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Quantitative determination of several synthetic corticosteroids by gas chromatography–mass spectrometry after purification by immunoaffinity chromatography

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Abstract

A study was conducted to test a multiresidue analytical procedure for detecting and quantifying several corticosteroids on which the European Union imposes maximum residue limits (MRLs). Primary extracts from different matrices (liver, milk, urine, faeces) were first purified on C_{18} cartridges. A new immunoaffinity clean-up step was included. The immunoaffinity gel was used to purify several corticosteroids simultaneously with enrichment of the corresponding fractions. The extracts were treated with an aqueous solution of pyridinium chlorochromate to fully oxidise all corticosteroids and to facilitate their extraction with dichloromethane. After evaporation, the final extract was reconstituted with toluene before injection into the GC–MS apparatus. The analysis was performed in the CI-negative ionisation mode using ammonia as the reactant gas. The estimated detection and quantification limits were, respectively, 0.25 and 0.5 ppb or lower. Overall, the method is reproducible to within 20%. Recovery is between 50 and 80% according to the corticosteroid. © 1997 Elsevier Science B.V.

Keywords: Corticosteroids; Dexamethasone; Flumethasone; Prednisolone; Methylprednisolone; Isoflupredone

1. Introduction

Corticosteroids are used to treat various diseases in cattle. There are plans to establish maximum residue limits [1] in the European Union for cattle treated with isoflupredone, a corticosteroid used to treat ketosis in dairy cows. It is therefore crucial to rely on accurate, sensitive and specific analytical methods to measure residues in biosamples. Standard methods detect a single product and lack overall sensitivity and reliability. The specificity of immunoassays has been improved by adding a preliminary HPLC or immunoaffinity purification step to the detection process [2–5]. The physico-chemical techniques developed for corticosteroids are often less sensitive. Additional purification steps combined with negative chemical ionisation appear efficient to improve the sensitivity [6,7]. Such techniques have been used mainly to detect dexamethasone in various biological samples [8–12]. The combined use of immunoaffinity chromatography with gas chromatography–negative ion chemical ionisation mass spec-

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trometry has been described as a means of confirming flumethasone abuse in equines [13]. TLC has also been used to detect corticosteroids at injection sites [14]. This paper describes the development and validation of an analytical procedure for identifying and quantifying most corticosteroids in a single test run during control screening. The test was validated for prednisolone, methylprednisolone, isoflupredone, flumethasone and dexamethasone in various biological matrices such as milk, liver, urine and faeces from cattle.

2. Experiinental

2.1. Materials

2.1.1. Reference compounds

Reference products were: isoflupredone (Isoflu, U6J39) from Upjohn (Puurs, Belgium) and prednisolone (Pred, P6004), 6-α-methylprednisolone (Methylpred, M0639), flumethasone (Flu, F9507), and dexamethasone (Dex, D1756) from Sigma (St. Louis, MO, USA). Isotopically labelled dexamethasone (2,4,6-[$^{2}H_{3}$]-dexamethasone – courtesy of Prof. J.M. Midgley, Glasgow, UK) was used as an internal standard for accurate GC–MS quantification; tritiated dexamethasone (Amersham, Little Chalfont, UK) was used as a carrier for the recovery controls. All stock solutions (1 ng/ml in ethanol) were prepared monthly and stored in the dark at −20°C; working solutions were prepared weekly.

2.1.2. Chemicals

Pyridinium chlorochromate from Sigma used to oxidise the corticosteroids was used without any further purification. Enzymatic hydrolysis was performed with *Helix pomatia* juice (Boehringer Mannheim, Mannheim, Germany, 127698). Other chemicals and reagents were of the highest purity grade. PBS (pH 7.4; 0.05 *M*) and sodium acetate buffer (pH 4.5; 0.2 *M*) were prepared daily. Solvents such as absolute ethanol, toluene, methanol, acetone, ethyl acetate, hexane and acetonitrile, used in the various steps of the extraction procedure (see below), were of the highest quality grade (pro analysis).

2.1.3. Biological specimens

Pooled samples of bovine milk, liver, urine and faeces from untreated animals were used as blanks and for calibration. Test samples were taken from two cows (Holstein), weighing about 500 kg each, treated simultaneously with a single intramuscular injection of each of the several corticosteroid specialities (5 ml Dexafort®: dexamethasone natrii phosphate 1.32 mg/ml and dexamethasone phenylpropionate 2.67 mg/ml; Cortexilar®: flumethasone 0.5 mg/ml; Predef®: isoflupredone acetate 2 mg/ml; Moderin® long acting: methylprednisolone acetate 20 mg/ml and 10 ml of a 2.5% solution of prednisolone).

2.1.4. Extraction and purification materials

The biological materials were subjected to a preliminary C_{18} column extraction step (6-ml and 3-ml capacity Baker columns; respectively ref. 7020-07 and ref. 7020-03). The materials were prepared as recommended by the supplier.

Immunoaffinity chromatography gel columns were used for one purification step. They were prepared by 50:50 (v/v) mixing of a dexamethasone-specific gel (batch TFF 12 Dexa 94) with a methylprednisolone-specific gel (batch TFF 12 MP 94). IgGs (3 mg per ml gel) were coupled to a Tresyl fast flow from Pharmacia (Uppsala, Sweden). The gels were produced by the Laboratory of Hormonology, CER (Marloie, Belgium).

2.1.5. Instrumentation

In addition to the usual laboratory equipment such as a Sorval Centrifuge, a sonicator (Ultrasonic) and shakers (Transsonic), we used the following instruments for analytical quantitation: an LKB 1212 β counter (LKB Pharmacia, Brussels, Belgium) for recovery determinations (scintillation cocktail: Aqualuma Plus) and a Trio 2000 gas GC–MS instrument from Micromass (Altrincham, UK) for the corticosteroid measurements. GC separations were carried out on a Carlo Erba 8060 gas chromatograph using fused silica capillary columns (HP5 MS 30 m×0.25 mm I.D. phase layer 0.25 μm from HP, Brussels, Belgium). The splitless injection mode was selected and the injection temperature set at 300°C. The oven temperature was programmed from 150 to 310°C at a rate of 15°C/min. The temperature of the

transfer line to the MS ion source was maintained at 310°C (and monitored during the whole process). MS quantitation was performed using a selected ion monitoring technique. A negative CI (NH_3) ionisation mode was selected to provide maximum sensitivity. Both the temperature source (190°C) and the reagent gas pressure in the ion source (from $4 \cdot 10^{-3}$ to $9 \cdot 10^{-3}$ Pa) are crucial experimental parameters. They must be frequently monitored to avoid any dramatic loss of sensitivity.

2.2. *Extraction procedure*

2.2.1. *Primary liquid–liquid extraction*

2.2.1.1. *Milk extraction.* Aliquots (10 ml) of pooled samples (biological, blank or spiked specimens) are added to a small volume (no more than 100 μl) of a standard solution containing both trideuterated and tritiated dexamethasone. The concentration of dexamethasone labelled with the stable isotope should not exceed 2 ppb. The amount of tritiated dexamethasone required to achieve sufficient statistical significance in the recovery estimate calculations is about 10 000 cpm. After equilibration at room temperature for 30 min, the samples are centrifuged for 20 min at 7200 *g* and 4°C. The floating fat layer is eliminated. The residual skimmed milk phase is diluted with an equivalent volume of water (10 ml). The whole sample is deposited on top of a preconditioned C_{18} cartridge.

2.2.1.2. *Liver tissues extraction.* A 5-g aliquot of a blank or spiked liver-tissue sample is mixed with equal amounts of both internal standards (the recovery determination standard and the corticoid quantitation standard, see above). After a 30-min equilibration period, 10 ml sodium acetate buffer and 100 μl *Helix pomatia* juice are added for deconjugation. The solid samples are placed in an Ultraturax device to be pulverised and homogenized. Mixing is continued for an additional 15 min in a sonicator. The homogenate is then rocked gently and horizontally for 2 h in a water bath maintained at 60°C. After this hydrolysis step, 20 ml acetonitrile are added to each sample and mixed for 30 min. After a 20-min centrifugation at 5000 *g* and 4°C, the supernatant (about 27 ml) is transferred to glass-screwed test tubes. After addition of 8 ml hexane and 2 ml dichloromethane, the samples are mixed under gentle shaking for 10 min and centrifuged at 2000 *g*. The middle layer (about 17 ml) is collected in new tubes to be finally evaporated under a gentle stream of dry nitrogen in a water bath maintained at 60°C. The final extracts are dissolved in 1 ml ethanol by vortexing for 10 s before the addition of 10 ml water. The next step (purification on the C_{18} column) is described below.

2.2.1.3. *Urine extraction.* Five ml of urine sample were treated with 2 ml acetate buffer. If required, the pH was adjusted to 4.8. The sample was then mixed with 50 μl *Helix pomatia* juice and incubated overnight at 37°C. PBS (5 ml) was added before the sample was run through the C_{18} column as described below.

2.2.1.4. *Faeces extraction.* Five grams of faeces were mixed vigourously for 2 h with 10 ml of a sodium acetate buffer and 35 ml ethyl ether. After centrifugation, the ether layer was evaporated under nitrogen and the solid residue dissolved in 3 ml ethanol by vortexing the mixture for 30 s and sonicating for 5 min. Water (12 ml) and hexane (5 ml) were added, the samples mixed for 15 min and centrifuged at 2700 *g* for 15 min. Hexane and solid material were removed by aspiration under a mild vacuum. Before corticosteroid extraction on the C_{18} column, 5 ml water were added to facilitate the elution.

2.2.2. *Solid-phase extraction*

2.2.2.1. *Procedure for milk, urine and faeces samples.* The 6-ml C_{18} extraction cartridges were prepared and conditioned by applying 10 ml methanol and 10 ml water. The biological samples were deposited on top of the column. Several washing volumes were applied before corticosteroid elution: 5 ml water; 5 ml of an acetone–water mixture (20:80, v/v); 5 ml of a methanol–water mixture (20:80, v/v); 5 ml of a dichloromethane–hexane mixture (20:80, v/v); 5 ml of an ethyl acetate–hexane mixture (10:90, v/v).

After the last washing step, the C_{18} columns were vacuum-dried during 30 s. The corticosteroids were

finally eluted with 3 ml ethyl acetate. The eluted organic fractions were evaporated to dryness under a stream of dry nitrogen and the residues finally dissolved in 0.5 ml ethanol plus 5 ml of PBS. These fractions were ready for the subsequent immunoaffinity purification step.

2.2.2.2. *Procedure for liver-tissue samples.* The preconditioning phase of the 3-ml C_{18} columns was as reported above. The washing step differed as follows: 5 ml water; 5 ml of an acetone–water mixture (20:80, v/v); 5 ml of a methanol–water mixture (20:80, v/v) and 5 ml of hexane. The columns were dried under mild vacuum before corticosteroid elution with 1 ml ethyl acetate. The further evaporation and reconstruction steps were performed as reported above.

2.2.3. *Clean-up step on an immunoaffinity column (IAC)*

The following procedure was applied to all the biological specimens.

A column was loaded with 1 ml gel and conditioned with two times 5 ml PBS and two times 5 ml water. The pH of the biological extract was adjusted to the optimal value of 7 to 7.5, after which the extract was loaded on the column. After loading, the column was washed three times with 3 ml water. The corticosteroids were eluted with 3 ml of a methanol–water (80:20, v/v) mixture. The extracts were evaporated to dryness under a stream of dry nitrogen and dissolved in 500 μl ethanol. The mixture was then vortex-mixed for 20 s and a 50-μl aliquot was set aside for recovery determination by liquid scintillation counting.

2.2.4. *Chemical transformation*

In order to increase the sensitivity of the analytical method [8], the following procedure, involving an oxidative reaction with pyridinium chlorochromate, was performed: (i) the remaining methanolic fraction (about 450 μl) was evaporated under a gentle stream of dry nitrogen; the final residue was dissolved in 50 μl acetonitrile and 200 μl of a 50 mg/ml pyridinium chlorochromate solution prepared in 2.5% sodium acetate. These extracts were heated in capped tubes for 3 h in an oven heated to 90°C; (ii) the oxidised corticosteroid derivatives are extracted with 3 ml dichloromethane to eliminate most reactive compounds. The organic layer, collected after centrifugation, was washed with 2 ml of the same solvent before evaporation under vacuum; (iii) the final extracts were reconstituted with 50 μl toluene and were ready for injection into the chromatographic column linked to the mass spectrometer (injection volume: 2 μl).

This oxidative modification step used to generate the final derivatives used for quantification by GC–MS tremendously modifies the structure of the original corticosteroids: the side chain at position 17 is removed and the hydroxyl group at position 11 is fully oxidised to a stable ketone function. The final derivative is a thermally stable trione which behaves very nicely, without adsorption, on an apolar gas chromatographic column. The stability of the derivative favours the generation of abundant ionic species, and hence a higher sensitivity in the selected ion recording mode.

3. Results and discussion

3.1. *Validation of the analytical technique*

3.1.1. *Reference mass spectra and selected ions*

The negative CI mass spectrum recorded for isoflupredone is reported in Fig. 1 as an example. It features very low fragmentation leading to higher sensitivity in the selected ion recording mode. The various ions traced for the different corticosteroids are reported in Table 1 and presented in Fig. 2.

3.1.2. *Selected ion tracer profiles*

As an example, Fig. 3 reports the experimental data collected for milk specimens (a blank and a 2 ppb spiked sample containing each of the tested compounds). Fig. 4 shows the corresponding data recorded for a liver-tissue sample. Dexamethasone (m/z 310) and methylprednisolone (m/z 312) are fully resolved. The signal-to-noise ratio appears sufficiently high to ensure maximum sensitivity detection. No matrix interference was detected in any of the samples.

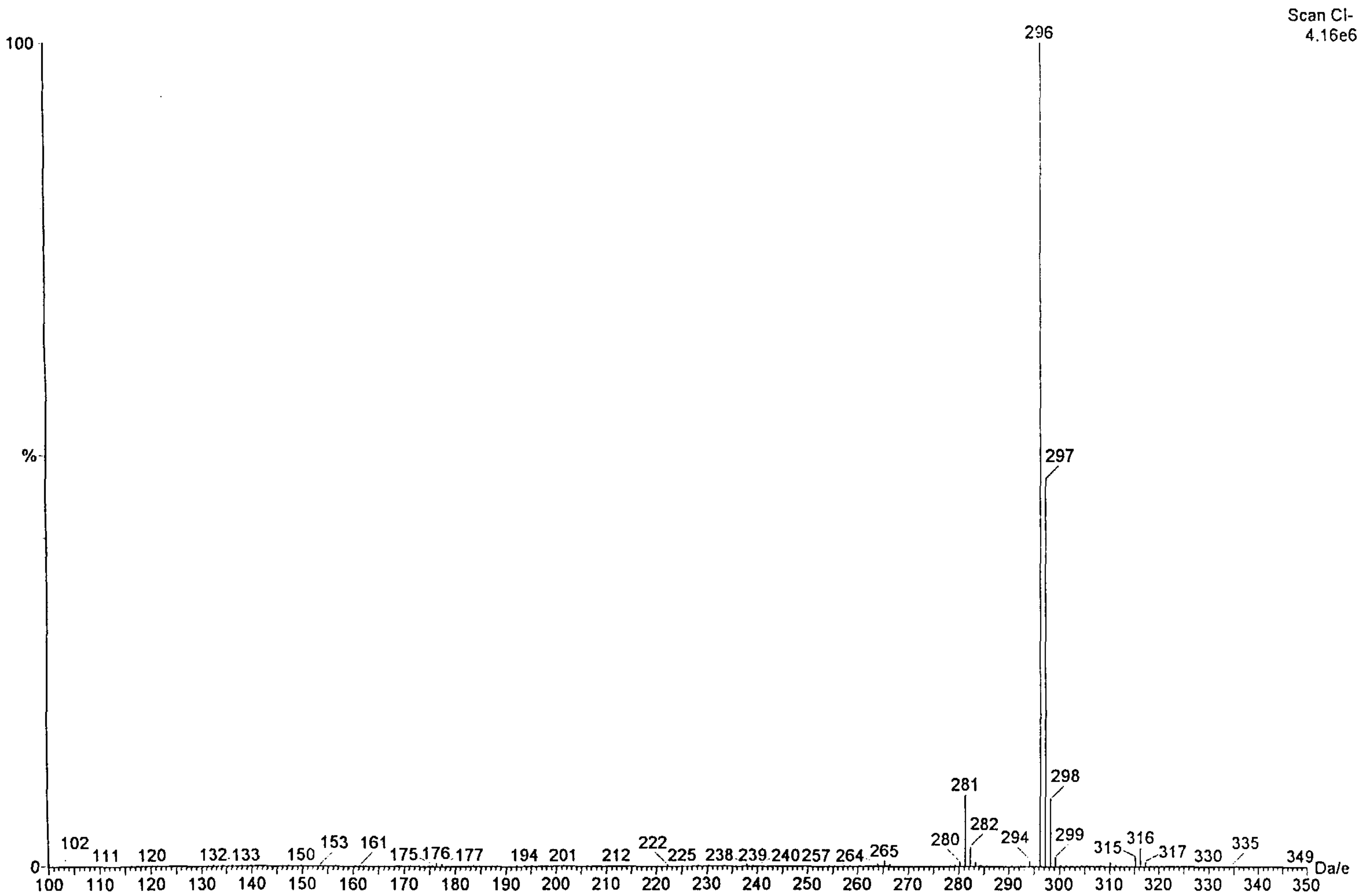

Fig. 1. Negative-CI mass spectrum of the oxydized form of isoflupredone.

Table 1
Selected ions for corticosteroid determinations

Corticosteroid	m/z values
Dexamethasone $^{2}H_3$	313
Dexamethasone	310
Flumethasone	328
Prednisolone	298
Methylprednisolone	312
Isoflupredone	296

3.1.3. Recovery test

The mean recovery values calculated for milk, liver, urine and faeces samples, treated on 4 different days, were respectively 75±7, 60±3, 80±5 and 50±8% (mean±S.D.). No significant difference in recovery was observed at any of the chosen concentrations of corticosteroids according to the Student-*t* test.

3.1.4. Reproducibility

The reproducibility and accuracy of the assay method were tested on four different days. Each day, new calibration curves were prepared as described above with samples obtained from blank biological specimens. Four quality control (QC) samples, covering the tested calibration range, were analysed by GC–MS after a single injection. For each tested corticosteroid, the QC sample concentration values were 0.5, 1, 2 and 4 ppb. The experimental data are reported in Table 2.

We conclude from these data that the reproducibility and accuracy of the assay method are excellent in the tested concentration range for the tested biological specimens.

3.1.5. Repeatability

Assay repeatability was tested on the same day by one technician analysing, with the same calibration curves, four different assay samples of a same spiked pooled biological specimen (milk, liver, urine or faeces). Four different concentrations were tested: 0.5, 1, 2 and 4 ppb. The results are reported in Table 3, where all data are expressed in ppb and are

Table 2
Reproducibility test for milk, liver, urine and faeces samples (all data are expressed in ppb)

Spiked value	Methylprednisolone (mean±S.D.)	Prednisolone (mean±S.D.)	Dexamethasone (mean±S.D.)	Flumethasone (mean±S.D.)	Isoflupredone (mean±S.D.)
Milk					
0.5	0.49±0.08	0.51±0.05	NA	NA	0.50±0.06
1	1.05±0.09	0.99±0.07	NA	NA	0.99±0.08
2	1.95±0.10	1.99±0.10	NA	NA	1.98±0.10
4	4.06±0.07	4.08±0.06	NA	NA	4.03±0.21
Liver					
0.5	0.48±0.02	0.46±0.03	NA	NA	0.49±0.04
1	1.04±0.10	0.97±0.11	NA	NA	0.98±0.07
2	1.99±0.14	2.08±0.07	NA	NA	1.99±0.11
4	3.93±0.23	3.91±0.02	NA	NA	4.01±0.17
Urine					
0.5	0.50±0.06	0.63±0.13	0.52±0.02	0.50±0.06	0.58±0.05
1	1.00±0.08	1.04±0.08	1.02±0.03	0.99±0.06	1.09±0.10
2	1.89±0.09	1.89±0.13	1.91±0.06	1.91±0.09	1.98±0.10
4	3.95±0.14	3.77±0.49	3.90±0.12	3.91±0.06	3.91±0.18
Faeces					
0.5	0.47±0.06	0.52±0.11	0.52±0.06	0.48±0.06	0.49±0.09
1	1.03±0.12	0.98±0.15	1.03±0.08	1.00±0.04	0.91±0.13
2	1.83±0.17	1.80±0.07	1.99±0.14	1.85±0.18	1.85±0.18
4	3.75±0.32	3.73±0.33	3.82±0.18	3.56±0.27	3.57±0.51

The number of determinations was 4 for each level of individual corticosteroid. NA: Not available.

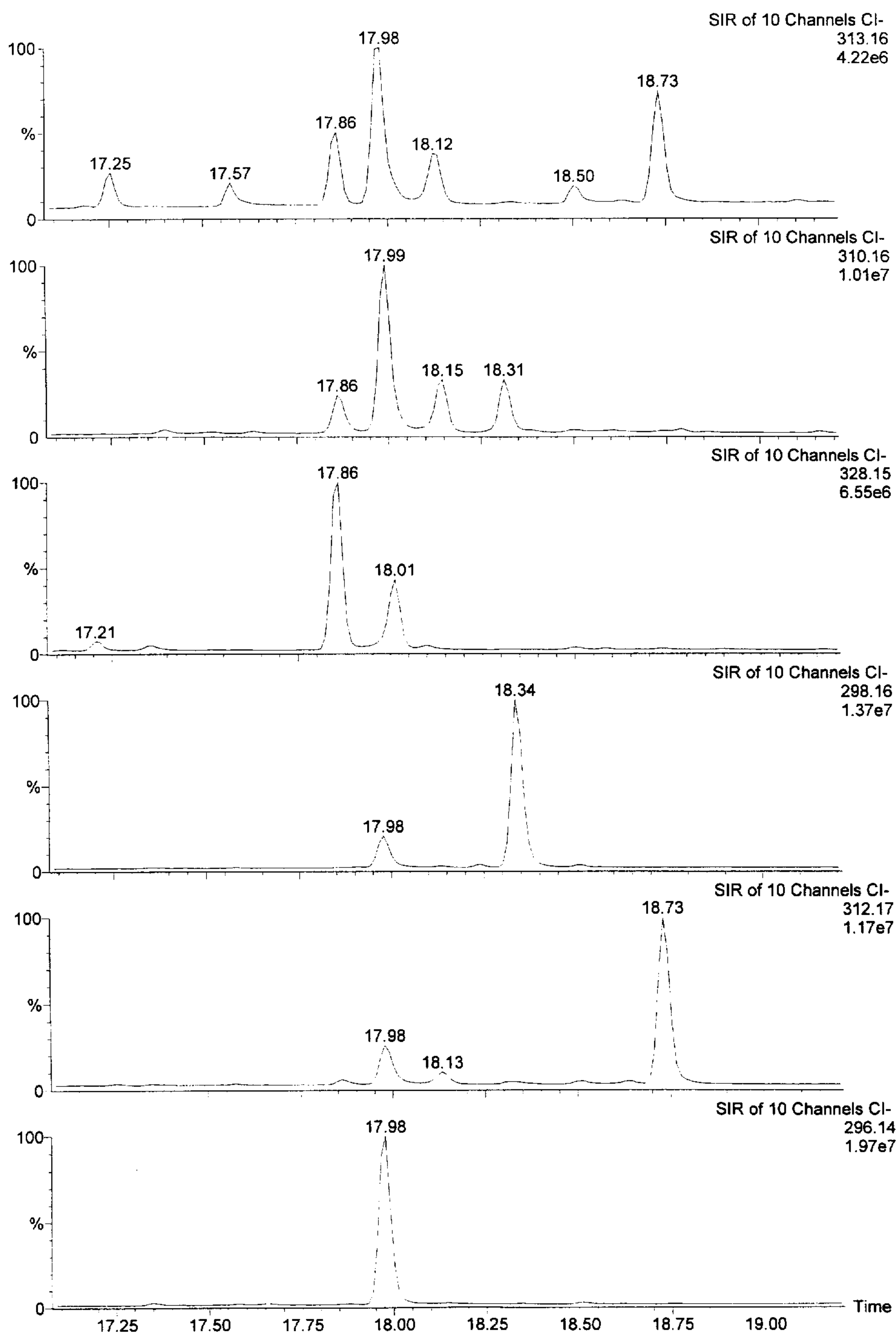

Fig. 2. Selected ions traced, recorded for the various derivatized corticosteroids. From top to bottom: m/z 313, t_R: 17:98/18:12 min for dexamethasone $^2H_3$ (IS); m/z 310, t_R:17:99/18:15 min for dexamethasone; m/z 328, t_R: 17:86/18:01 min for flumethasone; m/z 298, t_R: 18:34 min for prednisolone; m/z 312, t_R: 18:73 min for methylprednisolone; m/z 296 t_R: 17:98 min for isoflupredone.

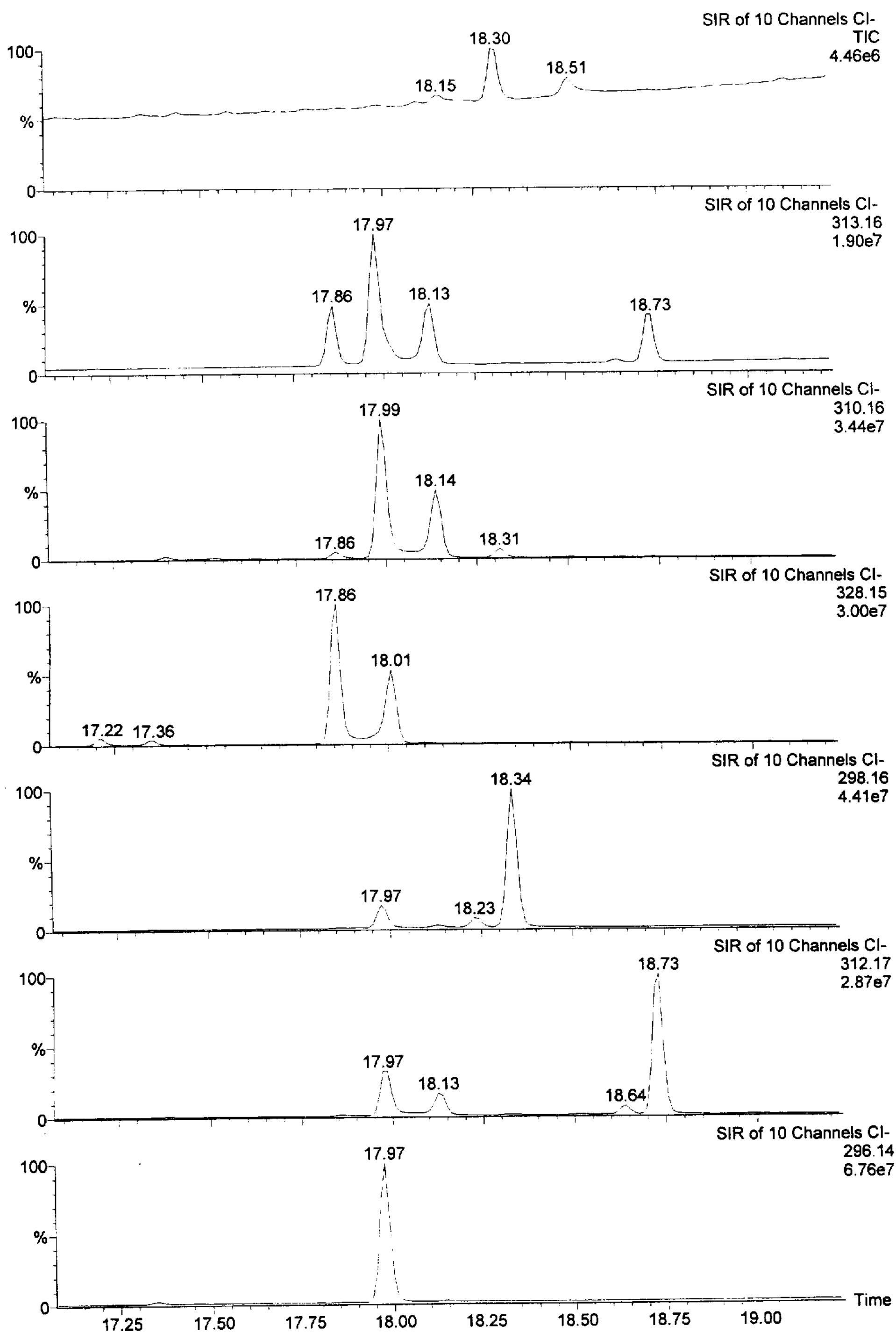

Fig. 3. TIC and SIR recordings collected from blank and spiked milk samples with 2 ppb of the various tested corticosteroids. From top to bottom: total ionic current; m/z 313, t_R: 17:97/18:13 min for dexamethasone $^2H_3$ (IS); m/z 310, t_R: 17:99/18:14 min for dexamethasone; m/z 328, t_R: 17:86/18:01 min for flumethasone; m/z 298, t_R: 18:34 min for prednisolone; m/z 312, t_R: 18:73 min for methylprednisolone; m/z 296, t_R: 17:97 min for isoflupredone.

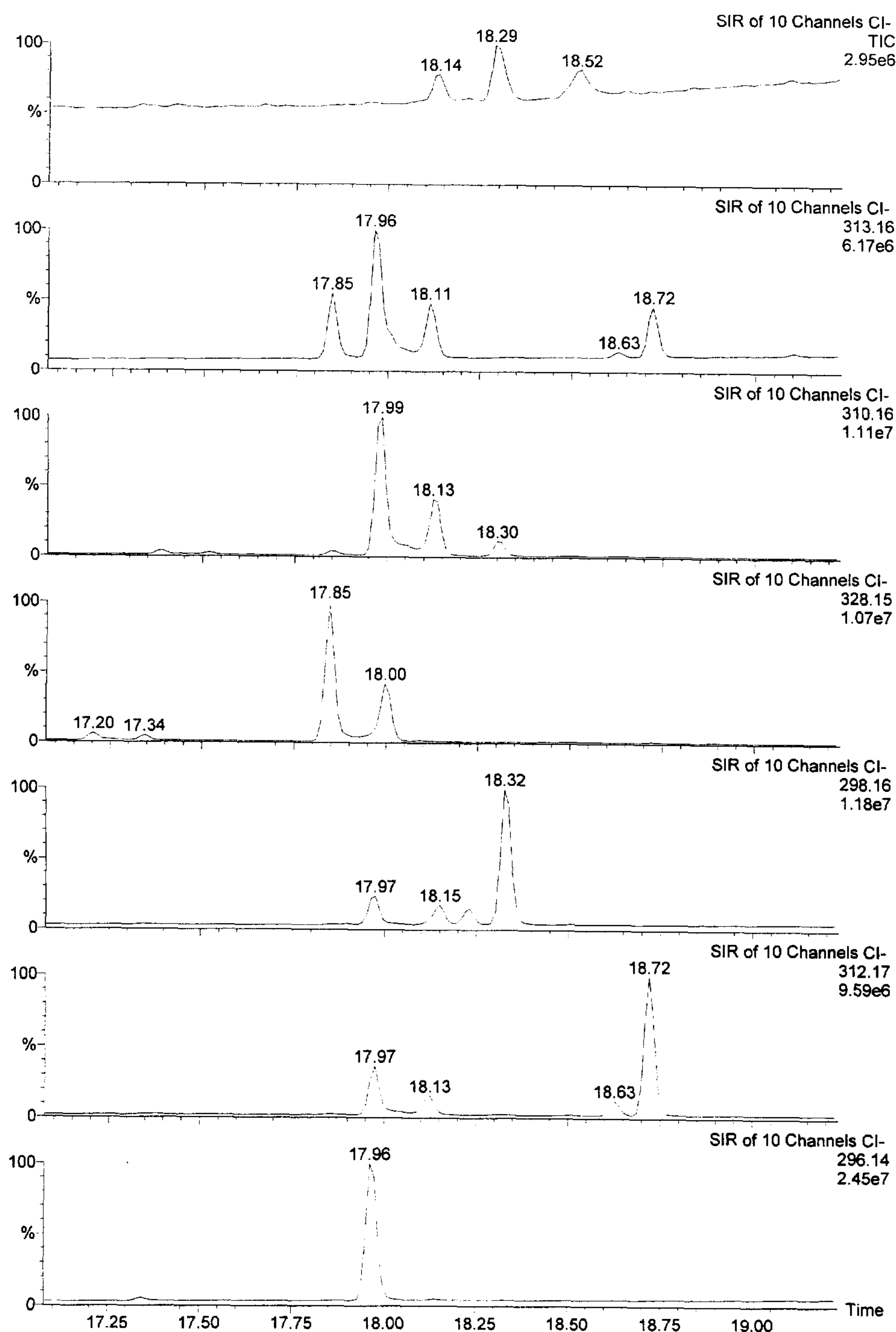

Fig. 4. TIC and SIR recordings collected form blank and spiked liver samples with 2 ppb of the various tested corticosteroids. From top to bottom: total ionic current; m/z 313, t_R: 17:96/18:11 min for dexamethasone $^2H_3$ (IS); m/z 310, t_R: 17:99/18:13 min for dexamethasone; m/z 328, t_R: 17:85/18:00 min for flumethasone; m/z 298, t_R: 18:32 min for prednisolone; m/z 312, t_R: 18:72 min for methylprednisolone; m/z 296, t_R: 17:96 min for isoflupredone.

Table 3
Repeatability test for milk, liver, urine and faeces samples (all data are expressed in ppb)

Spiked value	Methylprednisolone (mean±S.D.)	Prednisolone (mean±S.D.)	Dexamethasone (mean±S.D.)	Flumethasone (mean±S.D.)	Isoflupredone (mean±S.D.)
Milk					
0.5	0.54±0.05	0.52±0.01	NA	NA	0.53±0.01
1	0.96±0.10	0.96±0.13	NA	NA	1.00±0.04
2	1.94±0.08	1.96±0.09	NA	NA	2.02±0.08
4	4.08±0.09	4.05±0.04	NA	NA	3.94±0.07
Liver					
0.5	0.51±0.02	0.47±0.03	NA	NA	0.47±0.01
1	0.97±0.07	1.03±0.08	NA	NA	0.97±0.04
2	2.03±0.15	2.03±0.12	NA	NA	1.99±0.01
4	4.02±0.09	4.02±0.09	NA	NA	3.99±0.08
Urine					
0.5	0.54±0.08	0.70±0.07	0.59±0.06	0.55±0.03	0.60±0.05
1	1.03±0.05	1.04±0.12	1.06±0.09	1.06±0.09	1.04±0.07
2	2.03±0.09	1.82±0.11	2.04±0.10	1.92±0.08	1.92±0.07
4	3.95±0.12	3.39±0.29	3.87±0.12	3.87±0.08	3.84±0.09
Faeces					
0.5	0.45±0.06	0.55±0.06	0.48±0.03	0.56±0.06	0.50±0.07
1	1.00±0.06	1.04±0.10	1.05±0.10	0.99±0.08	1.02±0.11
2	1.82±0.09	1.87±0.10	2.03±0.15	2.06±0.13	1.99±0.17
4	3.36±0.63	3.80±0.25	4.04±0.18	3.84±0.16	3.91±0.09

The number of determinations was 4 for each level of individual corticosteroid. NA: Not available.

directly derived from the calibration curve. The method accuracy error is within 15 to 20%.

3.1.6. Limit of detection (LOD)

The LOD value was estimated using standard reference solutions prepared without any extraction step. The LOD is the lowest detectable (but not quantifiable) amount of corticoid that could be analysed in the selected ion recording mode. The experiment was repeated six times on different days. To better pinpoint the LOD values, we used eight different concentrations (plus blank specimens) so as to provide a broad data base in the lowest concentration range. The calculated mean concentrations are reported in Table 4.

Considering three times the standard deviation

Table 4
Values obtained for standard reference solutions (all data are expressed in ppb)

	Methylprednisolone (mean±S.D.)	Prednisolone (mean±S.D.)	Dexamethasone (mean±S.D.)	Flumethasone (mean±S.D.)	Isoflupredone (mean±S.D.)
0	0.02±0.02	0.01±0.01	0.02±0.03	0.01±0.02	0.02±0.02
0.1	0.10±0.01	0.10±0.01	0.10±0.01	0.01±0.01	0.10±0.01
0.2	0.20±0.03	0.20±0.03	0.19±0.01	0.20±0.01	0.19±0.01
0.5	0.51±0.03	0.53±0.04	0.56±0.01	0.54±0.01	0.57±0.05
1	1.00±0.08	1.00±0.09	1.05±0.04	1.06±0.03	0.99±0.07
2	2.03±0.14	2.03±0.11	1.94±0.08	1.94±0.10	1.91±0.19
3	2.92±0.13	2.96±0.07	2.88±0.04	2.90±0.05	3.00±0.11
4	3.92±0.11	3.85±0.11	3.86±0.05	3.87±0.03	3.86±0.14
5	5.08±0.11	4.98±0.16	4.85±0.13	4.90±0.14	4.92±0.16

The experiment was repeated 6 times on different days.

calculated from the mean background level as the minimum acceptable signal, we obtain an LOD value of 0.25 ppb. This experiment can also be used to test the linearity of the detection response which appears very acceptable throughout the calibration range.

3.1.7. Limit of quantification (LOQ)

Our quantifiability criteria were: accuracy to within 15 to 20% and variability below 15%. The corresponding LOQ value was estimated from the data collected for spiked samples in the reproducibility study. As our criteria were met in all cases, we consider the lowest tested concentration (i.e. 0.5 ppb) to be the LOQ value for each of the tested corticosteroids.

The immunoaffinity chromatography step tremendously improves the GC–MS profile by significantly increasing the signal-to-noise ratio and hence the sensitivity of the test, making this technique suitable for numerous routine determinations of corticosteroids in biological samples. This new analytical technique is suitable both for kinetic and residue analysis.

The combination of two antibodies facilitates the extraction and the purification of almost all corticosteroids, as demonstrated by the data reported in Table 5 showing the cross-reactivity of these antibodies.

The oxidative modification step applied to generate the derivatives quantified by GC–MS drastically lowers the limit of quantification. The thermostable trione behaves nicely on an apolar gas chromatographic column, avoiding any reduction in sensitivity due to peak-tailing and adsorption. Their respective stability is responsible for the low fragmentation reported in the mass spectra leading to the generation of abundant ionic species and consequently to higher sensitivity in the selected ion recording mode. The LOD value of 0.25 ppb for all tested compounds could be established. According to the precision and accuracy criteria applied to the data of the reproducibility tests, the corresponding LOQ value is around 0.5 ppb. We conclude that the reproducibility and repeatability of our assay method remain excellent in the tested concentration range.

3.2. Application to biological samples

To assess this technique in the field, we injected two cows with several corticosteroids (see Section 2.1). Levels of the different compounds were measured over a 36-day period in the urine and faeces (Table 6). In urine samples, methylprednisolone (used in a long-acting form) was detectable throughout the experiment. Prednisolone and flumethasone disappeared more rapidly than dexanmethasone and isoflupredone, which were detected for almost three weeks. In faeces, the overall elimination profiles were roughly identical despite the difficulty of recording the very low levels of the various corticosteroids.

The technique is also suitable for quantifying other corticosteroids. That it allows simultaneous identification of these compounds on the same samples during the same run is a major and decisive advantage not offered by other current methods.

Table 5
Cross-reactivity (%) of the anti-methylprednisolone and anti-dexamethasone antibodies tested with a dexamethasone conjugate in an ELISA test

Corticosteroid	Anti-methylprednisolone	Anti-dexamethasone
Dexamethasone	100	100
Prednisolone	50	42
Isoflupredone	74	27
Triamcinolone	30	10
Betamethasone	100	6
Cortisol	30	5
Flumethasone	45	2
Desoxycorticosterone	12	0.5
6 α-Methylprednisolone	120	0

Table 6
Excretion of various corticosteroids in urine and faeces for two cows (all data are expressed in ppb)

Day	Methylprednisolone				Dexamethasone				Isoflupredone				Flumethasone				Prednisolone			
	Urine		Faeces		Urine		Faeces		Urine		Faeces		Urine		Faeces		Urine		Faeces	
	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2
0	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
1	11.80	13.30	<LOQ	<LOQ	4.48	9.18	14.77	4.75	2.86	2.95	2.28	1.76	1.86	3.91	1.97	0.67	2.09	8.38	<LOQ	<LOQ
2	23.20	15.60	<LOQ	<LOQ	8.59	15.79	10.81	5.56	4.95	2.85	2.44	1.17	1.27	2.10	0.97	<LOQ	1.55	5.60	<LOQ	<LOQ
3	15.35	25.40	<LOQ	<LOQ	12.58	15.40	6.14	7.03	5.43	1.86	1.16	0.75	0.74	0.70	<LOQ	<LOQ	1.38	3.41		
4	22.70	24.55	<LOQ	<LOQ	25.59	14.71	7.74	5.53	5.57	0.97	0.96	<LOQ	0.57	<LOQ			0.94	1.17		
5	9.90	33.75	0.90	<LOQ	4.15	11.11	9.20	4.59	1.82	<LOQ	0.66		<LOQ				0.79	0.50		
6	13.50	43.10	0.89	<LOQ	9.91	16.45	2.52	5.72	1.94	0.57	<LOQ						0.54	0.53		
7	19.55	24.00	<LOQ	<LOQ	10.62	15.37	4.49	4.10	1.70	0.57	0.50						<LOQ	<LOQ		
8	21.15	29.50	<LOQ	0.67	9.76	7.29	6.86	3.97	1.36	<LOQ	<LOQ									
9	17.15	10.20	0.82	<LOQ	8.53	11.01	9.56	2.97	1.14		0.57									
10	18.60	5.20	<LOQ	<LOQ	5.07	7.24	4.89	2.65	0.87		<LOQ									
11	18.50	6.15	0.62	<LOQ	6.56	7.58	5.24	1.82	0.64											
12	15.10	7.30	<LOQ	<LOQ	5.08	5.95	4.15	2.47	0.55											
13	8.00	8.20	<LOQ	<LOQ	2.04	2.20	2.02	1.71	<LOQ											
14	8.50	8.10	<LOQ	<LOQ	1.47	2.13	1.08	1.00	0.44											
15	12.20	6.15	<LOQ	<LOQ	1.47	1.05	0.57	0.55	0.67											
16	7.35	4.70	<LOQ	<LOQ	0.84	0.52	0.56	0.54	<LOQ											
17	14.35	7.60	<LOQ	0.58	0.94	0.50	0.51	0.59	<LOQ											
18	15.20	6.15	0.68	0.64	1.01	0.69	<LOQ	<LOQ	0.97											
19	7.25	6.30	0.66	0.97	0.52	<LOQ			0.53											
20	6.20	4.25	<LOQ	0.86	<LOQ				<LOQ											
21	13.90	4.15	1.04	1.01	0.50				<LOQ											
22	19.65	4.50	<LOQ	0.97	0.61				0.59											
23	5.50	3.45	<LOQ	0.51	<LOQ				<LOQ											
24	8.65	3.95	0.88	0.66																
25	9.20	6.15	0.87	1.01																
26	6.40	4.75	<LOQ	<LOQ																
27	6.50	3.30	<LOQ	0.70																
28	4.45	2.40	0.61	0.63																
29	5.15	4.55	1.13	1.58																
30	3.00	2.10	0.59	0.83																
31	2.45	2.05	0.56	0.70																
32	2.00	2.90	1.54	1.81																
33	5.40	0.80	0.91	0.92																
34	2.30	2.35	0.71	0.80																
35	2.20	2.90	0.80	1.07																
36	1.85	4.65	<LOQ	0.60																

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