhere on the experimental design theory [18] or the application for hydrolysis optimisation [19]. The only observed significative effects were quadratics, corresponding to an optimum for each factor, on either side from which the response decrease noticeably. Only 17 assays permitted us to obtain the response surfaces presented in Fig. 2. The maximum amount of extracted dexamethasone was found for 3 ml of HCl and incubation for 4 h at 47°C. Acid hydrolysis resulting in clean samples compared to alkaline hydrolysis (that induced more drastic hair digestion), a C_{18} SPE extraction and a Na_2CO_3 liquid–liquid clean-up were sufficient to remove most interfering compounds. The obtained MRM ion chromatograms

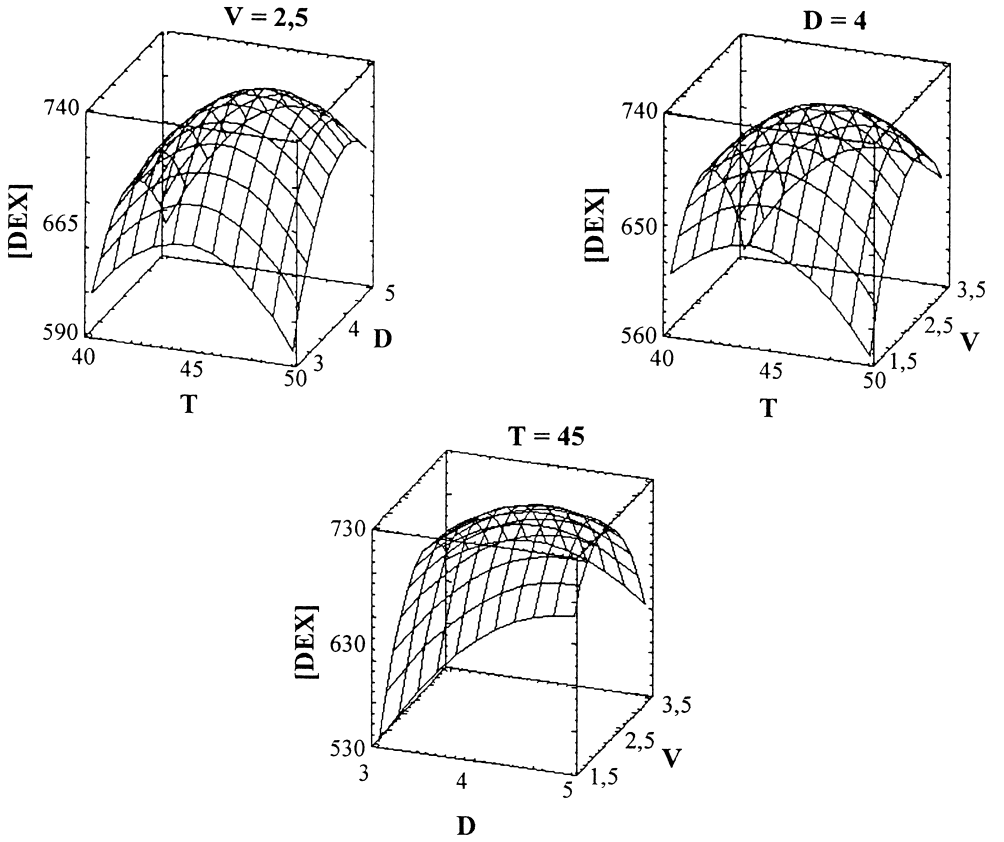


Fig. 2. Responses surface obtained after experimental design data analysis to optimise hair acid hydrolysis ([DEX]: absolute intensity of the 451>361 MRM trace proportional to the extracted dexamethasone amount; V: HCl volume in ml, D: incubation duration in h; T: incubation temperature in°C).

for a blank and a positive bovine hair sample are shown in Fig. 3.

The method validation process consisted, first, of the analysis of 20 blank samples from different matrices, in order to verify the absence of target analytes and potential interferring compounds. Specificity was found very satisfying, particularly for the $[M+acetate]^- > [M-H-CH_2O]^-$ trace, no interferences being detected in the analyte diagnostic chromatograms. Then, a pool of these blank samples was realised, and a calibration curve was built with spiked resulting samples at 5, 10, 15, 20 and 50 pg/mg (ppb). The coefficient of determination (R^2) of the calculated regression curves was 0.9687 for cortexolone and varied from 0.9988 to 0.9995 for other compounds, permitting to validate a good linearity. In a third step, the 20 blank samples are spiked both to 20 and 500 ppb, in order to determine the repeatability for these two concentrations as well as the detection (LOD) and identification limits (LOI). LOD was defined as the minimum concentration inducing one diagnostic MRM trace with $S/N>3$, and LOI as the minimum concentration inducing the two diagnostic MRM traces with $S/N>3$. The observed coefficients of variation for signal intensity varied from 6.8 to 14.4% at 20 ppb and from 6.8 to 16.5% at 500 ppb, except for triamcinolone that gave a higher variability (20.4 and 21.0%, respectively). The intensity ratio between the two MRM traces varied from 2.5 to 6.5%. Achieved detection limits were from 3 to 9 ppb and identification limits from 22 to 96 ppb. Only triamcinolone exhibited higher values (46 and 227 ppb, respectively). In a last step,three spiked samples at 500 ppb were analysed in order to estimate the recovery, with results varying between 31.7 and 66.6%. In conclusion of this validation process, the method performances were found to be very satisfactory, particularly

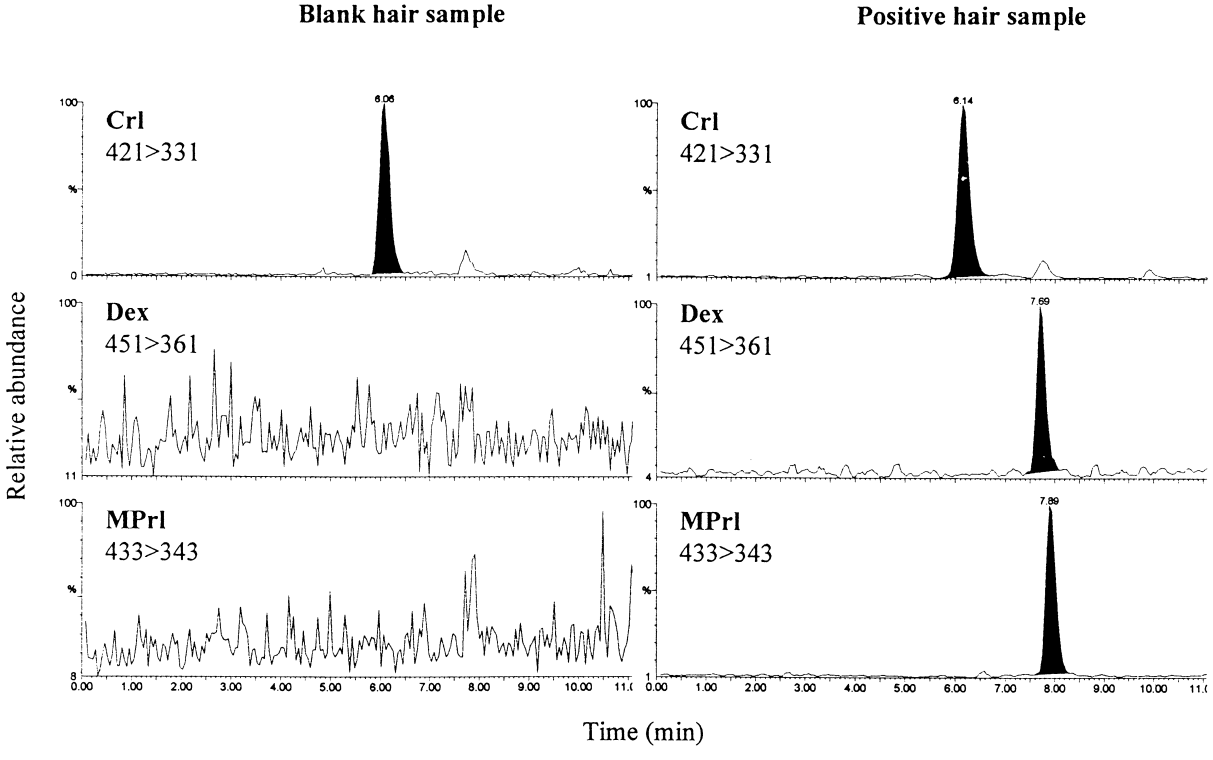


Fig. 3. MRM ion chromatograms of a blank (left) and a positive (right) bovine hair sample after treatment with dexamethasone (Dex) and methylprednisolone (MPrl) (Crl: endogenous cortisol).

in terms of specificity and sensitivity, with detection limits between three and ten times better than the values found in the literature.

3.2. Urine and meat samples

One example of the obtained MRM ion chromatograms for a 0.1 ppb spiked bovine urine sample is presented in Fig. 4. Focusing on corticosteroid physico–chemical properties permitted us to develop an analytical method with a high degree of specificity. Indeed, considering the C_{17} side-chain as the main characteristic of corticosteroids, both the purification procedure and the detection mode were adapted. In a first step, the SiOH SPE purification permitted us to remove a maximum of interfering compounds before eluting analytes with a highly eluotropic solvent, because of the strong interactions between the solid-phase silanol groups and the corticosteroid polar groups in C_{17}, C_{20} and C_{21}. In a second step, the corticosteroid characteristic cleavage in C_{20}–C_{21} in negative ESI with concomitant loss of formaldehyde gave to the $[M–H–CH_2O]^-$ diagnostic ion a high specificity.

Except for the first extraction steps, previously used for steroid residues [17] and consisting of lyophilisation, grinding and liquid–solid extraction with methanol and acetate buffer, the purification procedure for meat samples was identical to the one developed for urine samples. This applicability of a

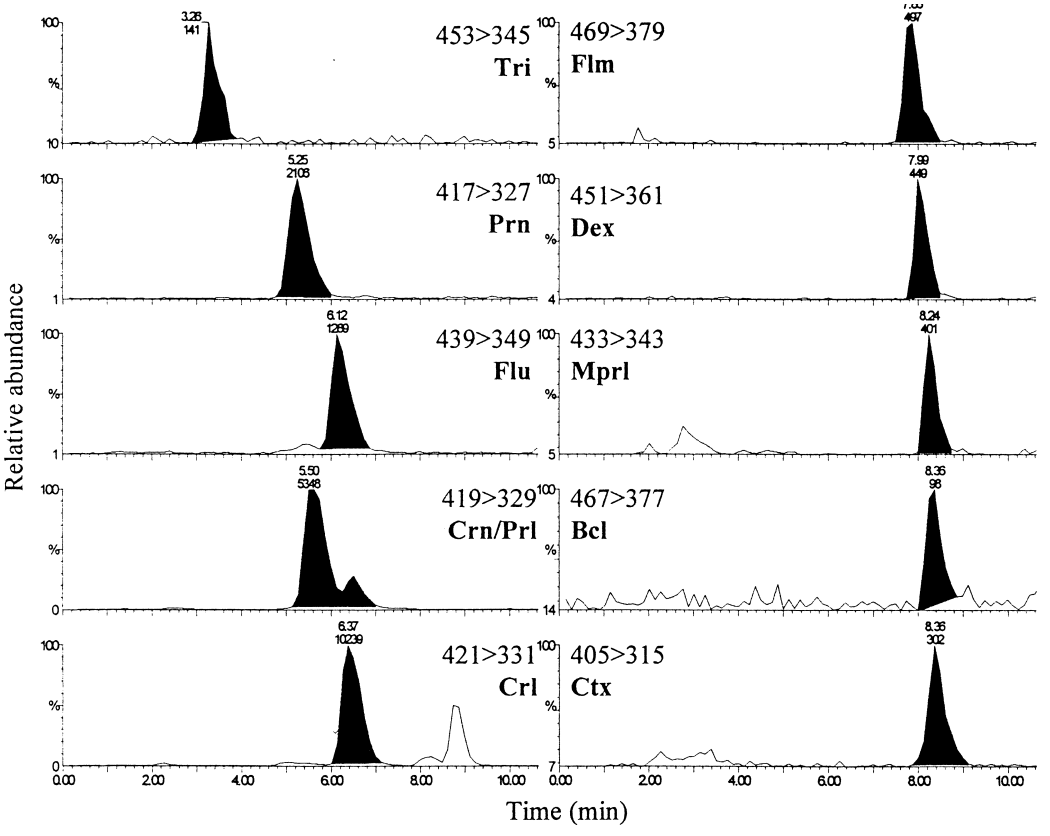


Fig. 4. $[M+acetate]^- > [M–H–CH_2O]^-$ MRM ion chromatograms of a spiked bovine urine sample at 0.1 ppb (0.5 ppb for triamcinolone and endogenous cortisol and cortisone). Tri=Triamcinolone, Prn=prednisone, Flu=fludrocortisone, Crn=cortisone, Prl=prednisolone, Crl=cortisol, Flm=fluméthasone, Dex=dexamethasone, MPrl=methylprednisolone, Bcl=beclomethasone, Ctx=desoxycortisone–cortexolone.

same method for different matrices was permitted by the physico–chemical approach focused on target analyte family characteristics rather than on matrices composition. The two diagnostic MRM traces for dexamethasone for 1-ppb spiked urine and meat samples are presented in Fig. 5, showing the high specificity of the $[M+acetate]^- > [M-H-CH_2O]^-$ diagnostic trace.

The same validation procedure used for hair samples was applied to urine and meat samples. Concentrations of spiked samples were 50, 100, 150, 200 and 500 pg/ml (ppt) for the calibration curve, and 0.2–100 ng/ml (ppb) for repeatability assays. The achieved performances for urine and meat samples were found to be very close, in particular with detection limits in the 40 – 70 pg/g (ppt) range, except for beclomethasone (380 ppt) and triamcinolone (500 ppt).

4. Conclusion

The objective of this work was to develop a fast and efficient extraction–purification procedure for corticosteroid residues in hair, urine and meat samples. The corticosteroid characteristic C_{17} side-chain was exploited both through purification and detection methods. For the first time, an efficient purification was performed by SiOH SPE because of strong interactions between solid-phase silanol groups and the corticosteroid polar groups in C_{17}, C_{20} and C_{21}. For a second time, the corticosteroid cleavage in C_{20}–C_{21} in negative electrospray ionisation mode led to a specific diagnostic fragment ion. Optimisation of hair acid hydrolysis, realised on an incurred sample using an experimental design, permitted us to achieve a very efficient extraction of the analytes. The proposed methods detection limits were between

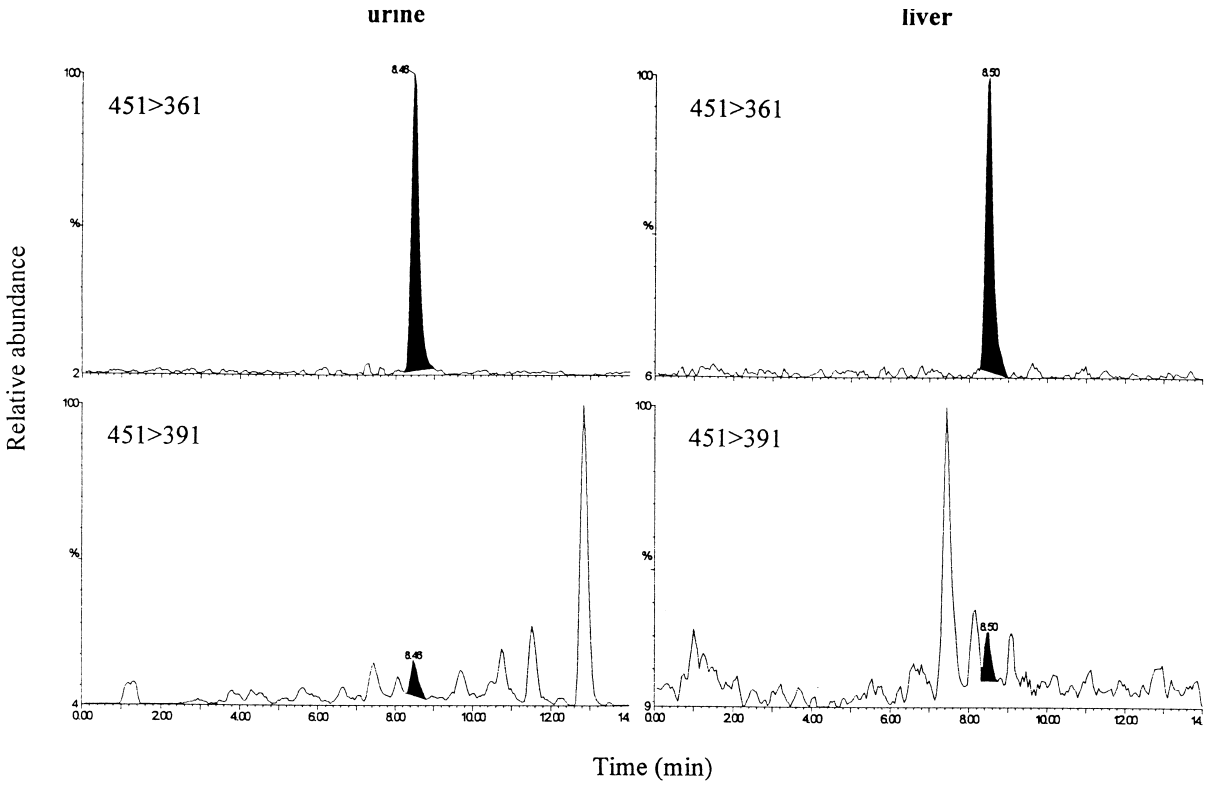


Fig. 5. Dexamethasone diagnostic MRM ion chromatograms for 1-ppb spiked bovine urine (left) and liver (right).

3 and 9 pg/mg (ppb) in hair and were in the 40 – 70 pg/g (ppt) range in urine or meat. This original multi-residue and multi-matrices methodology will be used in the future for metabolism studies of corticosteroids in meat producing animals.

References

[1] L. Istasse, V. De Haans, C. Van Eenaeme, B. Buts, P. Baldwin, M. Gielen, D. Demeyer, J.M. Bienfait, J. Anim. Physiol. An. Nutr. 62 (1989) 150.
[2] M.L.J. Rijckaert, H.P.J. Vlemmix, The Growth Promoting Effect of Glucocorticosteroids, Department of Chemical Engineering, Eindhoven University of Technology, 1992.
[3] J.P. Duchatel, J.M. Beduin, T. Jauniaux, F. Coignoul, H. Vindevogel, Ann. Med. Vet. 137 (1993) 557.
[4] K. De Wash, H. De Brabander, D. Courtheyn, C. Van Peteghem, Analyst 123 (1998) 2415.
[5] J.P. Antignac, B. Le Bizec, F. Monteau, F. Poulain, F. André, Rapid Commun. Mass Spectrom. 14 (2000) 33.
[6] E. Houghton, P. Teale, M.C. Dumasia, J.K. Welby, Biomed. Mass Spectrom. 9 (1982) 459.
[7] S. Rizea Savu, L. Silvestro, A. Haag, F. Sörgel, J. Mass Spectrom. 31 (1996) 1351.
[8] D. Courtheyn, J. Vercammen, M. Logghe, H. Seghers, K. De Wash, H. De Brabander, Analyst 123 (1998) 2409.
[9] L.G. Mc Laughlin, J.D. Henion, J. Chromatogr. 529 (1990) 1.
[10] E.T. Mallinson, J.S. Dreas, R.T. Wilson, A.C. Henry, J. Agric. Food Chem. 46 (1995) 140.
[11] J. Girault, B. Istin, J.B. Fourtillan, Biomed. Environm. Mass Spectrom. 19 (1990) 295.
[12] V.F. Fredline, P.J. Taylor, H.M. Doods, A.G. Johnson, Anal. Biochem. 252 (1997) 308.
[13] A. Ghulam, M. Kouach, A. Racado, A. Boersma, M.C. Vantyghem, G. Briand, J. Chromatogr. B 727 (1999) 227.
[14] D. Courtheyn, J. Vercammen, H. De Brabander, I. Vanderreyt, P. Batjoens, K. Vanoosthuyze, C. Van Peteghem, Analyst 119 (1994) 2557.
[15] V. Cirimele, A. Tracqui, P. Kintz, B. Ludes, J. Anal. Toxicol. 23 (1999) 225.
[16] V. Cirimele, P. Kintz, V. Dumestre, J.P. Goullé, B. Ludes, Forensic Sci. Int. 107 (2000) 381.
[17] P. Marchand, B. Le Bizec, C. Gade, F. Monteau, F. André, J. Chromatogr. A 867 (2000) 219.
[18] J. Goupy, La Méthode des Plans d'Expériences, Dunod, Paris, 1993.
[19] V. Ferchaud, P. Courcoux, B. Le Bizec, F. André, Anal. Chim. Acta, submitted.