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XEVO TQ MS: ADDRESSING NEW CHALLENGES IN THE FIELD OF GROWTH PROMOTERS IN BIOLOGICAL SAMPLES

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INTRODUCTION

The safety of our food supply can no longer be taken for granted. As the world changes and populations continue to grow, so will the responsibility of organizations to meet the demand of safe food supplies.

One route of human exposure to veterinary substances is through the food chain as a result of malpractice or illegal activities. Some substances of concern include corticosteroid hormones, β-agonists, and recombinant bovine somatotropin (rbST).

Fifty years after the discovery of natural corticosteroid hormones, many synthetic derivatives of these molecules are available today. In human and veterinary medicine, their legal use is strictly regulated. This includes withdrawal periods between treatment and animal slaughtering, as well as maximum residue limits (MRLs) in edible biological matrices for some compounds. Some of these substances have also been used as growth promoters in cattle, whereas these practices are banned in Europe. For many years, various analytical methods have been proposed for the identification of corticosteroid residues in edible tissues or urine samples. Most of these methods are based on liquid chromatography, coupled with multi-dimensional mass spectrometry with triple quadrupole, or ion trap mass analyzers. These present a high level of efficiency for analyzing urine, milk, muscle, or hair samples. Conversely, liver is a special case and remains more problematic due to the extreme complexity of this matrix. As a result, the identification and quantification of corticosteroids in liver with respect to the MRL fixed at European level (2 µg/kg for dexamethasone and betamethasone, and 10 µg/kg for methylprednisolone and prednisolone) still represent a significant analytical challenge. Recent discussions within the Joint FAO/WHO Expert Committee on Food Additives (JECFA) have underlined the lack of appropriate quantitative efficient methods for this matrix and have proposed a harmonized international MRL for dexamethasone fixed at 2 µg/kg for cattle, pig, and horse liver samples.[1]

In parallel of their regulated use for therapeutic purposes, β-agonists are potentially misused as growth- promoting agents in food producing animals.[2] As a result, efficient measurement methods based on mass spectrometry have been dedicated to these substances for many years. These methods rely on the direct measurement of drugs in a targeted mode, which only allows for the detection of a restricted number of compounds. Problems arise because there are many possible structures for β-agonists, which exhibit activity at the β2-adrenoreceptor level. Therefore, a range of compounds, either of known chemical structures but not yet included in the methods, or of unknown chemical structure, are missed during routine screening and confirmatory analysis. In addition, it has been reported that there are some cases where there has been the use of "cocktail" mixtures of very low amounts of several active substances. These factors make it a challenge to expose illegal practices. The possibility of extended multi-analyte monitoring with high sensitivity, as well as the capability of combined acquisition modes for structural elucidation of unknown compounds and/or unknown screening analyses, are examples of new challenges for the last generation of instruments in this field.

Recombinant bovine somatotropin (rbST), also known as growth hormone, is used in some countries as a general growth promoter in pigs and cattle, but also in lactating cows to increase milk production.[3,4,5] Different regulations exist regarding its use, but the lack of analytical methods for its detection makes it difficult to apply these regulations. It turns out to be an international issue in terms of animal doping, as well as food safety. Indeed, residues of rbST can be present in food produced by animals treated with this hormone. In order to detect residues of rbST in biological matrices, the analysis is targeted at the tryptic N-terminal peptide of the protein, specific to the difference between the endogenous and recombinant forms. The N-terminal amino acid alanine present in the endogenous form is replaced by a methionine in the recombinant one.

This application note describes the use of Waters® Xevo™ TQ MS to address some of the analytical challenges previously described when analyzing growth promoters in biological samples.

EXPERIMENTAL

Standards

All corticosteroids and β-agonists reference compounds were provided by Sigma (St Louis, MO, USA). The peptide used as a standard for the growth hormone application (= Nterm rbST), exhibits the following amino acid sequence: MFPAMSLSGLFANAVLR. This peptide was synthesized from Millegen (Labege, France).

Sample preparation

Sample extraction and purification procedures applied for corticosteroids in liver and β-agonists in urine have been described elsewhere.[6,7]

UPLC conditions

System: ACQUITY UPLC®
Runtime: 6.00 min
Column: ACQUITY® BEH C_{18}
1.7 µm, 2.1 x 50 mm

Mobile phase:

A: 0.1% formic acid (growth hormone) dissolved in water, or
0.1% acetic acid (corticosteroids and b-agonists) dissolved in water

B: CH_3CN + 0.1% formic acid (growth hormone), or
+ 0.1% acetic acid (corticosteroids and ß-agonists)

Flow rate: 0.8 mL/min
Injection volume: 2.0 µL

	Time (min)	Flow Rate	%A	%B
1.	Initial	0.800	100.00	0.00
2.	0.60	0.800	100.00	0.00
3.	4.00	0.800	0.00	100.00
4.	5.00	0.800	0.00	100.00
5.	5.10	0.800	100.00	0.00
6.	6.00	0.800	100.00	0.00

MS conditions

Instrument : Xevo TQ MS
Ionization mode: ESI+ (growth hormone and ß-agonists), ESI- (corticosteroids)
Capillary voltage: 1.50 kV
Cone voltage: 20.00 V
Source temp: 150 °C
Desolvation temp: 550 °C
Desolvation gas: 1000 L/hr

Collision gas flow: 0.15 mL/min

MRM method parameters

MRMs for β-agonists and rbGH were automatically generated using Waters' IntelliStart™ Software. IntelliStart includes a series of automated tools to streamline Xevo TQ operation and workflow.

	Charge State (z)	Precursor	Product	Cone V	Collision Energy
Corticosteroids					
Cortisone	1	329.30	137.10	45	20
Prednisolone	1	329.30	295.30	45	15
Cortisol	1	331.30	282.30	45	20
Methylpdrenisolone	1	343.30	309.30	45	15
Dexamethasone	1	361.30	307.30	40	15
Prednisolone/ Cortisone	1	419.40	329.30	20	10
Cortisol	1	421.40	331.30	15	10
Prednisolone-d6	1	425.40	335.30	15	10
Methylpdrenisolone	1	433.40	343.30	15	15
Dexamethasone	1	451.40	361.30	20	15
Dexamethasone-d4	1	455.40	365.30	20	15
β-agonists					
Terbutaline	1	226.14	107.01	22	28
	1	226.14	125.06	22	26
	1	226.14	152.08	22	16
	1	226.14	170.06	22	12
Salbutamol	1	240.14	120.95	20	30
	1	240.14	148.10	20	18
	1	240.14	166.07	20	12
Salbutamol d6	1	246.14	228.00	20	12
Metoprolol	1	268.20	116.12	30	18
	1	268.20	133.05	30	24
	1	268.20	159.09	30	20
	1	268.20	191.11	30	16
Clenbuterol	1	277.07	132.02	20	28
	1	277.07	140.02	20	46
	1	277.07	167.96	20	30
	1	277.07	203.04	20	16
Clenbuterol d6	1	283.07	255.00	20	16
Growth Hormone					
rbGH Nterm peptide	3	609.33	120.09	18	34
	3	609.33	141.12	18	50
	3	609.33	169.11	18	38
	3	609.33	251.21	18	20
	3	609.33	643.50	18	25
	3	609.33	774.27	18	20
	2	913.36	141.12	34	50
	2	913.36	169.11	34	46
	2	913.36	175.09	34	46
	2	913.36	774.27	34	26
	2	913.36	1047.70	34	25

RESULTS AND DISCUSSION

Corticosteroids

Figure 1 shows extracted MRM ion chromatograms of dexamethasone obtained from a liver sample spiked at various concentration levels. This figure clearly demonstrates the capability to unambiguously detect this target analyte at a concentration of more than 10 times lower than the MRL using Xevo TQ MS. Even if this level of sensitivity is not required for regulatory control purposes (efficient measurement at half MRL is sufficient), this is a very comfortable interpretation, and is clearly beneficial for the analyst. Moreover, it represents an answer to the current lack of analytical methods dedicated to this particularly complex matrix, as highlighted by JECFA. Another advantage linked to this instrumental sensitivity is a reduction of sample amount required for analysis, with subsequent reduction of matrix effects and direct positive impact on quantitative performances.

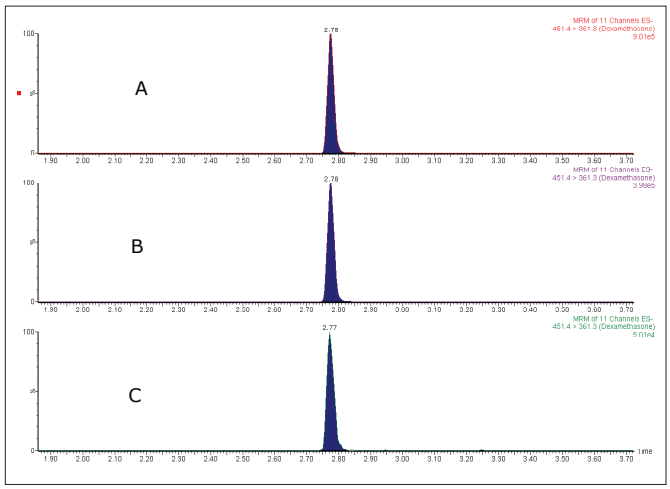

Figure 1. UPLC® (ESI-)/MS/MS MRM diagnostic signal of dexamethasone (451.4 > 361.3) obtained from a liver sample extract spiked at (A) 1 ng/g, (B) 0.5 ng/g, and (C) 0.1 ng/g.

Figure 2 shows MRM chromatograms of the three target exogenous corticosteroids for which a MRL is imposed (dexamethasone, methylprednisolone, and prednisolone), as well as two endogenous corticosteroids (cortisol and cortisone) obtained from a liver sample spiked at 0.1 µg/g. This figure illustrates the capability to unambiguously identify target compounds according to EU/2002/657 requirements, as well as the good chromatographic resolution of cortisone and prednisolone using the ACQUITY UPLC System. This is not always possible with conventional HPLC.

Instrument precision was assessed by injecting the same liver sample extract spiked at 0.5 ng/g six times. The obtained relative standard deviation (%RSD) of the absolute signal intensity was found to be lower than or equal to 5% for all the target analytes, indicating good analytical performance in heavy matrix.

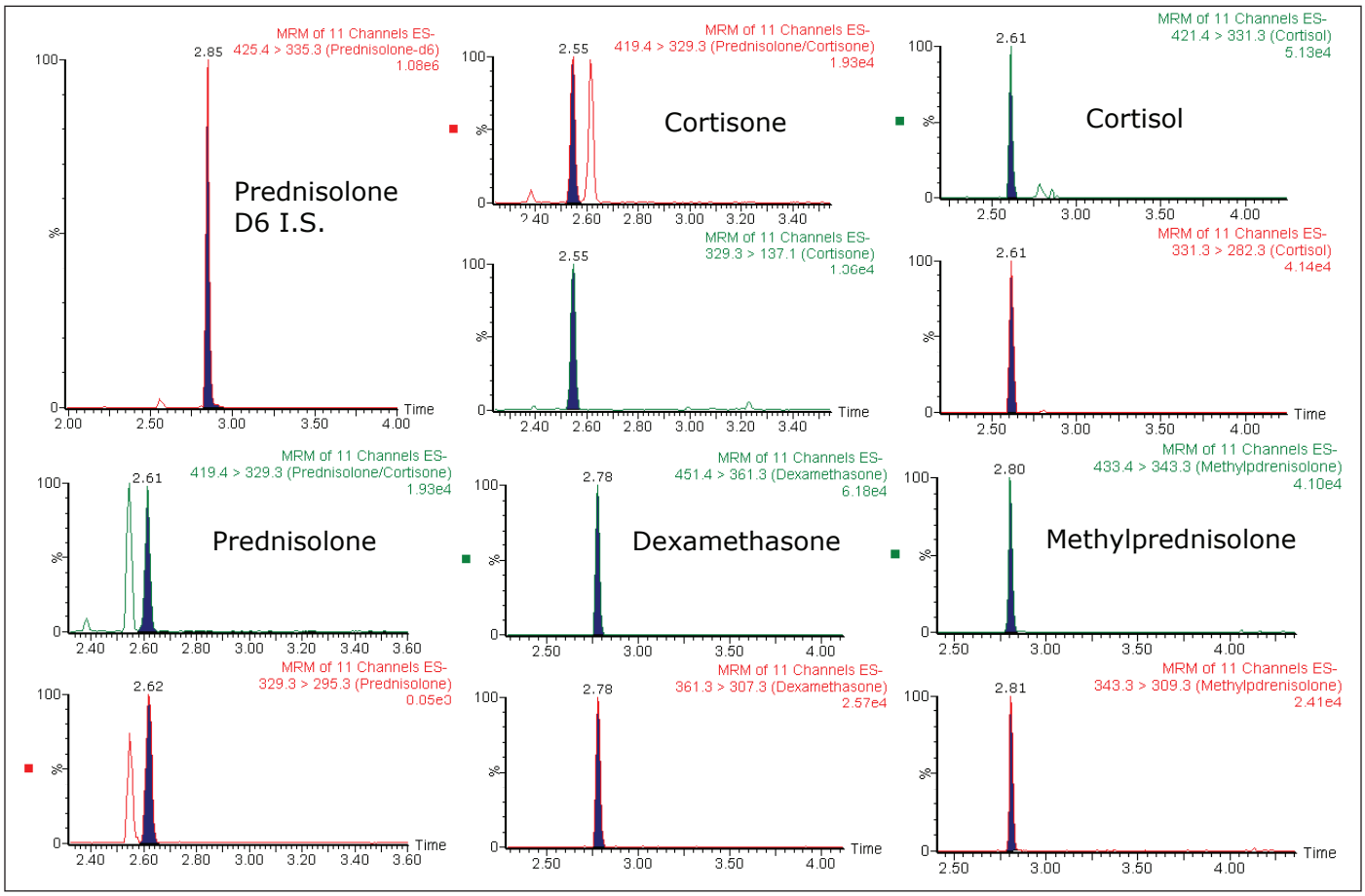

Figure 2. UPLC (ESI-)/MS/MS MRM diagnostic signals of prednisolone-d6 (I.S.), endogenous cortisol and cortisone, and prednisolone, dexamethasone and methylprednisolone, obtained from a liver sample extract spiked at 0.1 ng/g.

Experiments were also performed using alternative acquisition modes, such as neutral loss scan. This mode is particularly adapted for corticosteroids due to a loss of formaldehyde observed in negative electrospray ionization (CH_2O, m=30 mu), which is a characteristic of this family.[8] The results shown in Figure 3 demonstrate very good sensitivity in neutral loss mode, compared to other existing references, when expanding the scope of the analysis to unknown compounds belonging to the corticosteroid family. Additionally, Figure 3 shows the ability to monitor the background matrix with simultaneous MRM full-scan acquisition (Dual Scan-MRM matrix monitoring). This functionality allows real-time, qualitative information about the nature of the matrix to be acquired at the same time as routine quantitative analyses.

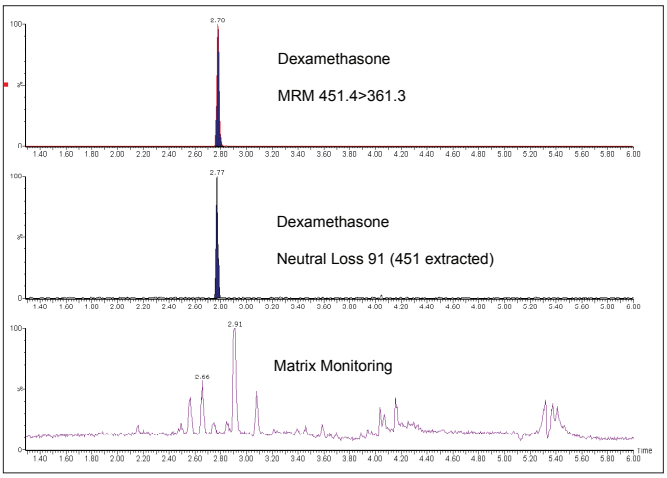

Figure 3. Specific extracted chromatograms of dexamethasone obtained from a liver sample extract spiked at 0.1 ng/g acquired in (A) UPLC (ESI-)/MS/MS in MRM mode, (B) UPLC (ESI-)/MS in neutral loss 90, and (C) UPLC (ESI-)/MS in simultaneous full-scan "matrix monitor" modes.

β-agonists

Initial instrument setup and MRM optimization of the β-agonists were automatically performed using IntelliStart Software. Compound information was entered and automatic method development was performed to generate multiple, fully optimized MRMs for each β-agonist. Figure 4 gives the extracts from the IntelliStart-generated method development report that shows the optimization of cone voltage, location of most abundant daughters, and optimization of collision energy for clenbuterol.

Figure 5 shows MRM chromatograms of clenbuterol obtained from a urine sample spiked at various concentration levels. These results indicate the clear capability to identify this target compound at concentrations as low as 10 pg/mL (ppt). These results provide a comfortable determination, considering the minimum required performance levels (MRPLs) currently in place, or being discussed at the European level. Moreover, it should be emphasized that the injected volume/final extract volume ratio in this case was equal to 2:50, which means there is potential for the sensitivity of the method to be improved by a factor of 5.

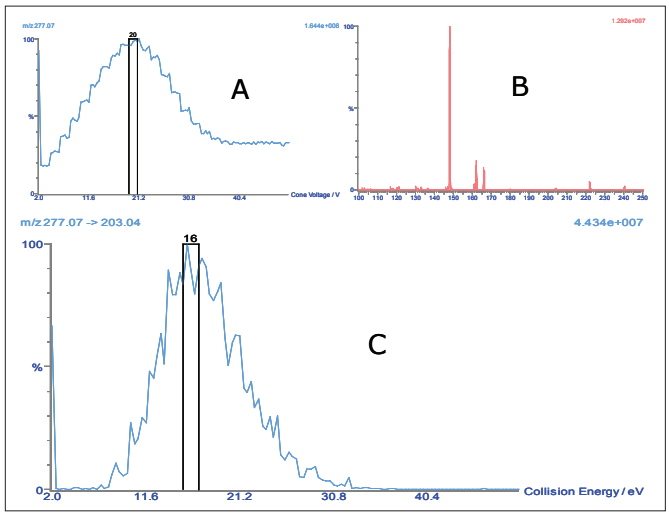

Figure 4: IntelliStart's automatic method development feature shows (A) optimization of cone voltage, (B) location of most abundant daughters, and (C) optimization of collision energy for clenbuterol.

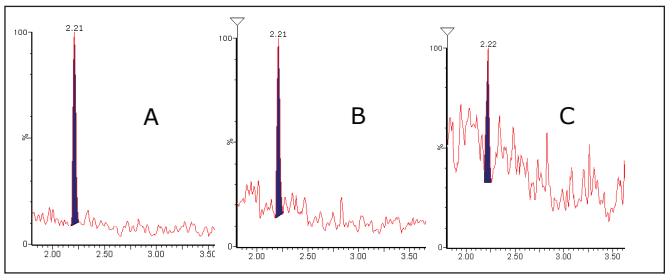

Figure 5. (A) UPLC (ESI+)/MS/MS MRM diagnostic signal of clenbuterol (277.1 > 203.0) obtained for a urine sample extract spiked at 20 pg/mL, (B) 10 10 pg/mL, and (C) 1 pg/mL.

Figure 6 results were obtained in other acquisition modes, including neutral loss (74 mass units), which corresponds to a cleavage typically observed for one sub-class of β-agonists compounds, and full-scan mode performed simultaneously with MRM mode (Dual Scan-MRM matrix monitoring). Once again, the sensitivity and specificity achieved using the Xevo TQ MS in neutral loss acquisition mode appears extremely promising for the development of generic screening approaches for detecting new β-agonists structures belonging to predefined sub-families.

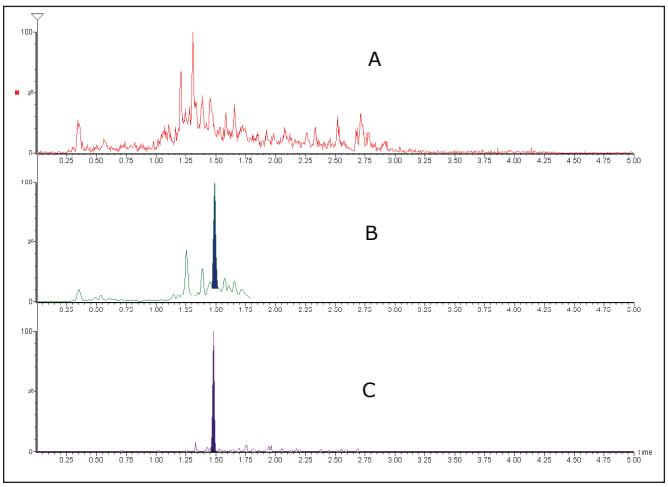

Figure 6. Specific extracted chromatograms of terbutaline obtained from a urine sample extract spiked at 20 pg/g acquired in (A) UPLC (ESI+)/MS (Dual Scan-MRM matrix monitoring); (B) UPLC (ESI+)/MS/MS (MRM 226.1 > 152.1); and (C) UPLC (ESI+)/MS (neutral loss 74).

Growth hormone

For this analysis, electrospray ionization in positive mode was used to detect a peptide specific to the recombinant form of the bovine growth hormone. This ionization mode served as a "soft" ionization technique that is optimal for peptides. The ionization of the N-terminal peptide rbST leads to two main forms (z=2 and z=3). Main transitions and related conditions were optimized using IntelliStart Software.

Some of the transitions generated by IntelliStart were those usually monitored for this compound (913.36 > 1047.7, and 774.27), and are known with respect to amino acid sequence and consequently were very specific for the target molecule. IntelliStart also proposed intense fragments corresponding to smaller ions (251.21, 175.09, 169.11, 141.12, and 120.09) which had not yet been identified or considered that were potentially specific to the peptide of interest (Figure 7). Application of the developed acquisition method to spiked bovine serum (5 ng peptide on column) led to very specific detection of the peptide without any interference on the chromatogram (Figure 7). Observed sensitivity and signal-to-noise ratios were very good when using the Xevo TQ for detecting expected levels of the protein in animal serum.

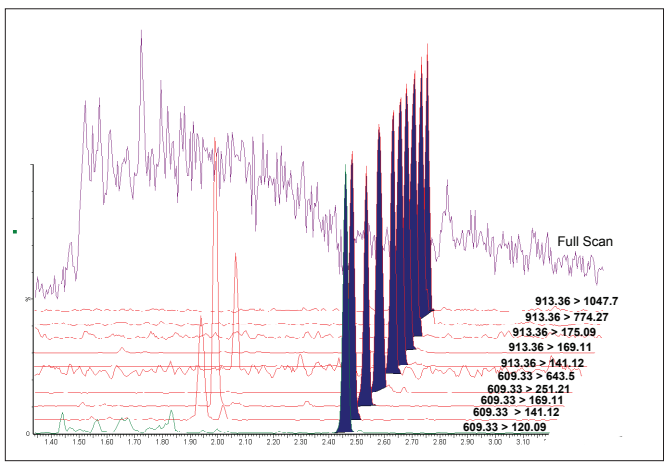

Figure 7. Extracted MRM chromatogram overlay (with offset) of serum spiked with growth hormone N-terminal tryptic peptide (5 ng on column equivalent). Also overlaid is a Dual Scan-MRM (matrix monitoring) TIC showing a high-matrix background.

CONCLUSIONS

- The high level of sensitivity offered by the Xevo TQ MS allows unambiguous determination of corticosteroids at the required MRL and offers an answer to the problem of lack of methodology in highly complex matrices, such as liver.
- The high sensitivity of the Xevo TQ MS allows for reduced samples sizes, and as a result, directly improves quantitative performance.
- The high sensitivity of Xevo TQ MS in neutral loss mode facilitates the development of new generic screening approaches in this field applied to corticosteroids and β-agonist analysis. This could potentially help identify new active substances.
- A high degree of specificity and sensitivity was observed for the analysis of recombinant bovine somatotropin. The use of IntelliStart Software on Xevo TQ MS allowed the discovery of MRM transitions not previously considered for this analysis.

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