AGRICULTURAL MATERIALS

Validation of Screening Method for Residues of Diethylstilbestrol, Dienestrol, Hexestrol, and Zeranol in Bovine Urine Using Immunoaffinity Chromatography and Gas Chromatography/Mass Spectrometry

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A method was developed, using commercially available immunoaffinity chromatography cleanup cartridges, followed by detection by gas chromatography/mass spectrometry, to screen for residues of the hormone growth promotants diethylstilbestrol, dienestrol, hexestrol, and zeranol in bovine urine. The single-laboratory, in-house validation included assessment of recoveries, repeatability, linearity of response, detection capability, and specificity (cross-reactivity) with a suite of antibiotics and other hormonal growth promotants. The method was validated for screening at a target concentration of 2.0 μg/L in urine. The detection capabilities for the analytes were diethylstilbestrol, 0.24; dienestrol, 0.15; hexestrol, 0.84; and zeranol, 0.28 μg/L.

Hormonal growth promotants (HGPs) are substances with anabolic properties that are sometimes administered to livestock to increase feed efficiency and formation of lean muscle mass, and accelerate attainment of market weight. Those HGPs with estrogenic or androgenic activity are also used as veterinary drugs to treat malignant neoplasms and to control lactation and the reproductive cycle (1). Among the HGPs used for these purposes are diethylstilbestrol (DES), dienestrol (DIEN), hexestrol (HEX), and zeranol (ZER). DES, DIEN, and HEX are structurally similar synthetic nonsteroidal estrogens, often collectively referred to as stilbenes. ZER, a derivative of benzoxacyclotetradecin, is synthesized from zearalenone, a mycotoxin produced by *Fusarium graminearium*. It is also a nonsteroidal estrogen. Side effects of the use of these substances include edema, alterations of liver functions, gastrointestinal disturbances, and (in males) thromboembolic risks (1). The structures of these compounds are presented in Figure 1.

Although the use of DES and other stilbenes is not permitted in food animals in North America, the use of all HGPs in food animals is banned in the European Union (EU), and no residues of these substances are allowed in meat products sold there. To meet EU requirements, cattle producers in a number of exporting countries have developed "hormone-free cattle" programs, where animals are certified to have been grown to market weight without the use of HGPs. National authorities are required to collect and test bovine urine for the presence of these HGPs in order to demonstrate that cattle raised for export to the EU are free of these substances (2, 3). For cattle to be acceptable to the EU, the HGPs must not be detectable in urine by a method capable of detecting residues at a concentration of 2.0 μg/L or higher (4). This level defines the minimum required performance limit (MRPL) for the analytical method. To meet the EU regulations, the Canadian Food Inspection Agency (CFIA) required a suitable screening method for these HGPs in bovine urine.

An existing CFIA method for DES, DIEN, HEX, and ZER in tissue, based on a method by Covey et al. (5), was extended to bovine urine. The original gas chromatography/mass spectrometry (GC/MS) determination of the analytes was retained, and a new sample preparation methodology, based on commercially available immunoaffinity chromatography (IAC) columns, was incorporated into the method. This approach was adopted to minimize the amount of time and resources needed to develop and validate the method, and reduce familiarization time for the technical staff. The new screening method was validated for the reliability of detection of the analytes at a concentration of 2.0 μg/L in fortified blank urine samples. The single-laboratory, in-house validation included assessment of recoveries, repeatability, linearity of response, detection capability (CCβ), and selectivity.

METHOD

Apparatus

(**a**) *GC/MS system.*—Agilent 5890 gas chromatograph equipped with splitless injector, Agilent 5972 mass selective detector (MSD), Vectra XM Series 4 computer, Chemstation software version B.01.00 (Agilent Technologies, Palo Alto, CA); CTC A2000SE autosampler (LEAP Technologies, Carrboro, NC).

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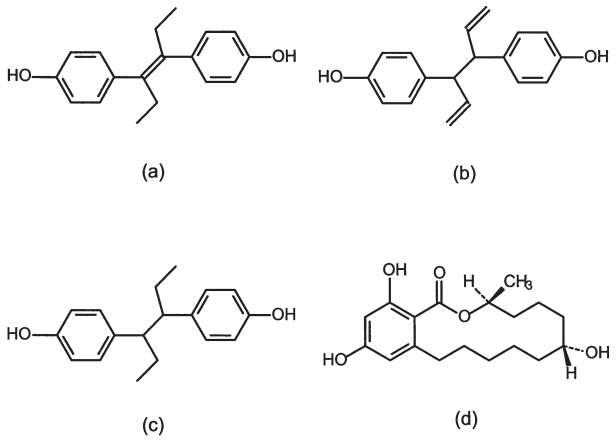

Figure 1. Structures of analytes: (a) diethylstilbestrol, (b) dienestrol, (c) hexestrol, (d) zeranol.

(**b**) *GC column.*—DB-5, 30 m × 0.25 mm id, 0.25 μm film thickness (Agilent Technologies).

(**c**) *Centrifuge.*—IEC Centra GP8R equipped with 20-place swing bucket and capable of 4000 × *g* (International Equipment Co., Needham Heights, MA).

(**d**) *Nitrogen evaporator.*—N-Evap (Organomation Associates, Inc., Berlin, MA).

(**e**) *Solid-phase extraction (SPE) manifold.*—SPE cartridge vacuum manifold (Supelco, Oakville, ON, Canada).

(**f**) *Mixer.*—Vortex-Genie (Fisher Scientific, Nepean, ON, Canada).

(**g**) *Centrifuge tubes.*—15 mL polypropylene, disposable (VWR, Mississauga, ON, Canada); 15 mL glass, disposable (VWR).

(**h**) *Syringes.*—10 and 250 μL (Hamilton, Reno, NV).

(**i**) *Repeater pipettor.*—Eppendorf with 50 mL combi-tip (VWR).

(**j**) *Autosampler vials.*—100 μL, conical, polypropylene with threaded caps (Supelco).

(**k**) *pH Test strips.*—pH 0.0–6.0 (Sigma Chemical Co., Mississauga, ON, Canada).

(**l**) *Stilbenes immunoaffinity columns kit.*—Contains immunoaffinity columns and stock buffer solutions (Cat. No. SJ 2154, Randox Laboratories, Crumlin, Antrim, UK).

(**m**) *ZER immunoaffinity columns kit.*—Contains immunoaffinity columns and stock buffer solutions (Cat. No. ZR 2420, Randox Laboratories).

Reagents

(**a**) *Water.*—Purified by reverse osmosis followed by deionization, absorption, and filtration.

(**b**) *Solvents.*—Ethyl acetate, ethanol, and methanol; high purity grade suitable for residue analysis (Caledon Laboratories, Georgetown, ON, Canada, and EM Science, Gibbstown, NJ).

(**c**) *70% Ethanol.*—Ethanol–water (70 + 30, v/v).

(**d**) *BSTFA.*—*N,O-bis*(trimethylsilyl)trifluoroacetamide in sealed 1 mL ampules (Pierce Chemical Co., Rockford, IL).

(**e**) *TMSI.*—*N*-trimethylsilylimidazole in sealed 1 mL ampules (Pierce Chemical Co.).

(**f**) *Derivatization agent.*—49 μL BSTFA and 1 μL TMSI combined in 100 μL autosampler vial. Not stable; prepare daily. *Note*: Syringes used for measurement of derivatization reagents must be rinsed with acetone before and after use.

(**g**) *Stock column wash buffers and column storage buffers.*—Contained in Randox kits.

(**h**) *Working column wash buffer.*—Stock column wash buffer–water (1 + 19, v/v). Not stable; prepare daily.

(**i**) *Working column storage buffer.*—Stock column storage buffer–water (1 + 4, v/v). Not stable; prepare daily.

(**j**) *Compressed gases.*—Nitrogen, prepure; helium, ultrahigh pure (Praxair, Scarborough, ON, Canada).

Standards

(**a**) *Zearalane.*—Not commercially available; a gift from Schering-Plough (Union, NJ); 99.8% purity as determined by supplier.

(**b**) *ZER.*—α-Zearalanol [55531-29-8] (Cat. No. Z-0292, Sigma Chemical Co.).

(**c**) *DES.*—Diethylstilbestrol [56-53-1] (Cat. No. D4628, Sigma Chemical Co.).

(**d**) *DES-d_8.*—Diethyl-1,1,1′,1′-d_4-stilbestrol-3,3′,5,5′-d_4 (Cat. No. D2849, CDN Isotopes, Pointe-Claire, PQ, Canada).

(**e**) *HEX.*—[84-16-2] (Cat. No. H7753, Sigma Chemical Co.).

(**f**) *DIEN.*—[84-17-3] (Cat. No. D3253, Sigma Chemical Co.).

(**g**) *DCB.*—Decachlorobiphenyl [2051-24-3] 200 mg/L in acetone (Cat. No. 48318, Supelco).

(**h**) *DES, DES-d_8, HEX, DIEN, ZER, zearalane stock solutions, 100 mg/L.*—Into separate 100 mL volumetric flasks, transfer 10.0 mg of each standard. Dissolve and dilute to vol-

Table 1. GC/MS operating conditions

Parameter	Settings
GC parameters	
Carrier gas	Helium at 1.0 mL/min (61 kPa gauge head pressure)
Injection port temperature	250°C
Splitless purge time	1 min
Oven temperature program	Initial temperature: 100°C, hold 1.5 min
	Ramp 1: 30°C/min to 220°C
	Ramp 2: 15°C/min to 290°C
	Final temperature: 290°C, hold 5.0 min
MS parameters	
Ionization mode	Electron impact, positive ion
Solvent delay time	8.0 min
Electron multiplier voltage	Autotune setting plus 500 V
Acquisition mode	Selected-ion monitoring

ume in methanol. Stock solutions are stable for 12 months if stored below 0°C.

(**i**) *DCB stock solution, 20 mg/L.*—Transfer 1.00 mL DCB 200 mg/L stock solution to a 10 mL volumetric flask. Dilute to volume with isooctane. Solution is stable for 12 months if stored below 0°C.

(**j**) *Mixed internal standards solution, 0.1 mg/L DES-d_8 and 0.2 mg/L zearalane.*—Transfer 100 μL DES-d_8 stock solution and 200 μL zearalane stock solution into 100 mL volumetric flask. Dilute to volume with methanol. Solution is stable for 2 months if stored below 0°C.

(**k**) *Mixed working standards solution, DES, DIEN, HEX, and ZER, 0.1 mg/L each.*—Transfer 100 μL each of DES, DIEN, HEX, and ZER stock solutions into a 100 mL volumetric flask. Dilute to volume with methanol. Solution is stable for 2 months if stored below 0°C.

(**l**) *DCB working solution, 2.1 mg/L.*—Transfer 1050 μL DCB stock solution to 10 mL volumetric flask. Dilute to volume with isooctane. Solution is stable for 2 months if stored below 0°C.

(**m**) *Fortified urine matrix standards, 0.0, 1.0, 2.0, and 4.0 μg/L urine equivalent.*—Fortify separate 5.0 mL portions of urine prepared as described below with 0, 50, 100, or 200 μL mixed working standards solution before extraction and cleanup.

(**n**) *Quality control (QC) urine sample, 2.0 μg/L urine equivalent.*—Fortify 5.0 mL portion of urine prepared as described below with 100 μL mixed working standard solution before extraction and cleanup.

(**o**) *GC/MS chemical standard, 1.0 μg/L urine equivalent.*—Transfer 100 μL mixed working standard solution to a 15 mL glass centrifuge tube just before evaporation of column eluates, as described below.

Sample Preparation

All urine samples are kept below 0°C until assayed. Two test portions of each sample are prepared separately for ZER and for stilbenes analyses using 2 different immunoaffinity columns.

Thaw 60 mL blank urine and 12 mL each sample overnight at 4°C. Transfer 2 × 6 mL test portions of each thawed sample urine and 10 × 6 mL portions thawed blank urine into separate 15 mL polypropylene centrifuge tubes. Centrifuge each at 4000 × *g* for 10 min. Decant 5.0 mL portions of supernatant from each tube into separate fresh tubes. Prepare 2 sets of fortified urine matrix standards and QC samples by fortifying portions of blank urine as described above. One set will be used for the ZER analysis, the other for stilbenes analysis.

Extraction and Cleanup

Load the ZER immunoaffinity columns into the vacuum manifold and let column storage buffer run through columns. Equilibrate each column with 15 mL diluted column wash buffer. Load first set of samples, QC sample, and matrix standards onto separate columns and let urine elute just to top of

Table 2. Selected-ion monitoring (SIM) parameters

Compound[a]	Retention time, min	SIM window, min	Ions, *m/z*	Dwell time, ms
cis-DES-$(TMS)_2$	9.0	8.0 to 9.2	412.2	50
			383.2	50
			397.3	50
cis-DES-d_8-$(TMS)_2$	9.0	8.0 to 9.2	420.3	50
trans-DES-$(TMS)_2$	9.5	9.2 to 10.3	412.2	30
			383.2	30
			397.3	30
trans-DES-d_8-$(TMS)_2$	9.5	9.2 to 10.3	420.3	30
DIEN-$(TMS)_2$	9.5	9.2 to 10.3	410.2	30
			395.3	30
			381.2	30
HEX-$(TMS)_2$	9.5	9.2 to 10.3	207.1	30
			191.1	30
			414.2	30
Zearalane-$(TMS)_3$	10.9	10.3 to 15.1	435.3	50
ZER-$(TMS)_3$	12.2	10.3 to 15.1	433.3	50
			523.4	50
			538.4	50
DCB	12.8	10.3 to 15.1	497.7	50

[a] DES = Diethylstilbestrol; DIEN = dienestrol; HEX = hexestrol; ZER = zeranol; DCB = decachlorobiphenyl; TMS = trimethylsilyl.

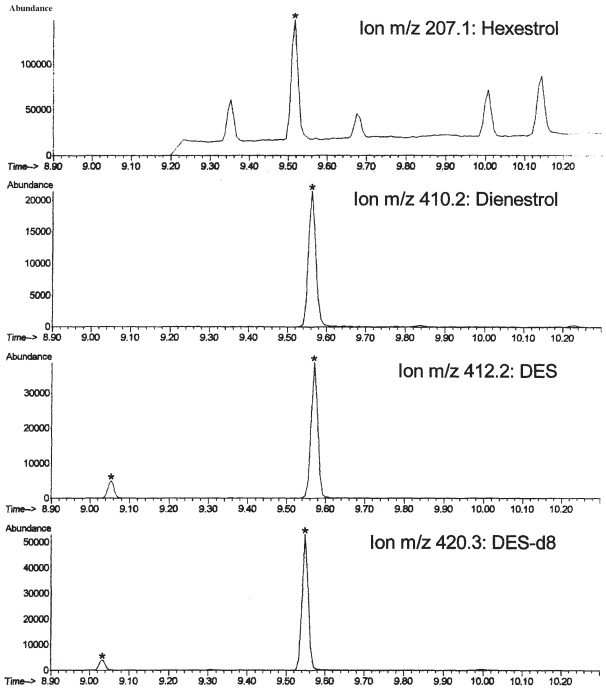

Figure 2. Typical SIM mass chromatograms from GC/MS determinations of hexestrol, dienestrol, *cis*- and *trans*-DES and *cis*- and *trans*-DES-d_8 in blank urine fortified at 2 μg/L. Relevant peaks marked with an asterisk.

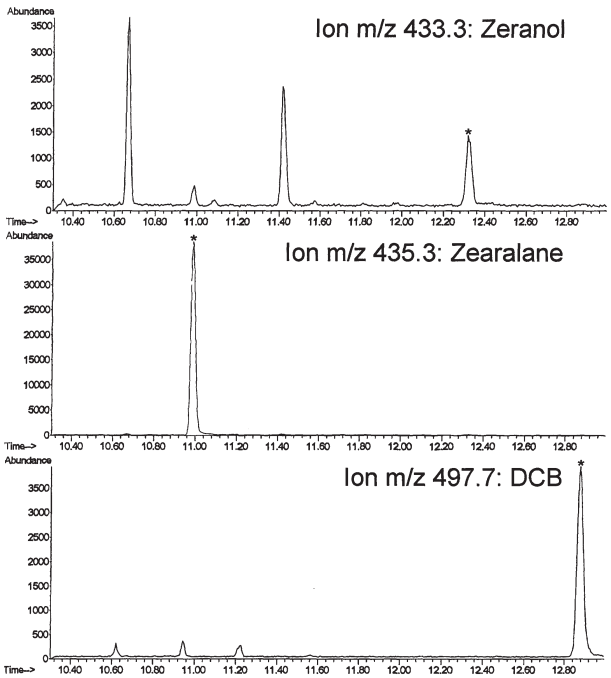

Figure 3. Typical SIM mass chromatograms from GC/MS determinations of zeranol, zearalane, and DCB in blank urine fortified at 2 μg/L. Relevant peaks marked with an asterisk.

bed under gravity flow only. Wash each column with 2 portions of 5 mL diluted wash buffer, followed by 5 mL water. Elute each column with 4 mL 70% ethanol under gravity flow only. Wash columns with additional 10 mL 70% ethanol in water before loading more samples. These columns can be used a total of 10 times before being discarded.

Stilbenes are extracted from the second set of samples, QC sample, and matrix standards using the same procedure as described for ZER, with the following change: stilbenes are eluted from stilbene immunoaffinity columns with 3 mL 70% ethanol under gravity flow only.

Prepare GC/MS chemical standard in centrifuge tube as described above. Evaporate contents of all tubes just to dryness at 60°C under gentle stream of nitrogen. Add 50 μL mixed working internal standard solution and 1 mL ethyl acetate to each centrifuge tube. Evaporate contents of all centrifuge tubes just to dryness. To each 100 μL autosampler vial add 2 μL DCB working solution. Add 25 μL ethyl acetate to each centrifuge tube, mix on a Vortex mixer 10 s, transfer contents to autosampler vials, and cap.

GC/MS Determination

GC/MS operating parameters are given in Tables 1 and 2. This method is applied both to detect ZER using zearalane as internal standard, and stilbenes using DES-d_8 as internal standard. The ion source of the MS is tuned using the Maximum Sensitivity Autotune function of the Chemstation software with perfluorotributylamine (PFTBA) as the mass calibrant. The source is cleaned whenever the ratio of the abundances of ion *m/z* 502 versus ion *m/z* 69 falls below 1.0%. The autosampler, for each sample and standard, draws up 2.0 μL derivatizing agent, 0.2 μL air, and 3.0 μL sample. Derivatization of analytes takes place in the heated injection port.

Calculations and Calibration

The GC/MS chemical standard is run to determine retention times for the analytes. Typical retention times are given in Table 2. Integrated areas (A_{mass}) for each target peak in each selected-ion monitoring (SIM) mass chromatogram are generated by the Chemstation software and entered manually into a Microsoft Excel spreadsheet. Sum ratios are generated for each analyte: SumRatio(ZER) = $(A_{538} + A_{523} + A_{433})/A_{435}$; SumRatio(DES) = $(A_{412}$ (*cis*) + A_{412} (*trans*))/(A_{420} (*cis*) + A_{420} (*trans*)); SumRatio(HEX) = A_{207}/(A_{420} (*cis*) + A_{420} (*trans*)); SumRatio(DIEN) = A_{410}/(A_{420} (*cis*) + A_{420} (*trans*)). Calibration curves are generated for each analyte by plotting SumRatios against concentration using results of the analyses of fortified matrix standards. The results of the analysis of the QC sample are used to construct a control chart for each analyte to monitor the day-to-day intralaboratory performance of the procedure. DCB is used to monitor day-to-day performance of the GC/MS.

Method Validation

Validation of the method was performed in 3 phases. The validation effort was divided into phases so that we could review the data generated in each phase and identify and correct problems before proceeding further.

The purpose of phase 1 was to confirm the linearity of the instrumental response to chemical standards over the concentration range of interest. Phase 1 consisted of chemical standard response curves generated from urine equivalent concentrations of 0, 1, 2, 3, 4, and 5 μg/L for each analyte, each concentration in duplicate, each curve generated on separate days.

The purpose of phase 2 was to assess the quality of the urine matrix-based calibration curves and accuracy and precision of determinations at the target concentration. Phase 2 consisted of 4 runs, each comprised a set of matrix standards fortified by the analyst at urine equivalent concentrations of 0.0, 1.0, 2.0, and 4.0 μg/L to generate calibration curves, plus 6 additional blank urine test portions fortified at 2.0 μg/L urine equivalent.

The purpose of phase 3 was to assess the accuracy and precision of the determination of analytes present in fortified urine blanks over the concentration range of interest. Phase 3 consisted of 2 batches of 6 spikes prepared by fortifying test portions of blank urine at multiple concentrations. Samples were provided as randomized blind samples to the analyst after preparation by a third party. Fortification concentrations were in the range of 1.0 to 4.0 μg/L urine equivalent. Six pairs of blind spikes were prepared for stilbenes, and 9 pairs of blind spikes were prepared for ZER. Calibration curves were generated as in phase 2.

The screening threshold concentration, above which a sample is declared suspect, was determined for each analyte according to the principles given in the drafts of the EU Commission Decision Document SANCO/1085/2000 (6) available when this work was done, which has been finalized and issued as EU Commission Decision 2002/657/EC (7), as follows. For each analyte, the determined concentrations of all phase 2 fortified samples, phase 2 matrix standards at 2.0 μg/L, and phase 3 blind spikes at 2.0 μg/L were sorted from lowest to highest values. The lowest value meeting or exceeding the 5th percentile was chosen as the screening threshold concentration.

The CCβ values for each analyte were determined according to the principles given in SANCO/1085/2000 (6). Decision limits (CCα) were calculated as intermediate values in the calculation of CCβ, but are not reported here. As defined in 2002/657/EC (7), CCα is " ...the limit at and above which it can be concluded with an error probability of α that a sample is noncompliant." CCβ is the lowest analyte concentration "...at which a method is able to detect truly contaminated samples with a statistical certainty of 1-β" (7). For banned substances, α is set at 0.01 (1% error rate) and β is set at 0.05 (5% error rate). The CCβ is required to be at or lower than the MRPL. In this study, the calibration curve procedure was applied using the matrix-based standards which were used to generate the 6 calibration curves from validation phases 2 and 3. The *y*-intercepts and the standard errors of the *y*-intercept

Table 3. Summary of phase 2 standard curves regression analyses

Analyte[a]	Curves	Correlation coefficient, R	Intercept, y_0'	Slope, b	Standard deviation, $s_{y/x}$
ZER	1	0.9997	0.0896	0.348	0.0172
	2	0.9998	0.120	0.332	0.0153
	3	0.9979	0.0162	0.132	0.0181
	4	0.9977	0.0156	0.126	0.0179
DES	1	0.9991	0.0484	0.642	0.0558
	2	0.9989	0.0730	0.842	0.0821
	3	0.9992	0.0602	0.800	0.0686
	4	0.9997	0.0362	0.884	0.0451
DIEN	1	0.9999	0.0104	0.334	0.0117
	2	0.9993	0.0054	0.392	0.0303
	3	0.9999	0.0116	0.450	0.0160
	4	0.9997	0.0154	0.448	0.0241
HEX	1	0.9962	0.450	2.76	0.508
	2	0.9915	0.634	2.59	0.710
	3	0.9717	1.05	2.31	1.17
	4	0.9972	0.486	3.47	0.543

[a] ZER = Zeranol; DES = diethylstilbestrol; DIEN = dienestrol; HEX = hexestrol.

Table 4. Measured concentrations of analytes in phase 2 urine blanks fortified at 2.0 μg/L

Analyte[a]	Run[b]	Average measured concentration, μg/L	Standard deviation, μg/L	CV, %[c]
ZER	1	1.97	0.14	7.1
	2	1.89	0.12	6.1
	3	1.98	0.16	7.8
	4	2.06	0.04	2.0
	Overall	1.97	0.13	6.5
DES	1	2.05	0.05	2.6
	2	2.03	0.04	2.1
	3	2.24	0.03	1.5
	4	2.00	0.06	2.9
	Overall	2.08	0.11	5.2
DIEN	1	1.97	0.24	12.4
	2	1.96	0.11	5.5
	3	2.00	0.04	2.2
	4	1.97	0.06	2.8
	Overall	1.98	0.12	6.2
HEX	1	2.12	0.06	2.7
	2	2.13	0.02	1.0
	3	2.23	0.04	1.8
	4	2.09	0.04	1.7
	Overall	2.14	0.07	3.1

[a] ZER = Zeranol; DES = diethylstilbestrol; DIEN = dienestrol; HEX = hexestrol.
[b] Values for each run are averages of single determinations of 6 fortified test portions.
[c] CV = Coefficient of variation.

(SE_{int}) were calculated from a linear regression analysis of the pooled calibration data. For each analyte, CCα was calculated as the corresponding concentration at the *y*-intercept plus $2.33 \times SE_{int}$. CCβ was calculated as the corresponding concentration at CCα plus $1.64 \times SE_{int}$. Although the strict interpretation of SANCO/1085/2000 (6) requires that, for banned substances, CCα be determined using standards with concentrations at or above the MRPL in equidistant steps, and that CCβ be determined using standards with concentrations at and below the MRPL in equidistant steps, the use of standards bracketing the MRPL was considered a practical compromise to reduce the number of analyses required for validation of the method.

The potential for interferences caused by the presence of other commonly encountered veterinary drug residues was assessed by conducting a selectivity study. Two test portions of blank urine were fortified with a mix of the following antibiotic compounds and HGPs to give a final concentration of 25 μg/L urine equivalent each: penicillin G, tilmicosin, tylosin, sulfathiazole, sulfamethazine, sulfadimethoxine, oxytetracyline, tetracycline, chlortetracycline, 17α-estradiol, testosterone, stanolone, boldenone, androstenedione, melengestrol acetate, methyltestosterone, pregnanediol, ethisterone, norgesterel, progesterone, and stanozolol. Two more test portions were fortified with the same mix to 25 μg/L urine equivalent each, and with ZER, DES, DIEN and HEX to 2.0 μg/L urine equivalent concentration each. A fifth test portion of blank urine was left unfortified. These 5 test portions were processed through the IAC columns and the concentrations of analytes determined by GC/MS.

Results and Discussion

Phase 1 Validation

The data used to generate the response curves were averages of single determinations of duplicate standards at each concentration. Linearity was assessed using the method of Cassidy and Janosky (8), in which the *y*-intercept-corrected slope at each data point, calculated as (response – *y*-intercept)/concentration, is plotted against concentration. Response curves are considered linear if the individual slopes deviate less than a predetermined value, typically 10%, from the average slope of the response curve and are randomly distributed above and below the average slope. The response curves were considered linear according to the criteria above, and the linear regression R values of the response curves (data not shown) were all >0.997.

Table 5. Summary of results from analyses of phase 3 blind spikes

Analyte[a]	Spike concentration, μg/L	*N*	Average measured concentration, μg/L	Average recovery, %	Standard deviation,[b] μg/L	CV, %[c]
ZER	1	2	0.97	97	0	0
	1.1	2	1.04	95	0.03	2.9
	1.3	2	1.35	104	0.07	5.2
	2	6	1.93	96	0.21	11
	2.4	2	1.73	72	0.04	2.3
	3.2	2	2.01	63	0.16	7.7
	3.5	2	3.32	95	0.03	0.8
DES	1.1	2	1.2	109	0.03	2.5
	1.2	2	1.24	103	0.04	3.2
	1.7	2	1.8	106	0.11	5.8
	1.8	2	1.8	100	0.04	2
	2.0	4	1.96	98	0.23	12
DIEN	1.1	2	1.18	107	0.04	3.4
	1.2	2	1.26	105	0.01	0.8
	1.7	2	1.78	105	0.13	7.3
	1.8	2	1.84	102	0.02	1.1
	2.0	4	1.62	81	0.65	40
HEX	1.1	2	1.12	102	0.04	3.6
	1.2	2	1.22	102	0.01	0.8
	1.7	2	1.70	100	0.04	2.4
	1.8	2	1.80	100	0.02	1.1
	2.0	4	1.99	100	0.08	4.0

[a] ZER = Zeranol; DES = diethylstilbestrol; DIEN = dienestrol; HEX = hexestrol.

[b] Where only duplicates are present, the standard deviation is actually the absolute value of the average deviation, i.e., $|(x_1 - x_2)/2|$.

[c] CV = Coefficient of variation.

Phase 2 Validation

Figures 2 and 3 present typical SIM mass chromatograms from GC/MS analyses of blank urine fortified with the analytes at 2 μg/L. Table 3 summarizes the results of the calibration regression analyses from phase 2. The data used to generate calibration curves were single determinations of 1 matrix standard at each concentration. For 11 of 12 curves, the correlation coefficients were >0.99. For all runs, the response curves were considered linear according to the criteria described above. Table 4 summarizes the results of determinations of the analytes in the fortified blank urine test portions. The average measured concentrations were within 12% of the fortification level. The coefficients of variation (CV) ranged from 1.0 to 12.4%. Matrix-based calibration curves were used for all subsequent analyses.

Phase 3 Validation

The results of the determinations of blind-spiked samples are summarized in Table 5. Average recoveries for individual analytes were ZER, 88.9%; DES, 103.2%; DIEN, 100.0%; HEX, 100.8%.

Selectivity Study

No significant responses were noted at expected retention times of analytes in the blank or in the 2 test portions fortified with the mix of antibiotic substances and HGPs. The response of the analytes was not affected by the presence of added substances.

Overall Method Performance

The ability of the method to maintain relative ion intensities over the term of the validation study is illustrated by the data presented in Table 6. The relative ion intensities were generated from analyses of the phase 2 and 3 matrix-based calibration standards and fortified blanks. In all but one case, the CV values of the relative ion intensities were within the permitted tolerances for relative ion intensities as recommended in the EU Commission Decision document 2002/657/EC (7). The ion peak ratio 414/207 (not shown) for HEX-$(TMS)_2$ was zero in all cases.

Because the method is primarily intended to be used for screening for residues present at a concentration of 2.0 μg/L or greater, the overall performance of this method in determining target analytes in blank urine fortified at this concentration

Table 6. Relative ion intensities generated from phases 2 and 3 calibration standards and fortified blank samples

	Zeranol-$(TMS)_3$		*cis*-DES-$(TMS)_2$		*trans*-DES-$(TMS)_2$		Dienestrol-$(TMS)_2$		Hexestrol-$(TMS)_2$
$(m/z)_1$ / $(m/z)_2$	523/433	538/433	383/412	397/412	383/412	397/412	395/410	381/410	191/207
x[a]	0.145	0.122	0.152	0.177	0.195	0.225	0.763	0.234	0.064
s[b]	0.017	0.018	0.018	0.182	0.011	0.021	0.047	0.047	0.022
CV, %[c]	12	15	12	10	6	9	6	20	35
Tolerance, %[d]	±20	±20	±20	±20	±20	±15	±10	±15	±50
N	36	36	38	31	51	52	53	52	52

[a] x = Relative ion intensities.
[b] s = Standard deviation of relative ion intensities.
[c] CV = Coefficient of variation.
[d] Maximum permitted tolerances for relative ion intensities according to ref. 7.

Table 7. Method performance summary for blank urine fortified at 2.0 μg/L

Analyte[a]	*N*	Average measured concentration, μg/L	Standard deviation, μg/L	CV, %[b]	Screening threshold concentration, μg/L[c]	CCβ, μg/L[d]
ZER	34	1.97	0.14	7	1.7	0.28
DES	31	2.06	0.13	6.2	1.8	0.24
DIEN	31	1.93	0.26	14	1.5	0.15
HEX	31	2.12	0.09	4.1	2.0	0.84

[a] ZER = Zeranol; DES = diethylstilbestrol; DIEN = dienestrol; HEX = hexestrol.
[b] CV = Coefficient of variation.
[c] Determined concentration above which samples are declared “suspect.”
[d] Detection capability, determined from combined data used to generate the 6 calibration curves from validation phases 2 and 3.

was assessed. The summary presented in Table 7 is derived from all phase 2 matrix standards at 2.0 μg/L, phase 2 fortified samples, and phase 3 blank spikes at 2.0 μg/L. Based on the screening threshold concentrations calculated for each analyte, a working screening threshold concentration of 1.5 μg/L was adopted for all analytes. The calculated CCβ values are all less than one-half the target concentration of 2.0 μg/L.

Conclusions

A screening procedure using IAC cleanup followed by GC/MS was developed and validated for the detection of DES, DIEN, HEX, and ZER in bovine urine at a target level of 2.0 μg/L. Average recoveries of each of the 4 analytes from blank urine fortified at the target level ranged from 96 to 106%, with CV values of 4.1–14.0%. The CCβ values ranged from 0.15 to 0.84 μg/L, less than one-half the target level. There were no interferences from a suite of antibiotic compounds and HGPs added to samples at a concentration of 25 μg/L.

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