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Quantitative determination of dexamethasone in bovine milk by liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry

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Abstract

Dexamethasone (DXM) is a synthetic glucocorticoid that is authorized for therapeutic use in veterinary medicine. The European Community (EC) fixed a maximum residue limit (MRL) at 2 ng/g for liver, 0.75 ng/g for muscle and kidney tissues, and 0.3 ng/ml for milk, while its use as growth-promoter is completely banned. The purpose of this study was to develop and validate a simple and reliable method to determine DXM residues in bovine milk. Milk proteins were removed by the addition of concentrated trichloroacetic acid and paper filtration. Solid-phase extraction clean-up on a C18 reversed phase column was performed to obtain an extract suitable for liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Chromatographic separation of DXM and the internal standard desoximetasone, was achieved on a PLRP-S polymeric reversed phase column, using a mixture of 0.1% (v/v) acetic acid in water (mobile phase A) and acetonitrile (mobile phase B) as the mobile phases. They were identified using the MS/MS detection technique, and were subsequently quantified. The method has been validated according to the requirements of the EC at 0.15, 0.30 and 0.60 ng/ml (being half the MRL, the MRL and double the MRL levels fixed by the EC). Calibration graphs were prepared in the 0.15–5 ng/ml range and good linearity was achieved ($r \geq 0.99$ and goodness of fit $\leq$10%). A limit of quantification of 0.15 ng/ml, i.e. half the MRL, was obtained. The limit of detection was 41 pg/ml. The decision limit (CCα) and detection capability (CCβ) were 0.48 and 0.76 ng/ml, respectively. The within-day and between-day precisions, expressed as R.S.D. values, were all below the maximum allowed R.S.D. values calculated according to the Horwitz equation. The results for accuracy fell within the −50 to +20% range. Recovery was 56%. The method was used for the quantitative determination of DXM residues in milk after intravenous administration of DXM to lactating cows to determine its depletion kinetics.

Keyword: Dexamethasone

1. Introduction

Dexamethasone (DXM; 9α-fluoro-11β,17α,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione) is a synthetic glucocorticoid derived from hydroxycortisone that has found widespread application in human and veterinary medicine. It is used in the treatment of metabolic diseases in ruminants, e.g. ketosis, and of inflammatory diseases in a number of animal species [1]. It is available as the free alcohol or in the form of different esters (phosphate, isonicotinate and phenylpropionate), and is usually administered either intramuscularly or intravenously at doses ranging from 20 to 60 μg DXM/kg body weight to horses, cattle and pigs [1]. They are also illicitly used for their growth-promoting effect [2]. To protect consumer's health, the latter use has been banned by the European Community (EC) [3], while maximum residue limits (MRLs) in milk and edible tissues have been established when DXM is used for its therapeutic indications only [4]. They are fixed at 0.3 μg/kg for bovine milk, 0.75 μg/kg for muscle and kidney, and 2 μg/kg for liver of bovine, porcine and equidae.

To determine DXM residues in milk or tissues for the purpose of depletion studies, the EC demands a validated method for the quantitative determination of the target compound in the different matrices [5]. The major challenge in the analysis of DXM residues in milk and tissue samples

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is of course that such a method has to be able to identify and quantify DXM at levels at least as low as half the MRL levels. Gas chromatography–mass spectrometry (GC–MS) methods with good sensitivity and selectivity exist at this purpose [6,7]. However, most of the corticosteroids are thermally labile and have a too low volatility for direct GC analysis, requiring derivatization of the analytes, which complicates considerably sample preparation. Until now, the papers presenting liquid chromatography–ultraviolet or mass spectrometry (LC–UV or MS) methods able to quantify DXM, or more generally glucocorticoids, in biological matrices, are not that numerous (see Table 1 for an extended overview). Some considerable effort has been dedicated to the confirmation of synthetic glucocorticoids in bovine or human urine in order to elucidate its misuse as a growth-promoting agent in livestock or its abuse as a doping agent in sports, with typical limit of detection (LOD) values around 0.2 ng/ml and limit of quantification (LOQ) values of 1 ng/ml [15–23]. Recently, also some papers have been published dealing with the analysis of glucocorticoids in animal tissue matrices [8–14], with some of them capable of quantifying DXM levels as low as 1 ng/g (MRL/2) in liver tissue. No report exists, to our knowledge, of a method capable of quantifying the even lower needed LOQs (0.375 ng/g, MRL/2) in muscle and kidney tissues, and in milk (0.15 ng/ml, MRL/2).

In this paper, we present the validation of an LC–atmospheric pressure chemical ionization (APCI)–tandem mass spectrometric (MS/MS) method using a minimal and simple sample preparation procedure that is able to quantify in a reliable manner DXM at 0.15 ng/ml in milk, being equal to half the MRL, which is the minimum requisite of the EC to be fulfilled. Further on, the use of the method to determine the depletion kinetics of DXM in milk, after intravenous administration to bovine, will be shown, since such data in the literature are very scarce.

2. Experimental

2.1. Standards and chemicals

Dexamethasone and desoximetasone (DSM)—used as the internal standard (IS)—were purchased from Sigma (Bornem, Belgium) (see Fig. 1 for their structure). Stock solutions of 1000 μg/ml of both components were prepared in methanol. Working solutions of DXM and DSM were prepared by appropriate dilution of the stock solutions with methanol. The working solutions were stored in a refrigerator at 0–8 °C and were stable for 1 month. Thereafter, they were replaced by fresh solutions. The stability of the stock solutions, stored at −20 °C, was not tested, but as a precaution the fresh working solutions were prepared using fresh stock solutions.

The solvents used for the mobile phase, water and acetonitrile (Acros, Geel, Belgium) were of HPLC grade. Acetic acid (Merck, Darmstadt, Germany), used as an additive to the mobile phase, was of analytical grade. All other chemicals used were also of analytical grade: hexane (Merck), hydrochloric acid (HCl) (Merck), sodium acetate (Vel, Leuven, Belgium) and trichloroacetic acid (TCA) (Merck). *Helix pomatia* juice, containing minimum 100,000 units of β-glucuronidase/ml and minimum 7500 units of sulfatase/ml, was purchased from Sigma.

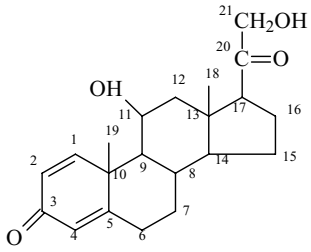

Fig. 1. General structure of synthetic glucocorticoids for DXM and DSM: –H at C6, –F at C9, –CH_3 at C16, and –OH (DXM) and –H (DSM) at C17.

2.2. Biological samples

Known DXM-free milk samples were obtained from lactating cows, which did not receive any medication. Incurred milk samples were obtained from 25 healthy, non-pregnant lactating cows (period between calving up to 3 months after calving, before next conception), which received an intravenous injection of a commercial preparation of DXM (as its sodium phosphate ester) at a dose of 40 μg/kg body weight, just after the morning milking at day 1. Milk samples were taken at the evening milking of day 1, and further at each morning and evening milking of days 2–5, and frozen at −20 °C until analysis. The respective daily milk productions were 9.9 ± 2.1, 8.7 ± 2.2, 9.7 ± 2.6, 10.9 ± 2.6 and 11.1 ± 2.3 kg.

2.3. Clean-up procedure

Ten milliliters of milk were transferred into a 16 cm × 1.2 cm glass tube and spiked with 50 μl of a working solution of 10 μg/ml of the IS (50 ng/ml). Samples used for the preparation of calibration curves were also spiked with appropriate volumes ranging from 30 to 60 μl of the working solutions of DXM. After vortex mixing for 15 s, 1 ml of a 20% (w/v) TCA solution was added. The tube was homogenized on a vortex mixer, and subsequently centrifuged at 4000 rpm for 10 min. The supernatant was filtered through a Whatman filter paper and set aside for further solid-phase extraction clean-up. A C18 column (500 mg/10 ml, Varian, Middelburg, The Netherlands) was installed on a vacuum-manifold and preconditioned with, respectively, 5 ml of methanol and 5 ml of water. The milk extract was allowed to pass slowly (±0.5 ml/min) through the C18 column. The column was washed with 5 ml of water and 5 ml of hexane (±1 ml/min). After drying, the analytes were eluted with 3 ml of methanol.

Table 1
Overview of LC methods capable of quantifying DXM residues in biological samples

Corticosteroids	Matrix	Species	Sample preparation	LC column	Mobile phase	Detection	Recovery (%)	LOD	LOQ	Reference
Tissues										
DXM	Liver, muscle	Bovine	Deconjugation *H. pomatia*, 0.04 M NaAc (pH 4.5), LLE ACN, hexane, DCM, coupled column LC (Spherisorb phenyl 50 mm × 4.6 mm + silica 12.5 mm × 4 mm)	Spherisorb cyano-propyl 100 mm × 4.6 mm, 3 μm	H_2O/CH_3COOH/2-propanol/hexane (0.1/0.1/5.8/94), 1.5 ml/min	UV 239 nm	Liver: 66–68%; muscle: 72–75%	Liver: 6 ng/g; muscle: 4 ng/g	>10 ng/g	[8]
DXM	Liver, kidney, muscle, fat	Bovine	LE 1 M NaOH, LLE ethyl acetate, ACN, hexane, SPE C18	Novapak C18 150 mm × 3.9 mm, 4 μm	H_2O/ACN/TEA (72/28/0.02), 1 ml/min	UV 254 nm	80%	10 ng/g	100 ng/g	[9]
DXM, BM	Liver	Bovine	LLE ASE, hexane, hexane/ethyl acetate (1/1)	Kingsorb C18 250 mm × 2 mm, 5 μm	ACN/5 mM AmmAc/MeOH (35/60/5), 0.1 ml/min	MS/MS PE-Sciex API III triple quadrupole APCI−	74–77%	–	1 ng/g	[10]
DXM, BM, etc. (12)	Meat, hair, urine	Bovine	LE MeOH, NaAc (pH 5.2), deconjugation *H. pomatia*, SPE C18, LLE Na_2CO_3 (10%), SPE SiOH	Nucleosil C18 50 mm × 2 mm, 5 μm	0.5% CH_3COOH/MeOH (60/40, 10/90, 60/40), 0.22 ml/min	MS/MS Quattro LC triple quadrupole ESI−	32–67%	Meat: 40–70 pg/g; urine: 40–70 pg/g; hair: 3–9 ppb	–	[11]
DXM, BM	Liver	Bovine	Deconjugation *H. pomatia*, 3 M NaAc (pH 4.6), LLE ACN, hexane, DCM, SPE C18	Hypercarb 100 mm × 4.6 mm, 7 μm	H_2O/ACN (90/10) + 0.3% HCOOH, 1 ml/min, split to 0.22 ml/min	MS/MS Quattro LCZ triple quadrupole ESI+	56–69%	0.2 ng/g	1 ng/g	[12]
DXM, BM, etc. (11)	Liver	Bovine	Deconjugation *H. pomatia*, 3 M NaAc (pH 5.2), LE MeOH, SPE C18	Hypercarb 100 mm × 2.1 mm, 5 μm	H_2O/ACN (90/10) + 0.3% HCOOH, 0.22 ml/min	MS/MS Quattro LCZ triple quadrupole ESI+/ESI−	75–95%	–	–	[13]
DXM, FLU	Liver	Bovine	Deconjugation *H. pomatia*, 3 M NaAc (pH 4.6), LLE ACN, hexane, DCM, SPE C18	Hypercarb 100 mm × 2.1 mm, 5 μm	H_2O/ACN (90/10) + 0.3% HCOOH, 0.22 ml/min	MS/MS Quattro LCZ triple quadrupole ESI+/ESI−	Liver: 82%; kidney: 89%; muscle: 71%; urine: 91%	Liver: 2.13 ng/g DXM; 0.19 ng/g FLU	–	[14]
Urine										
DXN, BM, etc. (14)		Human	LLE DCM	Hypersil 5-ODS 250 mm × 4.6 mm, 5 μm	H_2O/THF (72/28), 1 ml/min	UV-DAD 190–360 nm	–	0.02–0.14 ng	–	[15]
DXN, BM, etc. (14)		Human	LLE DCM, SPE C18, SPE Serdolit AD-2	Hypersil ODS C18 250 mm × 4.6 mm, 5 μm	H_2O/ACN (68/32), 1 ml/min	UV-DAD 190–360 nm	>90%	–	–	[16]
DXM, BM, etc. (11)		Bovine	Deconjugation *H. pomatia*, 5 M AmmAc (pH 5.0, 4.0), SPE C18	Spherisorb C18 250 mm × 4.6 mm, 5 μm	0.1 M AmmAc (pH 6.8)/ACN (60/40), 1 ml/min	MS/MS PE-Sciex API III triple quadrupole APCI−	80–86%	0.05 ng/ml	1 ng/ml	[17]
BM		Human	Deconjugation β-glucuronidase (pH 6–6.5), filtration 0.22 mm, coupled column LC (Miscrospher C18 50 mm × 4.6 mm)	Zorbax TMS 250 mm × 4.6 mm, 5 μm	MeOH/H_2O (50/50) + 0.1 M AmmAc, ACN/H_2O (37/63) + 0.05 or 0.15 M AmmAc, 1.3 ml/min	MS/MS TSQ 700 triple quadrupole ESI+	–	0.2 ng/ml	1 ng/ml	[18]
DXM, DM, etc. (23)		Equine	Deconjugation β-glucuronidase, 1 M NaAc (pH 5.0), LLE ethyl acetate, 1 M NaOH + 0.15 M NaCl	DB-8 75 mm × 4.6 mm, 3 μm	1% CH_3COOH/MeOH (100/0, 0/100, 100/0), 1 ml/min	MS/MS LCQ® ion trap APCI+	61–99%	0.2–05 ng/ml	–	[19]
DXM, BM, etc. (9)		Human	Addition of 10 mM ascorbic acid–ammonia buffer (pH 9.5), SPE XAD-7, LLE Extrelut-NT3	Inertsil 3 ODS-3 150 mm × 3 mm, 3 μm	1 mM AmmAc (pH 6.8)/ACN (60/40, 0/100, 60/40), 0.4 ml/min	MS PE-Sciex API 150 EX triple quadrupole ESI−	>90%	1–5 ng/ml	Limit of confirmation: 1–20 ng/ml	[20]

Table 1 (*Continued*)

Corticosteroids	Matrix	Species	Sample preparation	LC column	Mobile phase	Detection	Recovery (%)	LOD	LOQ	Reference
FLU	+Serum	Bovine	Deconjugation *H. pomatia*, NaAc (pH 5.2), SPE C18, LLE *tert*-butyl methyl ether	Nucleosil C18 250 mm × 2.1 mm, 5 μm	MeOH/1 mM Amm form (65/35), 0.15 ml/min	MS/MS PE-Sciex API 365 triple quadrupole Turboion Spray−	Urine: 87%; serum: 85%	Urine + serum: 30 pg/ml	Urine + serum: 0.1 ng/ml	[21]
DXM, FLU, TRIAC	+Animal feed	Bovine	Urine: deconjugation *H. pomatia* (pH 5.2), SPE HLB, IAC; feed: LLE *tert*-butyl methyl ether, SPE NH_2, PS-DVB, IACC	Superspher 100 RP 18e C18 125 mm × 4 mm, 4 μm	ACN/H_2O (35/65), 0.8 ml/min	MS/MS LCQ® ion trap APCI+	Urine: >63%; feed: 55–60%	Urine: 0.5 ng/ml; feed: 5 ng/g	–	[22]
DXM, FLU, etc. (5)		Bovine	Urine: deconjugation *H. pomatia* (pH 5.2), SPE HLB	C18 Alltima 150 mm × 2.1 mm, 5 μm	ACN/H_2O (40/60), 0.3 ml/min	MS/MS LCQ® Deca ion trap APCI+	>60%	–	1 ng/ml	[23]
Others										
DXM, BM, etc. (9)	Milk replacer		Dissolution in water, SPE C18	Supelcosil LC-8DB C8 150 mm × 4.6 mm, 5 μm	MeOH/1% CH_3COOH (70/30), 0.8 ml/min	MS SSQ 710 triple quadrupole APCI+	81–93%	5–12 ng/ml	–	[24]
BUD, FP	Plasma	Human	Protein precipitation with EtOH, SPE C18, derivatization with acetic anhydride	ODS Hypersil C18 100 mm × 2.1 mm, 5 μm	EtOH/H_2O (43/57), 0.5 ml/min	MS/MS TSQ 7000 triple quadrupole APCI+	BUD and FP: 88%	BUD: 0.025 ng/ml; FP: 0.01 ng/ml	BUD: 0.05 ng/ml; FP: 0.02 ng/ml	[25]
Cortisol	Saliva	Human	Protein precipitation with ACN + 0.5% CH_3COOH	Geneis C8 20 mm × 2.1 mm, 4 μm	H_2O/MeOH + 0.5% CH_3COOH (50/50; 0/100; 50/50), 0.2 ml/min	MS/MS MDS-Sciex API 3000 triple quadrupole Turboion Spray+	64%	0.2 ng/ml	–	[26]
DXM, BM, etc. (8)	Feces	Bovine	LLE ether, 10% $NaCO_3$/$NaHCO_3$ (pH 10.2), 1 M NaCl, SPE SiOH, SPE C18, preparative LC (ODS Ultrasphere C18 250 mm × 10 mm), derivatisation with ethoxyamine hydrochloride	Symmetry C18 150 mm × 3.9 mm, 5 μm	ACN/H_2O (60/40, 80/20, 100/0, 60/40), 0.6–1 ml/min	MS/MS LCQ® ion trap APCI+	13–55%	1 ng/g	>2 ng/g	[27]

The eluate was evaporated to dryness at 40 °C, under a gentle stream of nitrogen gas. The dry residue was redissolved in 200 μl of methanol and vortex mixed for 15 s. The reconstituted sample was then centrifuged in an Eppendorff cup (10,800 rpm, 10 min) and transferred into an autosampler vial. A 50 μl aliquot was injected onto the LC column.

2.4. Chromatography

The HPLC system consisted of a quaternary gradient pump P4000, an autosampler AS3000 with cooling device and a degassing kit using helium to sparge the eluents (all from Thermo Separation Products, ThermoFinnigan, San Jose, CA, USA). Chromatographic separation was achieved using a PLRP-S polymeric reversed phase column (250 mm × 4.6 mm i.d., 8 μm), in combination with a guard column of the same type (5 mm × 3 mm i.d.), from Polymer Laboratories (Shropshire, UK). The column was maintained at a temperature of 30 °C. The mobile phase A was a solution of 0.1% acetic acid in water, while the mobile phase B was acetonitrile. Mobile phase was delivered to the LC column at a flow rate of 1–1.5 ml/min for a total run time of 20 min. A gradient elution was performed: 0–11 min, 60% A, 40% B at 1 ml/min; 11.1–12.4 min, 10% A, 90% B at 1 ml/min; 12.5–16 min, 10% A, 90% B at 1.5 ml/min; 16.1–19 min, 60% A, 40% B at 1.5 ml/min; 19.1–20 min, 60% A, 40% B at 1 ml/min.

2.5. Mass spectrometry

The LC column effluent was pumped to an LCQ® ion trap mass spectrometer instrument (Finnigan MAT, ThermoFinnigan), equipped with an APCI ion source, which was used in the positive ion mode. A divert valve was used to divert the LC effluent to the waste during the first 4 min and the last 7.5 min of the chromatographic run. The instrument was calibrated with a solution of caffeine, L-methionyl-arginyl-phenylalanyl-alanineacetate·H_2O (MRFA) and Ultramark® 1621, according to manufacturers' instructions. Thereafter, the instrument was tuned by direct infusion of a solution of 10 μg/ml of DXM in the APCI source at 3 μl/min, first without and thereafter in combination with the LC mobile phase, using a T-union. The following tune parameters were retained for optimal DXM detection (at unit mass resolution): APCI corona discharge current, 7 μA; sheath gas flow rate, 90 (arbitrary units); auxiliary gas flow rate, 60 (arbitrary units); capillary voltage, 3 V; APCI vaporizer temperature, 500 °C; capillary temperature, 200 °C; tube lens offset, −40 V; octapole 1 offset, −1.5 V; interoctapole lens voltage, −16 V; octapole 2 offset, −16.5 V; octapole rf amplitude, 400 p.-p.V. These tune parameters were also suitable for DSM, due to the structural similarity between DXM and DSM. Optimal collision energy in MS/MS mode, corresponding to a (nearly) 100% fragmentation of the protonated molecule (collision-induced dissociation (CID)), was found to be 1 V for DXM and DSM. Under these conditions, typical product ions at m/z = 372.9, 355.0 and 337.0 were obtained for DXM, and at m/z = 357.0, 339.0 and 321.0 for DSM. Quantification was done with the LCQuan® software (ThermoFinnigan), using the above-mentioned transitions for both DXM (392.9 > 372.9) and DSM (376.9 > 357.0).

2.6. Validation criteria

The presented method for the quantitative determination of DXM was validated by a set of parameters which are in compliance with the recommendations as defined by the EC [5,28,29]. More particular, linearity, accuracy and precision (at the fixed MRLs, at half the MRLs and at double the MRLs), limit of quantification, limit of detection, decision limit (CCα), detection capability (CCβ) and specificity of the method have been evaluated.

3. Results and discussion

3.1. Mass spectrometry

Fig. 2 shows the mass spectra obtained after direct infusion of a standard solution of 10 μg/ml of DXM and DSM in the atmospheric pressure chemical ionization (APCI) source, in combination with the LC mobile phase. The positive ion mode was selected, since it produced a stronger signal than the negative ion mode. Preference was further given to the APCI interface rather than to the electrospray ionization (ESI) source, due to its greater sensitivity for the detection of corticosteroids, as also observed by others [23]. The mass spectra of DXM and DSM both show a major ion (Fig. 2, upper trace), at m/z 392.9 and 376.9, respectively, corresponding to the protonated molecules $[M + H]^+$ of DXM and DSM, respectively. For both components, some fragmentation was already observed in the MS mode. The CID product ion spectra (Fig. 2, lower trace) are for both DXM and DSM the result of a similar fragmentation mechanism: the predominant ions, at m/z 372.9 and 357.0, respectively, correspond to the loss of HF, while the ions at m/z 355.0 and 339.0, respectively, with a relative abundance below 20%, correspond to the combined loss of HF and H_2O. Further minor ions, at m/z 337.0 and 321.0, respectively, correspond to a supplementary loss of H_2O. For quantification, the product ions at m/z 372.9 and 357.0 were used for DXM and DSM, respectively. The ion ratio m/z 372.9/355.0 for DXM was used as its identification criterion, and was set at 0.19 ± 30% [28], which is the value found when a DXM standard solution was analyzed by the LC–MS/MS technique described.

3.2. Sample clean-up and chromatography

The sample clean-up procedure was kept as simple as possible, and consists of a deproteinization of the milk by the addition of concentrated TCA, followed by a paper filtration,

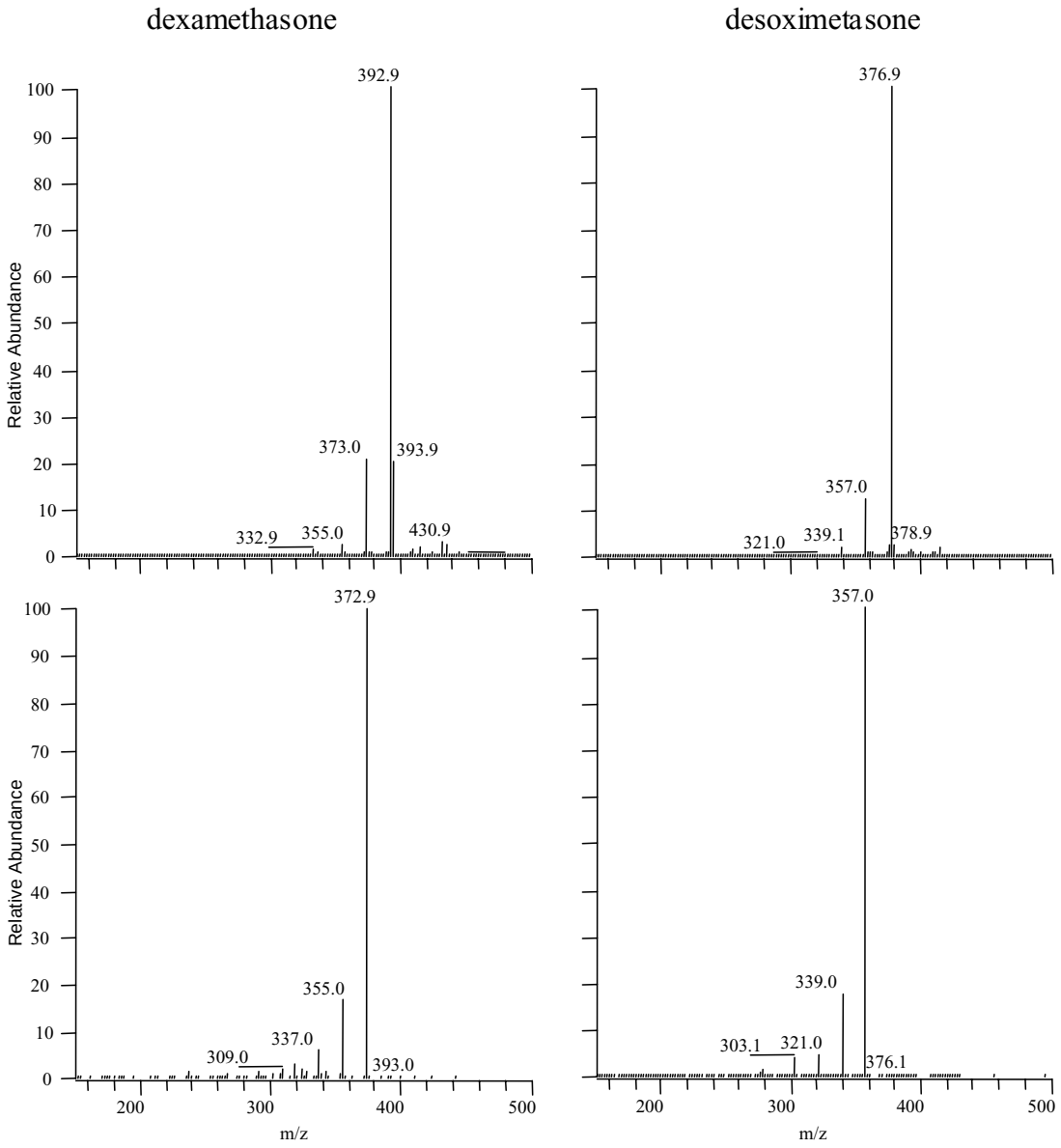

Fig. 2. Mass spectra of DXM and DSM (upper trace), and CID product ion spectra of their protonated molecules (lower trace), obtained after direct infusion of standard solutions at 10 μg/ml, in combination with the LC mobile phase (APCI, positive ion mode, collision energy in MS/MS = 1 V for DXM and DSM).

and a further SPE clean-up on a C18 column. This yielded an extract that was suitable for subsequent LC–MS/MS analysis: during this study about 500 milk samples were analyzed without the need to clean the MS instrument (no drop in sensitivity was observed, as revealed by the injection ($n = 3$) of a standard solution of DXM and DSM before each batch of milk samples), or without the need to change the LC precolumn or analytical column, since retention time and peak shape remained unchanged. The recovery of DXM was found to be 56%, as determined by comparing the DXM signal of six samples spiked with DXM at the MRL level prior to extraction, and the DXM signal of six blank extracts spiked at the same level just prior to injection on the LC–MS apparatus. The inclusion of a deconjugation step to deconjugate phase II metabolites of DXM that might be present in incurred milk samples, recommended by the EC [1], was found to be not necessary: an evaluation on six of the incurred milk samples of the depletion study of Fig. 5 did not result in higher DXM levels after such a deconjugation step. Therefore, compared to the procedure described in Section 2, 5 ml of a 0.2 M sodium acetate buffer pH 5.5 (mix of 0.2 M sodium acetate solution and 0.2 M acetic acid solution, 89/11, v/v) was added to the 10 ml milk samples, followed by 200 μl of *H. pomatia* juice. After an incubation overnight at 42 °C, clean-up was further performed as described earlier, with the exception that more 20% (w/v) TCA solution had to be added (1.5 ml) and an additional 100 μl HCl to acidify and deproteinize correctly the buffered milk samples.

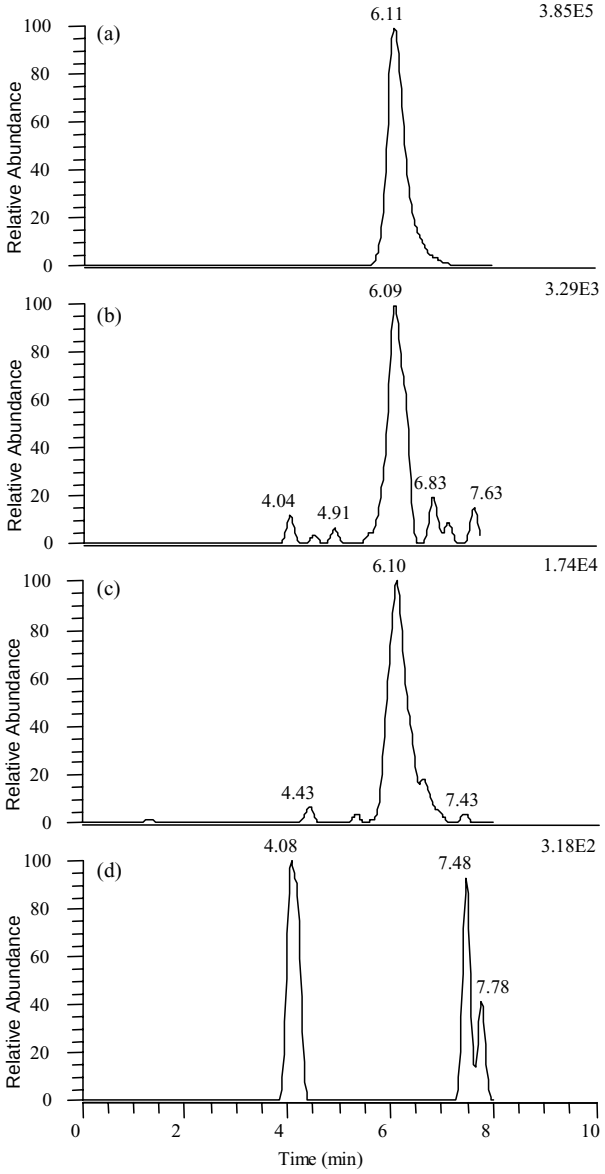

Fig. 3. MS/MS mass chromatograms for DXM (m/z = 372.9 ion), for a standard solution at 5 μg/ml (a), a milk sample spiked at the MRL level (0.30 ng/ml) (b), an incurred milk sample (first milking after DXM administration; DXM concentration found: 4.1 ng/ml) (c), and a blank milk sample (d).

Fig. 3 shows the MS/MS mass chromatograms for a standard solution of DXM, a milk sample spiked with DXM at the MRL level (0.30 ng/ml), an incurred milk sample (DXM concentration found: 4.1 ng/ml), and a blank milk sample. The retention time is, for spiked as well as for incurred milk samples, the same as compared to an injection of a standard solution: DXM eluted at 6.1 min, while DSM eluted at 8.8 min (not shown). As can be seen, the blank sample chromatogram is clean and free from endogenous interferences at the elution time of DXM (and DSM, not shown), demonstrating the specificity of the LC–MS/MS technique.

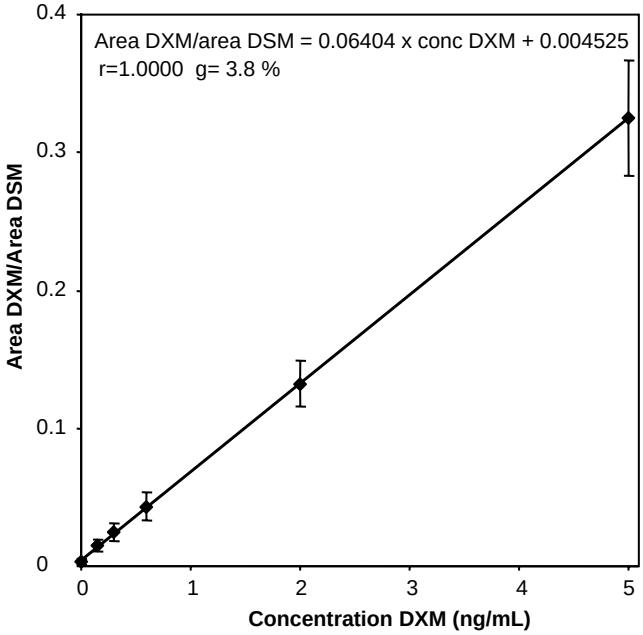

Fig. 4. Calibration curve for DXM in the 0.15–5 ng/ml range, being the means of 11 calibration curves extracted and analyzed on different days over a period of 45 days.

3.3. Method validation

3.3.1. Linearity

The results of the linearity evaluation are summarized in Fig. 4 for a calibration curve in the 0.15–5 ng/ml range, including the 0.30 ng/ml MRL level, evaluated over a period of 45 days for a total of 11 calibration curves. As can be seen, an excellent linearity was observed: the correlation coefficient of the calibration curve is above 0.99, while the goodness of fit coefficient (g) is below 10%, indicating the good quality of the calibration curve [30].

3.3.2. Accuracy and precision

The accuracy and "within-day" precision of the method were determined using six independently spiked blank milk samples at three different spike levels: at the MRL, at half the MRL and double the MRL. The results are summarized in Table 2. The accuracy fell within the range of −50 to

Table 2
Results of the accuracy and the "within-day" and "between-day" precision evaluation of milk samples spiked with DXM at different levels

Concentration spiked (ng/ml)	Mean concentration found (ng/ml)	Accuracy (%)	R.S.D. (%)[a]	R.S.D.$_{max}$ (%)[b]
0.15	0.18	+17.6	31.1	40.1
0.30	0.31	+4.3	32.3	36.2
0.60	0.46	−22.6	31.6	32.6
0.30[c]	0.27	−9.7	23.8	36.2

[a] R.S.D.: relative standard deviation (precision).
[b] R.S.D.$_{max}$ = $2/3 \times 2^{1-0.5 \log c}$ (with c the analyte concentration in g/ml) [28].
[c] Evaluated on 52 samples over a period of 45 days.

+20%, at the three levels tested, testifying of the good accuracy of the method [28]. The precision, expressed as R.S.D. values, at all three levels tested, fell within the maximum R.S.D. values, calculated with the Horwitz equation according to [28] and listed in Table 2. The "between-day" precision was determined using blank milk samples spiked at the MRL level and analyzed on different days. The results, also summarized in Table 2, show that the "between-day" precision fell also within the calculated maximum values. Accuracy fell also within the −50 to +20% range.

3.3.3. Limit of quantification and limit of detection

The LOQ was established by analyzing six blank milk samples, which were spiked with DXM at half the MRL level, i.e. 0.15 ng/ml. As discussed earlier, this level could be quantified fulfilling the criteria for accuracy and precision [28], and was therefore set as the LOQ of the method. It is ≤MRL/2 as required [5]. The LOD was determined using the signal-to-noise (S/N) = 3/1 criterion [29]. The mean S/N value of the above-mentioned six LOQ samples was determined: it was found to be 11, giving an LOD of 41 pg/ml.

3.3.4. Decision limit and detection capability

For MRLs substances, the decision limit CCα is the concentration above which it can be decided with a 95% statistical certainty that the measure is truly above the MRL. According to [28], it was calculated as the concentration found plus 1.64 times the standard deviation, when analyzing blank milk samples spiked at the MRL level ($n = 6$). A value of 0.48 ng/ml was obtained for the analysis of DXM in milk. The detection capability CCβ for MRL substances is the concentration at which the method is able to quantify the substance with a 95% statistical certainty. It is calculated according to [28] in the same way as CCα, but using spiked blank milk samples at the CCα level. These experiments were not carried out. Instead CCβ was estimated by extrapolation to the CCα levels, using the rule of three, of the data (mean and S.D., $n = 6$) obtained at the MRL level (and used for the calculation of CCα). CCβ was then found to be 0.76 ng/ml for the analysis of DXM in milk.

3.3.5. Specificity

Blank milk samples extracted and analyzed with the above-mentioned method were free of endogenous interferences at the elution times of DXM and DSM, testifying of the good specificity of the method.

3.4. Results of a residue depletion study

The mean DXM concentration in milk of cows treated with an intravenous dose of DXM (under the form of its sodium phosphate ester) at 40 μg/kg body weight is represented in Fig. 5. As can be seen, the maximum concentration (3.8 ± 0.8 ng/ml) was measured at the first milking after treatment. DXM residues where then quickly eliminated: at the fourth milking after treatment the measured concentration (0.21 ± 0.16 ng/ml) was already below the MRL level, while at the fifth milking it was far below the LOQ. This finding is consistent with [1].

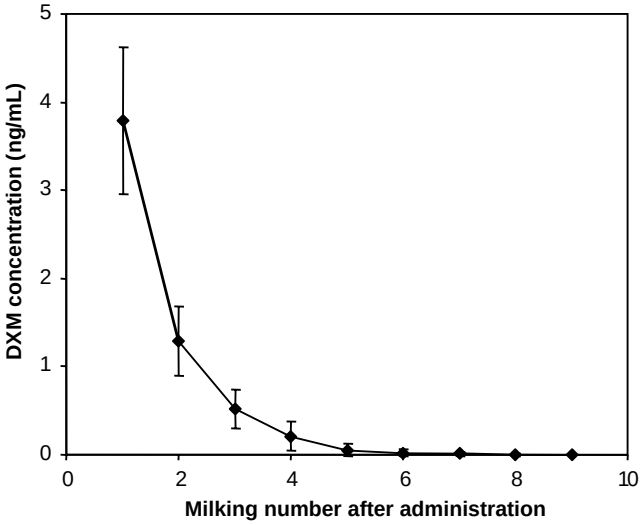

Fig. 5. Residues of DXM, expressed in ng/ml milk per milking, of 25 cows treated with an intravenous dose of DXM at 40 μg/kg body weight.

4. Conclusion

In this paper, an LC–APCI–MS/MS method was presented which is capable of quantifying DXM residues in milk at a level that is as low as 0.15 ng/ml, which is half the MRL fixed by the EC. The sample preparation is simple and quick, and consists merely of a deproteinization step with TCA, followed by SPE clean-up on a C18 column after paper filtration. The method was validated according to the requirements of the EC, and was therefore suitable for monitoring DXM residues in milk of lactating cows that were treated with an intravenous injection of DXM, in order to determine the withdrawal time. It was found that DXM was quickly eliminated from the milk, and that DXM levels were below the MRL at the fourth milking after treatment.

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