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Differentiation between dexamethasone and betamethasone in a mixture using multiple mass spectrometry

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Abstract

The objective of this study was to provide LC and GC–multiple mass spectrometry (MS^n) data in positive and negative ion modes to prove the distinction between dexamethasone and betamethasone in a mixture of both components. Using GC–MS, the differentiation was based on a difference in the ratio of the ion traces of the two chromatographic peaks of the α and β epimer with m/z 310 and 330. A minimum of 15% dexamethasone should be present in a mixture of both to detect it as present with a probability of 95%. In the same way betamethasone can be detected from 15% on. Because of the very similar structures of the dexamethasone and betamethasone epimers, no reversed-phase (RP) separations have been reported. Normal-phase separations have been reported in other studies. However because of the compatibility of RP mobile phases in the coupling with MS, the latter was the method of choice. In LC–MS^n positive ion mode the product ion 355 was plotted against the sum of 337 and 319. With this combination dexamethasone and betamethasone could be discriminated in a mixture of 20 to 80% of each combination of analytes. In negative ion mode only two product ions were formed from the fragmentation of the acetate adduct, $[M\text{-}H]^-$ and $[M\text{-}H\text{-}CH_2O]^-$. The intensity of the fragment 391 ($[M\text{-}H]^-$) was determined in the discrimination of the two epimers.

Keywords: Pharmaceutical analysis; Dexamethasone; Betamethasone; Glucocorticoids

1. Introduction

Betamethasone (Btm) and dexamethasone (Dxm) are potent synthetic glucocorticoids that are widely used in the treatment of inflammation, allergies and other diseases related to glucocorticoid deficiency. Their effects also include stimulation of gluconeogenesis, increased catabolism of proteins, and mobilization of free fatty acids. At first sight and/or from a pharmacological point of view the illegal use in cattle fattening was a surprise because corticosteroids are catabolic agents and their use in animal fattening is contra-indicated. However, in practice and also in the literature, indications of the growth promoting effect of corticosteroids was found [1–3]. Dexamethasone is licenced for therapy in veterinary practice but the use of betamethasone is not registered in Belgium. Since the consequences for the owner of the ''positive'' animal can be less or more severe depending on the analyte detected, the analyst should make an effort in determining the difference between dexamethasone and betamethasone.

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This is illustrated with two examples. First in a Dxm result of 3 μg kg^{-1} in liver (MRL=2.5 μg kg^{-1}) 0.6 μg kg^{-1} Btm is included. The final result for Dxm (2.4 μg kg^{-1}) is lower than the maximum residue limit (MRL) but a forbidden product is detected. If (second example) a positive result was found for Btm and a part of the response was due to Dxm the final situation stays the same: a forbidden product is detected. However, if in the future both Dxm and Btm have an MRL of 2.5 μg kg^{-1}, a better differentiation between both components is needed.

Many papers describe the determination of corticosteroids with gas chromatography–mass spectrometry (GC–MS) [4–6]. However the chemical oxidation can cause different corticosteroids to produce the same oxidation product, hence lacking specificity. Keh-Ren et al. reported a high-performance liquid chromatography (HPLC) method with UV–Vis detection [7]. Dexamethasone and betamethasone were separated with a normal-phase compatible mobile phase. Reversed-phase (RP) HPLC is less suitable than normal-phase HPLC for positional isomers (Fig. 1). The universal use of RP-HPLC and the compatible solvents allow the coupling with a mass spectrometer. Using ion trap technology full scan mass spectra are very specific in MS^n (multiple mass spectrometry) positive ion mode. A large number of ions is produced that can be used to compare ion ratios between dexamethasone and betamethasone. In negative ion mode only two product ions were formed from the fragmentation of the acetate adduct, $[M\text{-}H]^-$ and $[M\text{-}H\text{-}CH_2O]^-$, but the ratios also differ depending on the analyte [8].

Using GC–MS the ratios of the two epimers were calculated towards the ratios of deuterated dexamethasone.

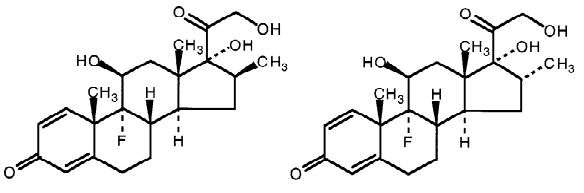


Fig. 1. Structural formulae of dexamethasone and betamethasone.

2. Experimental

2.1. Chemicals

The chemicals for extraction were of analytical grade. The solvents for preparation of the mobile phase were of HPLC grade. Both were obtained from Merck (Darmstadt, Germany). The standards were obtained from Sigma (St. Louis, MO, USA) [dexamethasone (Dxm; Sigma D1756) (9-fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione); betamethasone (Btm; Sigma B7005) (9-fluoro-11β, 17, 21-trihydroxy-16β-methylpregna-1, 4-diene-3,20-dione)].

$[^2H_3]$Dxm (Dxm-d_3) was a generous gift of the RIVM (Bilthoven, The Netherlands).

2.2. Solutions

Stock solutions were prepared at 200 ng μl^{-1} in ethanol. The working solutions were prepared in mobile phase.

2.3. Instrumentation

2.3.1. LC–MS–MS

The HPLC apparatus comprised of a TSP P4000 pump and a Model AS3000 autosampler (TSP, San Jose, CA, USA). Separation was carried out on a Symmetry C_{18} column (5 μm, 150×2.1 mm; Waters, Milford, MA, USA). Analysis was carried out using an LCQ^{DECA} Ion Trap Mass Analyzer (ThermoQuest, San Jose, CA, USA), with an electrospray interface and Xcalibur 1.1 software. The analytes were detected in MS–MS-full scan positive ion mode and MS–MS-full scan negative ion mode.

An isocratic mixture of 0.5% acetic acid in methanol–water (60:40, v/v) was used at a flow-rate of 0.3 ml min^{-1}.

2.3.2. GC–MS

GC–MS analyses were performed using a Hewlett-Packard Model 5989A MS Engine and a Model

5890 Series II gas chromatograph. The injector type used was split/splitless with a split ratio of 1:25. A Hewlett-Packard HP-5 MS fused-silica capillary column (25 m×0.25 mm, 0.25 μm film thickness) was used. The carrier gas was high-purity helium (Air Liquide, Liège, Belgium) at a flow-rate of 0.8 ml min^{-1}. Methane was used as modifying gas at an ion-volume pressure of 133.3 Pa.

The oven temperature was initially held at 100°C for 2 min, then raised to 280°C at a rate of 20°C min^{-1}, then kept constant for 10 min and raised again at a rate of 10°C min^{-1} to 300°C. The final oven temperature was held for 3 min. The split valve was opened after 2 min.

2.4. Detection

Using LC–MS, because of the coelution of Dxm and Btm one scan event was used. In this scan event the precursor ions of Dxm or Btm [393 in positive ion mode (isolation width=3.5) and 451 in negative ion mode (isolation width=2.5)] were isolated. The isolation width was manually tuned to obtain the maximum intensity of precursor ion in the trap. The relative energy applied was 25% in both ion modes. The product ions were acquired in full scan and are listed in Table 1 for positive and negative ion modes.

GC–MS measurements were made in selected ion monitoring (SIM) mode. The mass peak width for the m/z values used was 0.5 u. The dwell time was 50 ms. The source temperature was held at 150°C. The ionization current was 300 μA and the ionization voltage was 230 eV.

3. Results and discussion

3.1. LC–MS–MS

3.1.1. Positive ions

The following mixtures of Dxm and Btm were prepared in mobile phase: 10 ng μl^{-1} Dxm, 10 ng μl^{-1} Btm, 9+1 ng μl^{-1} Dxm+Btm, 1+9 ng μl^{-1} Dxm+Btm, 5+5 ng μl^{-1} Dxm+Btm. One microliter was injected on column. The relative intensities of the diagnostic product ions are summarized in Table 2. Average values with the standard deviations are given for 10 injections. In positive ion mode a large number of product ions were formed (Fig. 2). In negative ion mode the acetate adduct is fragmented, resulting in only two product ions; $[M\text{-}H]^-$ and $[M\text{-}H\text{-}CH_2O]^-$ (391, 361) for Dxm and Btm, respectively. Positive ion mode gives more possible combinations to optimize the discrimination between Dxm and Btm. In Fig. 3 the different combinations are given. In the abscis the relative intensities of the product ion 355 is given for the different combinations of concentrations of Dxm and Btm. In the *y*-axis the sum of product ions 337 and 319 is given. Two times the standard deviation in the *x*- and *y*-axes was calculated to form an ellipse around the datapoints. This ellipse contains 95% of the datapoints of the corresponding concentration. From the equation of the ellipse the intersection with the regression line through the average datapoints can be calculated. For the ellipses of 100% Dxm, Dxm–Btm (90:10), 100% Btm and Dxm–Btm (10:90), the intersections were calculated and there was an overlap of 100% Btm and Dxm–Btm (10:90).

Table 1
Diagnostic product ions of Dxm and Btm in positive and negative ion modes

Analyte	Precursor ion	Collision energy (%)	Product ions
Positive ion mode			
Dexamethasone	393	25	373, 355, 337, 319, 309, 291, 279, 237, 267
Betamethasone	393	25	373, 355, 337, 319, 309, 291, 279, 237, 267
Negative ion mode			
Dexamethasone	451	25	361, 391
Betamethasone	451	25	361, 391

Table 2
Average relative intensities and standard deviations of the diagnostic product ions of Dxm, Btm in positive ion mode

	373	355	337	319	309	291	237	279	267
Dxm, 10 ng μl^{-1}	100.0	34.96	16.26	9.75	5.58	4.30	2.97	0.87	2.20
		±0.78	±0.43	±0.35	±0.41	±0.24	±0.21	±0.20	±0.10
Dxm+Btm, 9+1 ng μl^{-1}	100.0	32.56	14.62	8.67	4.79	3.74	2.59	1.25	2.08
		±1.14	±0.39	±0.28	±0.22	±0.15	±0.28	±0.48	±0.18
Dxm+Btm, 5+5 ng μl^{-1}	100.0	27.19	10.93	5.99	3.24	2.32	1.70	1.22	1.06
		±0.46	±0.30	±0.34	±0.24	±0.21	±0.18	±0.18	±0.13
Dxm+Btm, 1+9 ng μl^{-1}	100.0	22.57	7.45	3.59	1.93	1.02	0.71	1.36	0.37
		±0.65	±0.26	±0.24	±0.13	±0.10	±0.08	±0.20	±0.05
Btm, 10 ng μl^{-1}	100.0	21.92	7.15	3.17	1.78	0.83	0.56	1.65	0.23
		±0.47	±0.28	±0.14	±0.15	±0.10	±0.10	±0.60	±0.04

$n=10$.

100% Dxm was compared with Dxm–Btm (90:10). If the lowest point of the intersection of the 100% Dxm ellipse with the regression line is higher than the highest point of intersection with the 90:10 ellipse, there is no overlapping. In this case (34.06; 25.07) is higher than (33.49; 24.38). In the case of 100% Btm and Dxm–Btm (10:90) (21.91; 10.33) is lower than (22.18; 10.67), the two ellipses overlap and there can be no discrimination of Dxm and Btm for a 10:90 ratio.

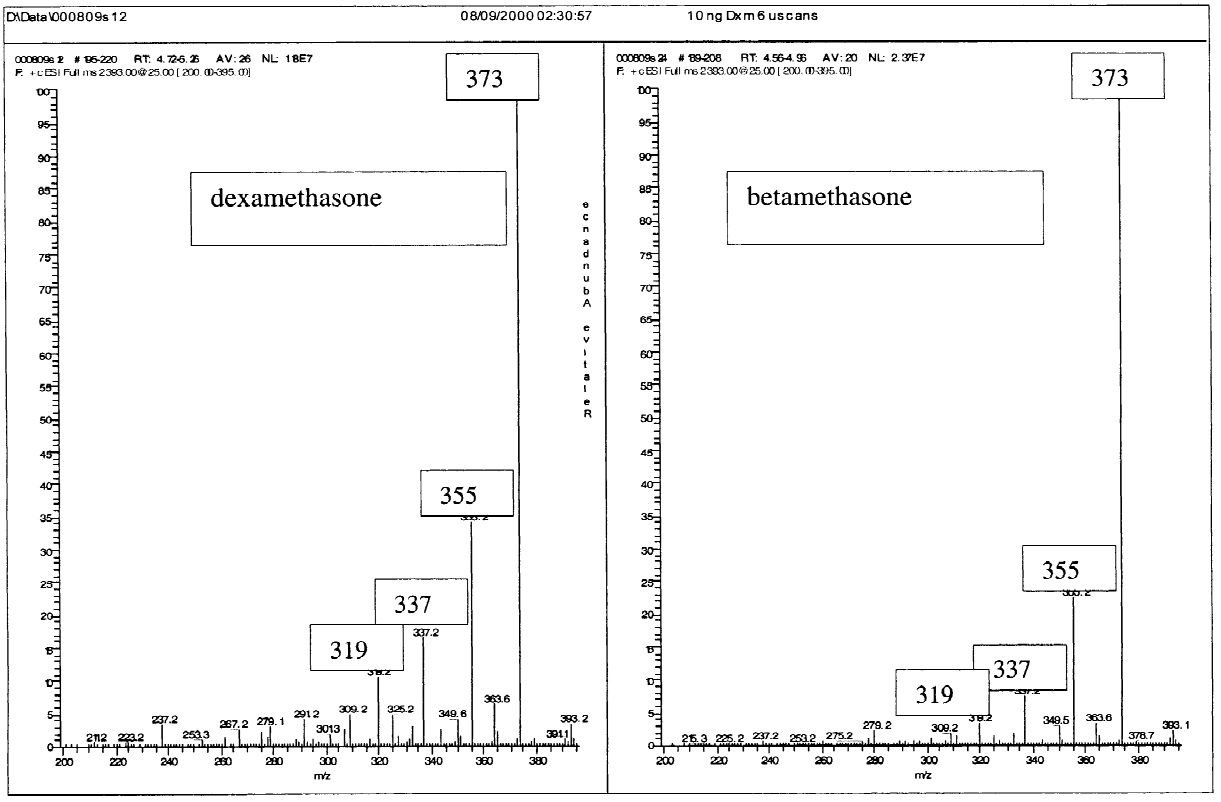
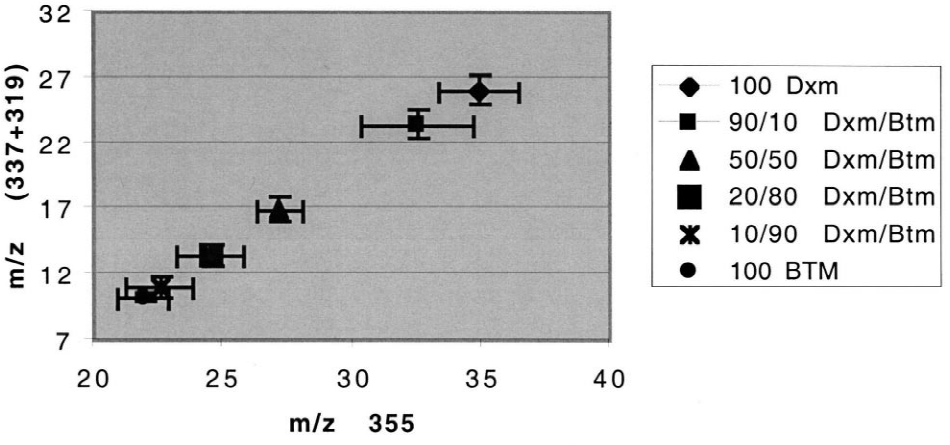


Fig. 2. Spectra of dexamethasone and betamethasone in positive ion mode LC–MS–MS.



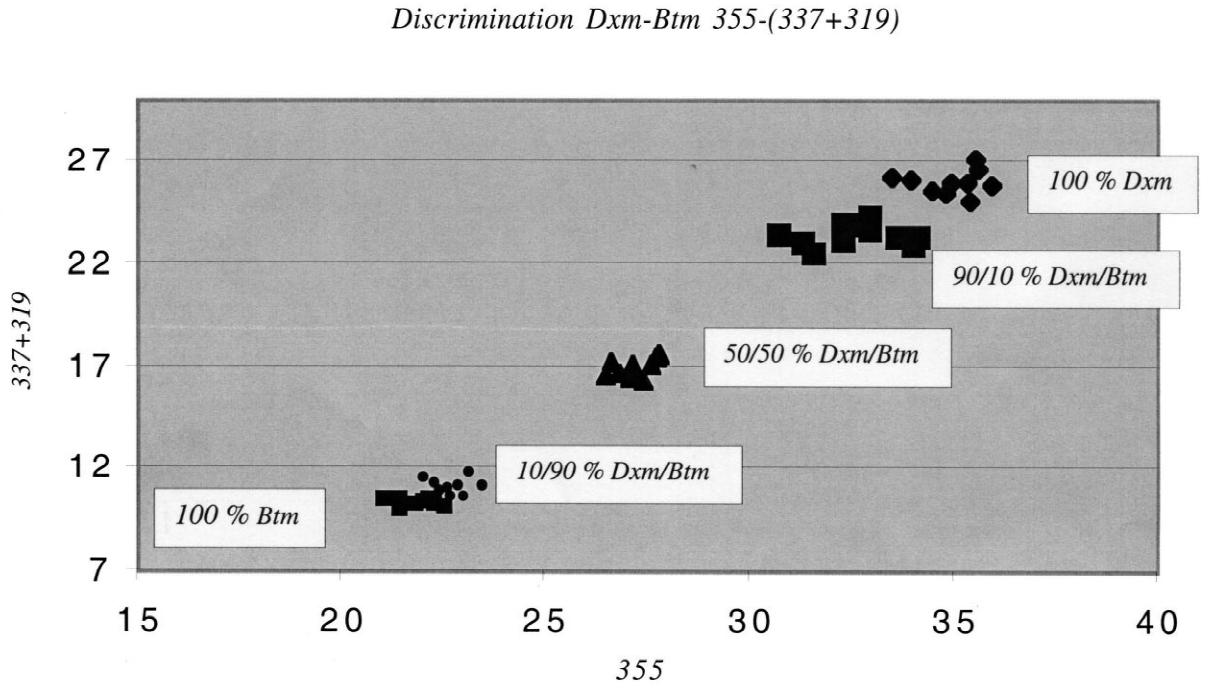


Fig. 3. Discrimination between Dxm and Btm based on three product ions.

In a mixture of 20:80 of either analyte, both could be discriminated with a probability of 95%. Details of the calculations are given in Table 3.

3.1.2. Negative ions

Similar calculations were performed for negative ions (spectra given in Fig. 4). Since there is only one ratio, the upper and lower limit around the average were calculated by adding or substracting twice the standard deviation.

There was an overlap of the lower limit of 100% Dxm and the upper limit of Dxm–Btm (90:10). But

Table 3
Ellipse equations and coordinates of the intersection with the regression curve of the different mixtures of Dxm and Btm

	Ellipse equation	$(x_1, y_1)^a$	$(x_2, y_2)^a$
100% Dxm	$1.34x^2-94x+2.42y^2-126y+3280$	(35.69; 27.04)	(34.06; 25.07)
Dxm–Btm (90:10)	$1.17x^2-76x+4.75y^2-222y+3838$	(33.49; 24.38)	(31.84; 22.38)
Dxm–Btm (20:80)	$1.17x^2-57x+4.75y^2-128y+1559$	(25.31; 14.46)	(23.66; 12.46)
Dxm–Btm (10:90)	$0.68x^2-30x+1.67y^2-37y+547$	(23.11; 11.79)	(21.91; 10.33)
100% Btm	$0.13x^2-5.9x+0.88y^2-18.25y+159$	(22.18; 10.67)	(21.61; 9.97)

[a] Intersection with regression curve: $y=1.2123x-16.22$.

not for the Dxm–Btm (80:20) mixture. There was no overlap for the upper limit of 100% Btm and the lower limit of Dxm–Btm (10:90). Details are given in Table 4.

3.2. GC–MS

Residues of dexamethasone and betamethasone in biological matrices are still frequently determined by employing negative chemical ionization mass spectrometry (NCI-MS) after oxidation [5,9]. Btm, differing from Dxm only in the β-position instead of the α-position of the 16-methyl group, on oxidation gives rise to the same two reaction products as dexamethasone: 9α-fluoro-16α-methyl-1,4-androstadiene-3,11,17-trione and its 16β-methyl epimer. Both products can easily be separated by GC. The retention time of the α-epimer is lower than that of the β-epimer. In the spectra of both compounds, the most intense ion is at m/z 310 $[M\text{-}HF]^-$, while the second is the molecular anion. This ion at m/z 330, has an intensity of about 30% and 8% against the first, for the α-epimer and β-epimer, respectively. As described earlier distinction between Dxm and Btm

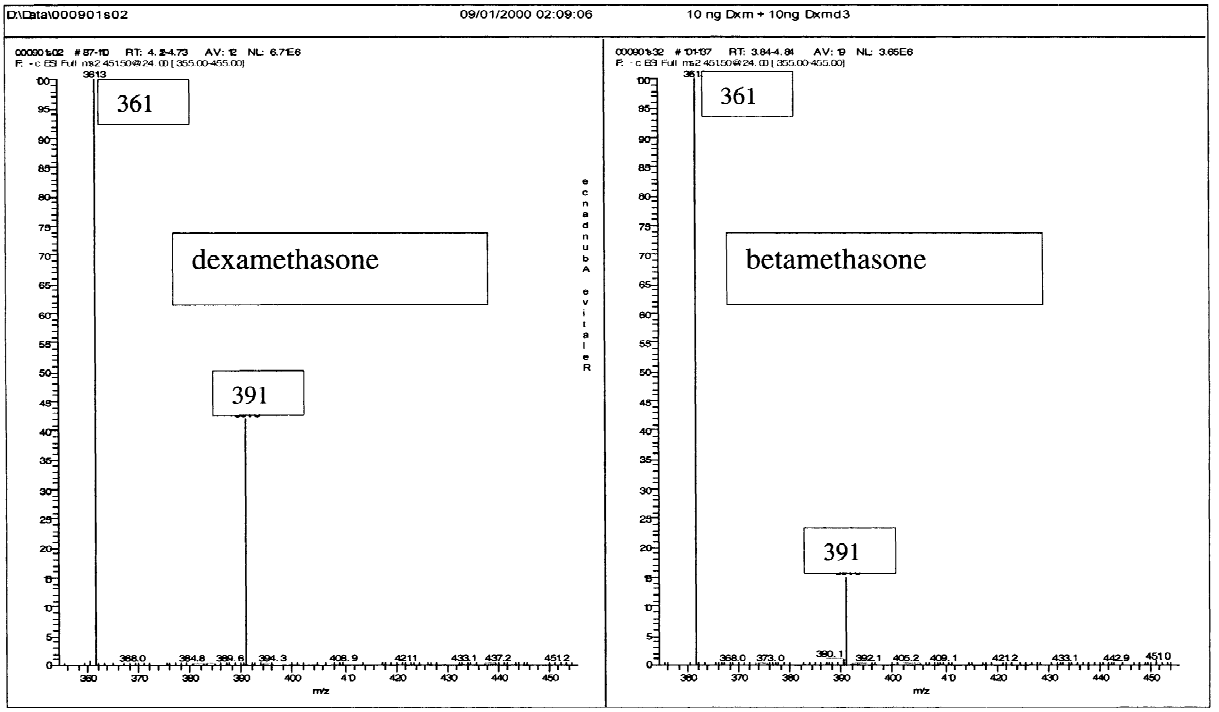


Fig. 4. Spectra of dexamethasone and betamethasone in negative ion mode LC–MS–MS.

Table 4
Average relative intensities and standard deviations of the diagnostic product ions of Dxm, Btm in negative ion mode

	361	391	Lower limit	Higher limit
Dxm, 10 ng μl^{-1}	100.0	41.21±0.84	39.53	42.88
Dxm+Btm, 9+1 ng μl^{-1}	100.0	40.32±1.46	37.39	43.24
Dxm+Btm, 8+2 ng μl^{-1}	100.0	35.95±1.46	33.03	38.87
Dxm+Btm, 5+5 ng μl^{-1}	100.0	29.47±0.57	28.34	30.60
Dxm+Btm, 2+8 ng μl^{-1}	100	20.17±0.49	19.19	21.14
Dxm+Btm, 1+9 ng μl^{-1}	100.0	17.47±0.49	16.49	18.44
Btm, 10 ng μl^{-1}	100.0	14.91±0.39	14.13	15.69

$n=10$.

is possible by the large difference in the ratio of the two oxidation products obtained: the ratio of α/β obtained is larger than 2 for Dxm and about 0.2 for Btm [9].

In this study five different solutions were analyzed in sixfold on 2 different days. The solutions contained 12.5 μl of Dxm-d_3 and 12.5 μl of 1 ng μl^{-1} of Dxm, Btm, Dxm–Btm (90:10), Dxm–Btm (50:50) or Dxm–Btm (10:90). Oxidation was performed as described earlier and 5 μl out of 25 was injected. Ratio R was obtained by dividing the α-epimer/β-epimer ratio of ion 310 by the α-epimer/β-epimer ratio of ion 313 for Dxm-d_3. Fig. 5 shows the correlation between the ratio R and the percentage of Dxm in the mixture. From the prediction limits it can be concluded that Btm is detected if $R \leq 0.18$, while Dxm is detected if $R \geq 0.86$. If $0.18 < R > 0.86$ then a mixture of both compounds is present. The percentage of Dxm (P_{DXM}) can be obtained from the following equation:

$$P_{DXM} = -6.205 + 108.8R$$

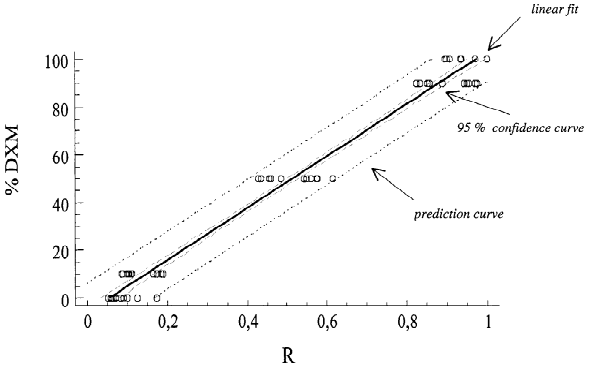


Fig. 5. Correlation between the ratio R and the percentage of Dxm in the mixture.

Out of the prediction limits (Table 5), it can also be concluded that a minimum of 15% of Dxm should be present in a mixture of both to detect it as present with a probability of 95%. In the same way Btm can be detected from 15% on. During routine control 10 R-values of blank samples spiked with 2 ppb of Dxm (Table 5) were all found to be above the calculated value of 0.86, proving the presence of Dxm.

4. Conclusion

Using LC–MS, dexamethasone and betamethasone can be differentiated using positive or negative ion modes in a mixture of 80–20% of either analyte. For GC–MS the percentages become 15% for both.

The ability to differentiate the two analytes fulfill the current needs of the inspection services.

Table 5
Calculation of R for 10 samples spiked with Dxm at the level of 2 pbb

Predicted values		95% Prediction limits		95% Confidence limits	
x	y	Lower	Upper	Lower	Upper
0.98	100.69	88.49	112.88	98.20	103.17
0.95	96.98	84.81	109.16	94.61	99.36
1.01	103.83	91.61	116.04	101.25	106.41
0.99	101.74	89.54	113.94	99.22	104.26
0.89	90.44	78.30	102.58	88.25	92.63
1.00	102.16	89.96	114.37	99.63	104.70
1.05	108.16	95.92	120.41	105.45	110.88
0.90	91.38	79.23	103.52	89.16	93.59
0.93	95.31	83.14	107.47	92.98	97.63
1.02	104.88	92.66	117.10	102.26	107.49

Given in this value, the percentage of Dxm was predicted according to the curve shown in Fig. 5.

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