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Journal of Chromatography B, 788 (2003) 137–146
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of dexamethasone in urine by gas chromatography with negative chemical ionization mass spectrometry

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Received 3 April 2002; received in revised form 4 December 2002; accepted 19 December 2002

Abstract

Dexamethasone, as some other synthetic corticosteroids, is licensed for therapy in veterinary practice, but its misuse as a growth promotor, often in combination with beta-agonists, is forbidden. In this report an analytical method is described for the detection and confirmation of very low concentrations of dexamethasone in urine. The influence of enzymatic hydrolysis time of samples with glucuronidase was studied. The proposed method consisted of the enzymatic hydrolysis of urine samples, which were then extracted and concentrated using solid-phase cartridges with mixed reversed-phase materials (OASIS). No further clean-up step was found to be necessary. Eluates were derivatized following a previously described method [Analyst 119 (1994) 2557]. Detection, identification and quantification of residues of this compound was carried out by gas chromatography with mass spectrometry in the negative chemical ionization mode. The proposed procedure permits the determination of dexamethasone in urine at levels as low as 0.2 ng ml^{-1}

Keywords: Dexamethasone

1. Introduction

Dexamethasone is a synthetic glucocorticosteroid used in cattle to treat inflammatory diseases. The misuse of dexamethasone in livestock production has been clearly demonstrated, sometimes in combination with clenbuterol in mixtures or cocktails to achieve growth promotion of food producing farm animals, with the aim to reduce meat fat, to increase the appetite of the animals and to increase the efficiency of the use of beta-agonists. The use of these compounds may also have adverse effects, such as degeneration of the thymus in calves [2] and the production of meat of inferior quality. The legal or illegal use of these substances may give rise to the presence of residues in food of animal origin, which has to be controlled.

Their therapeutic use is restricted by means of the establishment of maximum residue limits (MRLs) in muscle, liver, kidney, milk and fat for dexametha-

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1570-0232/03/$ – see front matter
doi:10.1016/S1570-0232(02)01039-5

sone [3], betamethasone [4], prednisolone [5] and methylprednisolone [6], but otherwise no residues may be present, as their use as growth promotors is forbidden. Therefore, sensitive analytical detection and confirmatory methods are needed to control its presence in food producing animals.

To detect or control the possible illegal or unauthorized use of corticosteroids on a farm, tissues with a MRL, such as liver, kidney or muscle, are not available, so target samples of living animals have to be taken, for example urine, blood or faeces. As urine samples are often taken on farms to control for the presence of other banned veterinary drugs such as hormones, this matrix may be the sample of choice for the screening and confirmation of substances such as corticosteroids.

Several researchers have devised methods for the detection of dexamethasone or other glucocorticosteroids in different matrices: thin-layer chromatography [7–9], high-performance liquid chromatography [8,10–13], enzyme immunoassay [14] and GC–MS methods [14–17]. Recently, various authors have described LC–MS and also LC–MS–MS methods [18–24] for the detection and identification of different corticosteroids. Unfortunately, this technique is not always available in laboratories.

A few analytical methods based on GC–MS are available for the determination of corticosteroids in urine. Sensitive methods have been published by Bagnati et al. [15], who used an ''on-line'' immunoaffinity HPLC system as clean up previous to GC–MS detection, and Courtheyn et al. [1], who used a three organic phase solvent extraction, solid-phase clean-up and HPLC fractionation, but these extraction and clean-up procedures are not always commercially available or are rather tedious.

The aim of this work was to develop a rapid and simple analytical method for the determination and confirmation of dexamethasone in urine at low levels by means of gas chromatography with negative chemical ionization mass spectrometry (GC–NCI-MS). Dexamethasone was chosen as representative of the classical groups of synthetic corticosteroids, as it is the most frequently used.

In this paper, a new, rapid extraction, clean-up and quantification procedure for the detection of dexamethasone in urine is presented. Detection is based on the work of Courtheyn et al. [1].

2. Experimental

2.1. Materials and reagents

All solvents used for extraction and clean-up were HPLC grade: methylene chloride (BDH, UK), methanol and acetonitrile (LabScan, Dublin, Ireland), *tert*.-butyl methyl ether and toluene (Merck, Darmstadt, Germany). Pyridinium chlorochromate and the standards were obtained from Sigma (St. Louis, MO, USA): dexamethasone (DEX) (9-fluoro-11β, 17, 21 - trihydroxy-16α-methylpregna-1, 4-diene-3, 20-dione) and methylprednisolone (MP) (6α-methyl - 11β, 17α, 21 - trihydroxy-1,4-pregnadiene-3,20 - dione).

De-mineralized water was prepared with an AlphaQ system (Millipore, Bedford, MA, USA). Solid-phase extraction OASIS HDL (300 mg) and SepPak C_{18} (360 mg) cartridges and an extraction manifold were used (Waters, Milford, MA, USA). Plastic syringes (20 ml) (Monojet, Millipore) were used for the SepPak cartridges. Sodium acetate (analytical-reagent grade) and the β-glucuronidase of *Helix pomatia* were obtained from Merck. Sulphuric acid and glacial acetic acid (analytical grade) were obtained from Merck.

Evaporation of organic solvents was achieved in a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA). Incubations and oxidations of the corticosteroids were carried out in an oven (range between 10 and 90 °C) (Pacisa, Madrid, Spain).

The carrier gas was helium at a flow-rate of 1.0 ml min^{-1} and methane was used as modifying gas (both high-purity grade) (Air Liquide, Madrid, Spain). Urine samples obtained from untreated and treated calves were analyzed in our laboratory and untreated urine samples were used as ''blank'' samples for the spiked calibration curves.

Centrifugations were carried out with a Macrotonic centrifuge (Selecta, Madrid, Spain) and a centrifuge for 10 ml tubes (HermLe Z200A, Wehingen, Germany).

2.2. Instrumental

GC–MS analysis was performed on a gas chromatograph (HP 5890A) automatic injector (HP 61513A) in splitless mode, with a mass spectrometer

(HP 5988A) in NCI mode (Hewlett-Packard). Samples were analyzed using a SPB-5 column (Supelco; 5% diphenyl, 95% dimethylpolysiloxane column, 30 m×0.25 mm).

2.3. Standard solutions, sample preparation and extraction

In this study, methylprednisolone was used as the internal standard, as the method is used for dexamethasone alone, although other corticosteroids may also be analyzed using the proposed method. Stock standard solutions of dexamethasone and methylprednisolone (0.04, 8 and 400 ng μl^{-1}) were prepared in methanol and stored in the dark at ≈4 °C (range 2–10 °C) for a maximum of 12 months for 400 ng μl^{-1}, 1 month for 8 ng μl^{-1} and 2 days for 0.04 ng μl^{-1}.

Different amounts (100, 250, 500 and 1250 μl) of a dexamethasone standard solution of 0.04 ng μl^{-1} and a constant amount (500 μl of 0.04 ng μl^{-1}) of internal standard were added to 10 ml of thawed blank bovine urine for the preparation of the calibration curves. Aliquots of the urine samples were adjusted to pH 4.6–5.0 by the addition of 1 ml 1.0 *M* pH 4.8 acetate buffer and with 20% acetic acid, if necessary. Samples were hydrolyzed by the addition of 30 μl glucuronidase and with overnight incubation in an oven at 37 °C to cleave the glucuronide conjugates. This step was omitted for non-hydrolyzed samples. The optimal hydrolysis time was studied by varying the incubation time from 0 to 24 h. Samples have to be centrifuged prior to the clean-up procedure.

2.4. Sample clean-up procedure

As the objective of this study was the development of a fast and efficient extraction–purification procedure for dexamethasone residues in urine samples, two types of extraction columns were compared for the clean-up of urine samples. Recoveries of dexamethasone standard were studied by applying clean-up procedures based on solid-phase C_{18} cartridges and mixed-phase C_{18} filling (OASIS). Extraction and clean-up of urine samples was carried out by applying the following procedures.

(1) SPE program for the purification step on the C_{18} cartridge. The cartridge was pre-treated with 5 ml methanol and 5 ml demineralized water and the centrifuged urine sample was loaded onto the cartridge with a plastic syringe and washed with 4 ml H_2O. The cartridge was dried under a stream of nitrogen for 30 min, 4 ml of hexane–methylene chloride (4:1) was added and the corticosteroids were eluted with 12 ml hexane–ethyl acetate (1:1). The obtained eluates were evaporated to dryness under a slow stream of nitrogen at 45 °C.

(2) SPE program for the purification step on the OASIS cartridge. The cartridges were placed in the extraction manifold (using vacuum) and pre-treated with 3 ml methanol and 3 ml demineralized water. The centrifuged urine sample was loaded onto the cartridge and washed twice with 3 ml methanol–0.02 *M* NaOH (40:60) and then with 3 ml water. The corticosteroids were eluted with 3 ml methanol. The obtained eluates were evaporated to dryness under a slow stream of nitrogen at 45 °C.

2.5. Oxidation step

Oxidation of the dry extracts was carried out using the procedure described by Courtheyn et al. [1]. The residue obtained after evaporation of the eluate was taken up in 50 μl acetonitrile and 200 μl of an aqueous solution containing 50 mg ml^{-1} pyridiniumchlorochromate and 25 mg ml^{-1} sodium acetate. The mixture of acetonitrile and pyridiniumchlorochromate was thoroughly mixed and heated in hermetically closed vials for 3 h at ≈92 °C. Courtheyn et al. [25] also published a faster and shorter oxidation procedure with stronger oxidation agents, but that method was not used in this study.

After cooling, the oxidized corticosteroids were extracted twice with 3 ml *tert.*-butyl methyl ether–methylene chloride (2:1). The mixture was frozen in an ice–salt mixture and the organic phases decanted. The joint organic extracts were evaporated to dryness in a Turbovap evaporator under a gentle stream of nitrogen at 45 °C and the dry residue was dissolved in 30 μl toluene.

2.6. GC–MS analysis

The use of mass spectrometry after adequate

clean-up of urine samples was examined in order to allow the detection of dexamethasone in urine at the sub-ppb level. The analysis was performed by means of SIM analysis of the oxidized urine extracts in the NCI mode, observing the presence of the m/z 310, 330 and 295 fragments of oxidized dexamethasone. The m/z 312 ion of methylprednisolone, as internal standard, was also measured. Aliquots of the final extracts (2 μl) were injected into the GC–MS system in the splitless mode by means of an automatic injector, the temperature of which was set at 260 °C. The oven temperature was programmed as shown in Table 1.

2.7. Calibration curves

Calibration curves were constructed on different days: blank urine samples (10 ml) were spiked at different levels with dexamethasone standard (0.4, 1.0, 2.0, and 5.0 ng ml^{-1}) and analyzed on different days with the selected extraction and clean-up method. A constant methylprednisolone concentration (2.0 ng ml^{-1}) was added to every sample.

The linearity of the response was evaluated via the coefficient of variation (C.V.) of the response factor, defined as the ratio between the abundance of the m/z 310 ion and the abundance of the m/z 312 ion at different dexamethasone concentrations in ng ml^{-1} (with a constant internal standard concentration of 2 ng ml^{-1}).

The correlation coefficient of the obtained calibration curve, represented by the equation $y = ax + b$, where x is the dexamethasone concentration and y the ratio between the abundance at m/z 310 and the abundance at m/z 312, was calculated from the obtained results and the influence of different urine samples on the values for the slope.

Table 1
Oven temperature program of the GC–MS system

Time (min)	Total time (min)	T (°C)	Ramp (°C min^{-1})
Initial		100	
2	2	100	
9	11	280	20
10	21	280	
2	23	300	10
3	26	300	

2.8. Detection

Detection of the presence of dexamethasone in the samples was carried out by checking for the presence of a peak at the retention time (±0.5%) of the dexamethasone standard. Samples with a chromatographic peak at the same retention time as the dexamethasone standard and with the presence of the diagnostic ions 310, 330 and 295 were submitted to the identification procedure. The detection capability was studied by analyzing 20 different urine samples spiked at the 0.2 ng ml^{-1} level.

2.9. Quantification

Quantification was carried out by relating the ratio between the abundance of m/z 310 (most abundant fragment of dexamethasone) and m/z 312 (most abundant fragment of methylprednisolone) (I.S.) and the daily response factor of the calibration curve. A calibration curve has to be constructed every time for each batch of samples. The concentration of dexamethasone in samples with the presence of a dexamethasone peak was calculated by means of the response factor of the calibration curve obtained on the same day. Samples with an assumed dexamethasone concentration of >0.2 ng ml^{-1} were submitted to the identification procedure.

2.10. Repeatability and reproducibility of the quantification procedure

In order to evaluate the repeatability of the method, the analysis of five replicates at the same level and on the same day was performed. Blank samples were spiked with 1 ng ml^{-1} of dexamethasone and 2 ng ml^{-1} of methylprednisolone as I.S. Repeatability was estimated as the coefficient of variation (C.V.) of the mean response factor.

To evaluate the reproducibility of the method, 10 ml aliquots of urine samples were analyzed on different days and at different levels. The samples were spiked with dexamethasone at 0.4, 1.0, 2.0, and 5.0 ng ml^{-1} and again with methylprednisolone as I.S. at the 2.0 ng ml^{-1} level. The results obtained were evaluated.

2.11. Confirmation: identification and specificity

For confirmation of the identity of the detected peaks, the criteria of European Decision 2002/657/CE have to be fulfilled [26]. Therefore, the repeatability and reproducibility of the ratios between the obtained abundances of the different ions (310, 330 and 295) were determined for the spiked blank samples, at different levels, on the same day and on different days.

For identification purposes, ratio values of unknown samples were compared with the mean ratio value of the spiked samples (of the calibration curve). The maximum permitted tolerances for relative ion intensities between 20 and 50% have to be within ±25%, whereas the tolerances for relative ion intensities of 10–20% have to be ±30% [26].

Blank urine samples were spiked with 0.2 ng ml^{-1} dexamethasone and a constant amount of I.S. (MP) to check the applicability of the proposed method for the detection and identification of this compound at this level.

The analysis of 20 blank samples of urine was performed in order to verify the absence of dexamethasone or the presence of potential interfering compounds.

3. Results and discussion

3.1. Selection of the solid-phase extraction procedure

Two different cartridges (C_{18} and OASIS) were studied for the clean-up of dexamethasone. The OASIS column, with the conditions described in Experimental, was selected, considering its speed, obtained recoveries (about 90% or more with standards) and the reduction of background signals from the matrix in the chromatogram, producing an increase in detection capability.

3.2. Influence of incubation time

Optimization of the incubation time for enzymatic hydrolysis of urine samples with *Helix pomatia* juice was tested. Positive urine samples were used for this study, as they contain glucuronic acid bound dexamethasone. Overnight incubation was found to be best, as can be concluded from Fig. 1, and which was also confirmed by other studies [27].

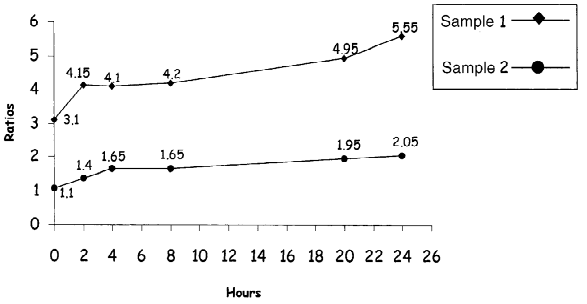

Fig. 1. Influence of incubation time for the enzymatic hydrolysis of dexamethasone-positive urine ($n=4$).

3.3. Detection

Dexamethasone and methylprednisolone standard were oxidized and analyzed using the GC–MS system in the SIM mode. Typical chromatograms obtained for the standards and the different ion fragments for these substances are presented in Fig. 2. As can be seen, dexamethasone is a corticosteroid which produces two isomers, the 16α- and 16β-methyl epimers, which can be separated chromatographically. In this study, only the 16α-isomer was considered.

When analyzing urine samples with the described GC–MS equipment and conditions, a much greater response was found when the same amount of dexamethasone standard alone was analyzed. This matrix effect was studied by analyzing the same amount of dexamethasone (20 ng) in the presence of different amounts of the same urine sample (0, 1, 3, 6 and 10 ml) and submitting these mixtures to the complete analytical procedure. The results obtained in this experiment are presented in Fig. 3 and, as can be seen, the greater the concentration of urine, the greater the response of the ion current, although all samples contained the same amount of dexamethasone (20 ng).

This matrix effect, already described for other substances, for example in pesticides analysis, could have been caused by a loss of the dexamethasone standard alone during the oxidation and extraction step, but may also have its origin in the GC–MS

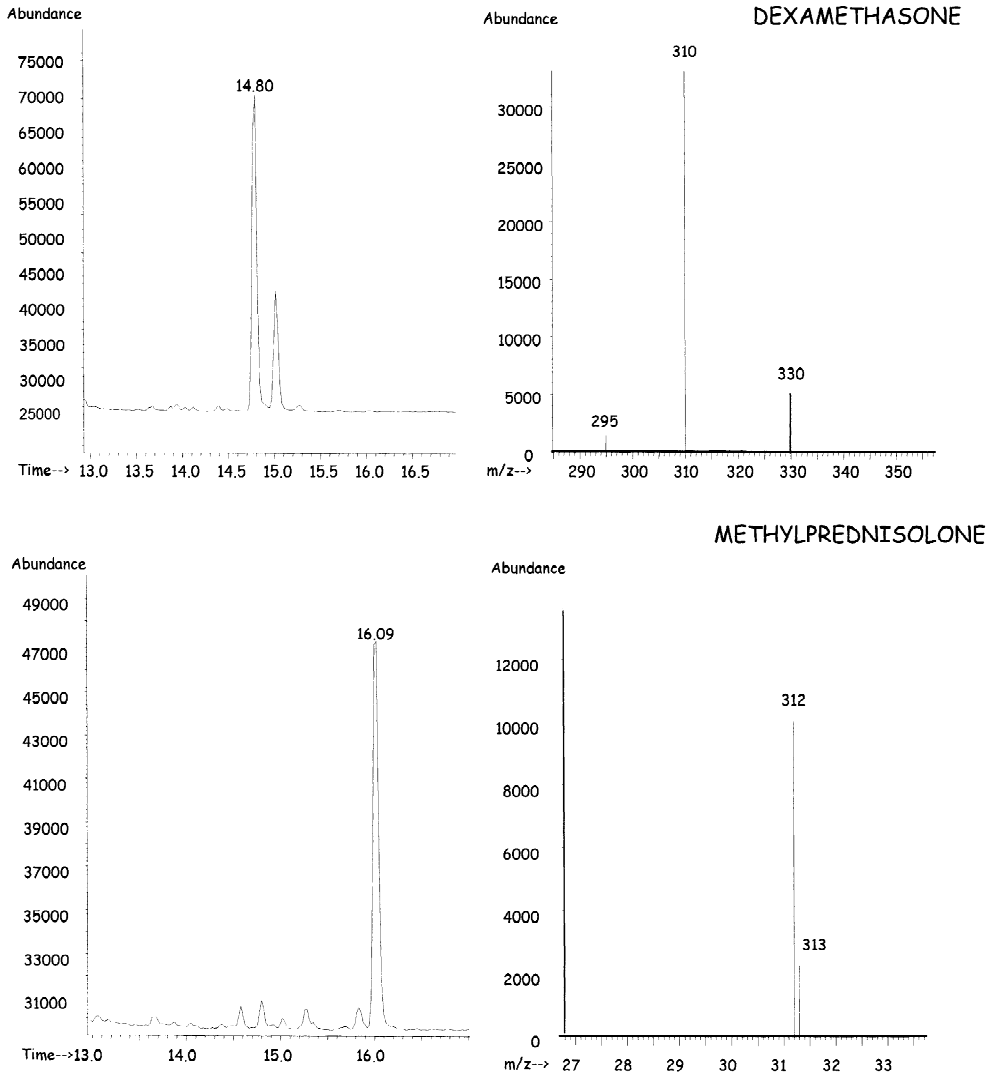

Fig. 2. Typical GC–MS chromatograms of 5 ng dexamethasone and methylprednisolone standard, and their MS spectra.

system itself. To verify which of these possibilities was the cause of the observed matrix effect, the following assay was performed: 0.64 and 1.28 ng of standard dexamethasone alone (B and D) and an extract of 10 ml blank urine sample after the complete extraction and clean-up procedure (A) were derivatized separately and each of them suspended in 30 μl toluene. The extracts were then injected into the GC–MS system with a double volume of a mixture of the standard and urine extract (1 + 1). The results of the obtained abundances are presented in Table 2, where the derivatized urine extract exhibited no response at all, dexamethasone alone had a rather low response, but dexamethasone in the presence of the blank urine extract gave a 10 times higher response.

In conclusion, with the mass detector used, the presence of urine considerably increased the response of dexamethasone. It is worthwhile mentioning that this matrix effect was considerably smaller with a recently aquired GC–MS instrument.

An example of the ion chromatograms obtained at the 0.5 ng ml^{-1} spiked bovine urine level is presented in Fig. 4.

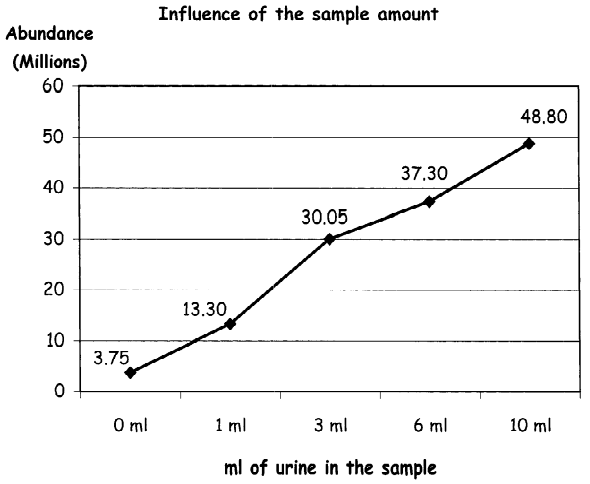

Fig. 3. Influence of the presence of different amounts of urine sample extracts on the monitored abundance of 20 ng injected dexamethasone.

Table 2
Influence of the presence of the derivatized urine extract on the monitored abundances

	Derivatized extract (30 μl)	Injected (μl)	Obtained abundances
A	Blank urine	1	<10
B	0.64 ng DEX standard	1	3532
C	A+B (1+1)	2	43 063
D	1.28 ng DEX standard	1	8811
E	A+D (1+1)	2	90 392

As the response depends not only on the kind of sample but also on the state of the equipment, the presence of an internal standard was required and methylprednisolone (MP) was chosen, although this compound is a corticosteroid used in food producing animals, and, in this case, the quantification of dexamethasone could give lower results.

3.4. Calibration curves, linearity, repeatability and reproducibility

Blank urine samples were spiked with dexamethasone and methylprednisolone to obtain the daily response factor, defined as

$$\frac{\text{abundance 310/abundance 312}}{\text{concentration DEX/concentration MP}}$$

The repeatability (five times, same sample, on the same day, and on the same level, 0.5 ng ml^{-1} DEX) of this response factor was determined and yielded a coefficient of variation of 14.1%. The results are presented in Table 3.

The possible influence of the type of matrix on the response factor was studied in three different intra-day assays: different urines samples (adult and young bovine and pork urine) were spiked at different levels (0.2–1.0 ng ml^{-1} DEX/ng ml^{-1} MP) and analyzed on three different days. As can be seen from the data of Table 3, the relationship between the DEX/MP concentration and abundance ratio

Table 3
Influence of different parameters on the repeatability and reproducibility on the response factor

Repeatability/reproducibility	Mean response factor	C.V. (%)	*n*
Same day, same urine sample (spiked with 0.5 ng ml^{-1})	2.63	14.1	5
Day A, different urine samples (spiked with 0.4–2 ng ml^{-1})	4.29	13.1	5
Day B, different urine samples (spiked with 0.4–2 ng ml^{-1})	1.73	15.5	7
Day C, different urine samples (spiked with 0.4–2 ng ml^{-1})	2.14	13.4	8
Different days, different urine samples (spiked with 0.4–2 ng ml^{-1})	2.52	41.3	5[a]

The response factor was defined as abundance 310/abundance 312 in relation to ng ml^{-1} of dexamethasone/ng ml^{-1} of internal standard (methylprednisolone).

[a] Daily mean values.

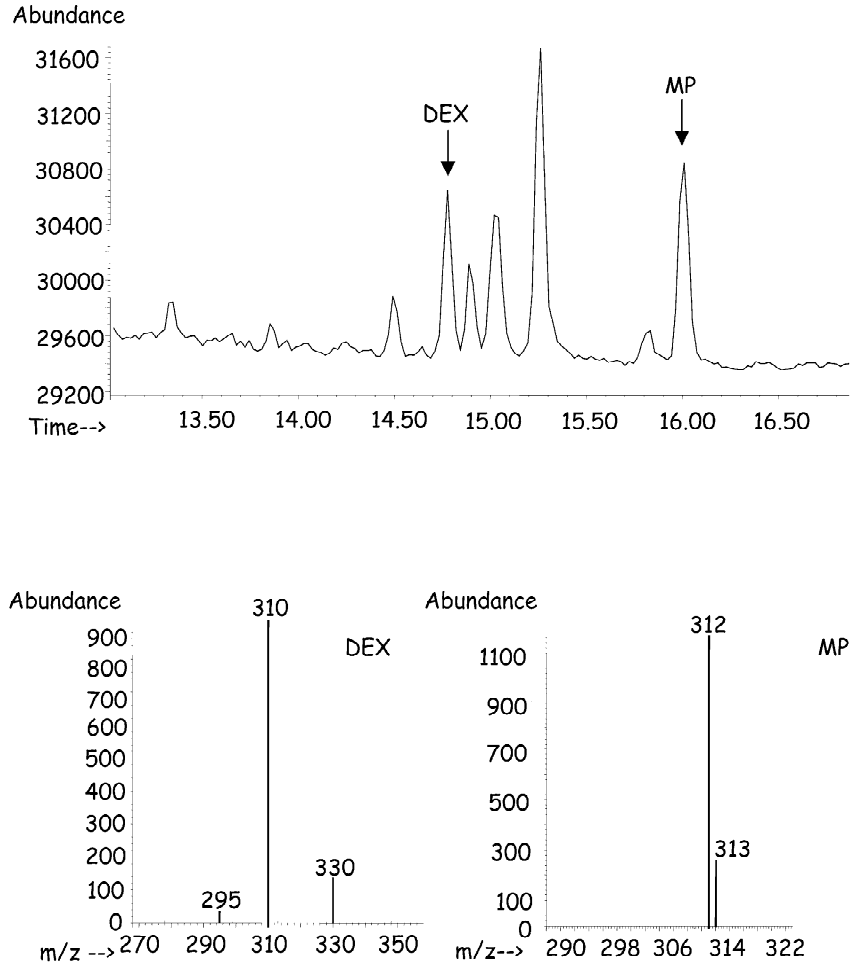

Fig. 4. Typical GC–MS chromatogram of a urine sample spiked with 0.5 ng ml^{-1} dexamethasone and 1 ng ml^{-1} methylprednisolone.

yielded different values, depending on the day and not on the kind of sample: intra-day assays have coefficients of variation of $<16\%$. The between-day variation of the response factor may depend on the state of the instrument.

The mean value of all obtained daily mean response factors yielded a very high coefficient of variation (C.V.) (41.3%). Therefore, for quantitative purposes, the analysis of every batch of unknown samples always has to include three spiked blank urine samples for the intra-day calibration curve, from which the daily mean response factor may be calculated.

The correlation coefficient (r) of the calibration curves (see Section 2.7) was calculated from the results obtained for 20 different urine samples at different concentration levels analyzed on the same day, showing an acceptable correlation:

$$Y = 1.85x + 0.01 \ (r = 0.9926)$$

(x and y, see Section 2.7).

3.5. Quantification and detection limits

As the reproducibility of the response factor over a rather long period (see Table 3) yielded a high coefficient of variation (C.V.), the daily mean factor obtained from the spiked blank urine samples was always used for quantitative purposes. Response

factors may undergo variation, for example after cleaning of the source. Ratios of obtained abundances of m/z 310 and 312 of the unknown samples were used for calculation of the dexamethasone content by means of the daily response factor.

The detection capability was estimated as being 0.2 ng ml^{-1}, as samples spiked at that level could be detected and confirmed. This CCβ (Commission Decision 2002/657/EC) could not be calculated mathematically due to the matrix effect. However, all urine samples spiked at the 0.2 ng ml^{-1} level could be detected and confirmed (CCβ).

3.6. Identification and specificity

Identification of dexamethasone peaks was achieved by comparing the obtained ratio with the corresponding ratios of the spiked samples. Dexamethasone gives three fragments and, therefore, two ratios, yielding three identification points [26], which are sufficient for a compound with a MRL.

The repeatability (intra-day) and reproducibility (inter-day) of the ratios in spiked samples over a long period were calculated and are presented in Table 4. High repeatability and also reproducibility of the ratios were obtained. A maximum permitted tolerance for relative ion intensities of ±25% for ratios between 20 and 50%, and of ±30% for ratios between 10 and 20% (both for GC–CI-MS) is recommended [26]. Nevertheless, as the state of the instrument may influence these ratios, the ratios of assumed dexamethasone peaks always have to be compared with those of spiked blank urine samples in the daily batch of samples.

Specificity was found to be satisfactory, particularly as no interferences were detected in the analyte diagnostic chromatograms. A problem may be posed by the presence of betamethasone, as it has the same structure as dexamethasone, except for the 16β position of the CH_3 group. Both compounds produce two identical oxidation products, the α- and β-epimers, which are well separated in gas chromatography. However, the difference between dexamethasone and betamethasone lies in the relation of the isomers: in the case of dexamethasone, the α-isomer is more abundant than the β-isomer and in betamethasone this relation is reversed. However, this difference between dexamethasone and betamethasone may change from day to day in the same, already injected, vial. The ratios between the abundance of m/z 310 and 330 fragments also yielded different values for the α- and β-epimers: the first has a ratio of about 15% and for the β-epimer a mean ratio of about 1.8% was observed. Similar differences were also found by De Wasch et al. [28], who found different ratios in mixtures of standards (30 and 8%). Studies of the differences between these corticosteroids have also been carried out by other workers [15,29]. Therefore, dexamethasone and betamethasone can be distinguished, but a mixture of both will not fulfill the identification criteria.

Table 4
Repeatability and reproducibility of the ratio of the abundance of dexamethasone fragments during a 3-month period

Day	m/z 295/310		m/z 330/310	
	Mean	C.V. (%)	Mean	C.V. (%)
1	0.0187	4.8	0.1617	2.1
2	0.0180	2.7	0.1570	2.0
3	0.0200	2.5	0.1700	4.4
4	0.0180	5.0	0.1750	4.1
5	0.0195	6.6	0.1730	3.0
6	0.0170	13.5	0.1880	5.3
7	0.0315	7.3	0.1820	2.3

4. Conclusion

In conclusion, the proposed method, which offers a rather rapid and newly developed clean-up of the sample, was found to have acceptable reproducibility, high specificity and sensitivity and a detection capability which allows the detection of the illegal use of dexamethasone in food producing animals by means of equipment (GC–MS) generally available in most routine laboratories involved in the National Residue Plans.

Acknowledgements

The financial support of the fellowship of O. Huetos of the Foundation of Health Research (FIS) and the QLT1-CT-1999-00122, EU project is greatly acknowledged.

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