Simultaneous Determination of Zilpaterol and Other Beta Agonists in Calf Eye by Gas Chromatography/Tandem Mass Spectrometry

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Adrenergic drugs for growth promotion have been outlawed in European meat production; however, molecules such as Ractopamine and Zilpaterol are licensed for feeding swine and cattle in the United States, Mexico, and South Africa. Analysis of bovine retinal extracts has recently shown considerable extension in the detection period following withdrawal. Previous studies demonstrated that residual concentrations of Clenbuterol and related substances in retinal tissue were >100 ng/g at day 50 of withdrawal. A method was developed to identify and simultaneously quantify Clenbuterol-like substances with anilinic moieties and drugs with phenolic and catecholic moieties, such as Ractopamine and Zilpaterol, in retinal tissue. The method was validated according to SANCO/1805/2000. After extraction in 0.1N HCl, samples were cleaned up on C_{18} non-endcapped solid-phase extraction columns and analyzed as trimethylchlorosilane derivatives by gas chromatography/tandem mass spectrometry, electron impact mode. At concentrations of agonists between 62.5 and 250.0 ng/g in bovine retina, mean recoveries ranged from 85.3 to 94.8%, repeatability was <9.6%, and within-laboratory reproducibility was <10.5%. The decision limits (CC_α) were within the range of 66.3–70.4 ng/g, and the detection capability (CC_β) varied from 73.9 to 79.8 ng/g. Results are discussed in terms of a multiresidue approach to improve reliability of the monitoring strategy.

The increasing number of adrenergic agonist drugs of potential use as growth promoters in animal production and their continuous turnover can pose a risk for food safety and consumer protection (1). The use of β-agonists for growth promotion has been banned in the European Union (EU), while molecules such as Ractopamine and Zilpaterol are approved as repartitioning agents in finishing swine and cattle feed in the United States, Mexico, and South America (2, 3). Moreover, Ractopamine and Zilpaterol are currently administered to feedlots at doses 10-fold higher (10–20 mg/kg) than those suggested for Clenbuterol, because of their lower efficiency (4).

The accumulation of such drugs at residue levels in biological complex matrixes makes mandatory the development of multiresidual methods with high sensitivity and specificity on alternative samples able to document previous drug exposure (5, 6). Investigations on Clenbuterol have shown that it accumulates more persistently in the bovine eye than in urine and liver and is still detectable in, such tissue 50 days after withdrawal, as a result of its high affinity with melanin and mucopolysaccharides usually present in relevant concentration in the choroid/pigmented retinal epitelium (7–12). It has been recently reported that the panel of molecules able to accumulate in bovine retina is not only limited to Clenbuterol-like substances, with anilinic moieties, but also includes compounds such as Ractopamine and Ritodrine, which are characterized by phenolic moieties (Figure 1; 13).

A multiresidue gas chromatography/tandem mass spectrometry (GC–MS/MS)-based procedure has been developed and validated to identify those agonists of possible accumulation in the eye, after a simple C_{18} cleanup step, for surveillance purposes, according to the available pharmacokinetic data in cattle (14, 15).

Experimental

Reagents and Materials

(**a**) *Clenbuterol, Ritodrine, and Ractopamine as hydrochloride salts*.—Sigma Italia (Milan, Italy).

(**b**) *Clencyclohexerol, Clenpenterol (NAB 760), and Zilpaterol hydrochloride*.—Kindly gifted by the EU Reference Laboratory for Residues of Veterinary Drugs (Berlin, Germany).

(**c**) *N1-(2-(4-amino-3,5-dichlorophenyl)-2-hydroxyethyl)-N1-isopropyl-propanamide* (*Compound A*).—Synthesized as pure standard by G. Boatto (University of Sassari, Sassari, Italy; 16).

(**d**) *Methanol, hydrochloric acid, sodium hydroxide pellets, and diethylamine*.—Merck (Darmstadt, Germany).

(**e**) *Phosphate buffer solution* (*PBS*).—0.066M, pH 7.4. Produced by mixing 196 mL potassium dihydrogen orthophosphate (KH_2PO_4), obtained by weighing 9.08 g/L

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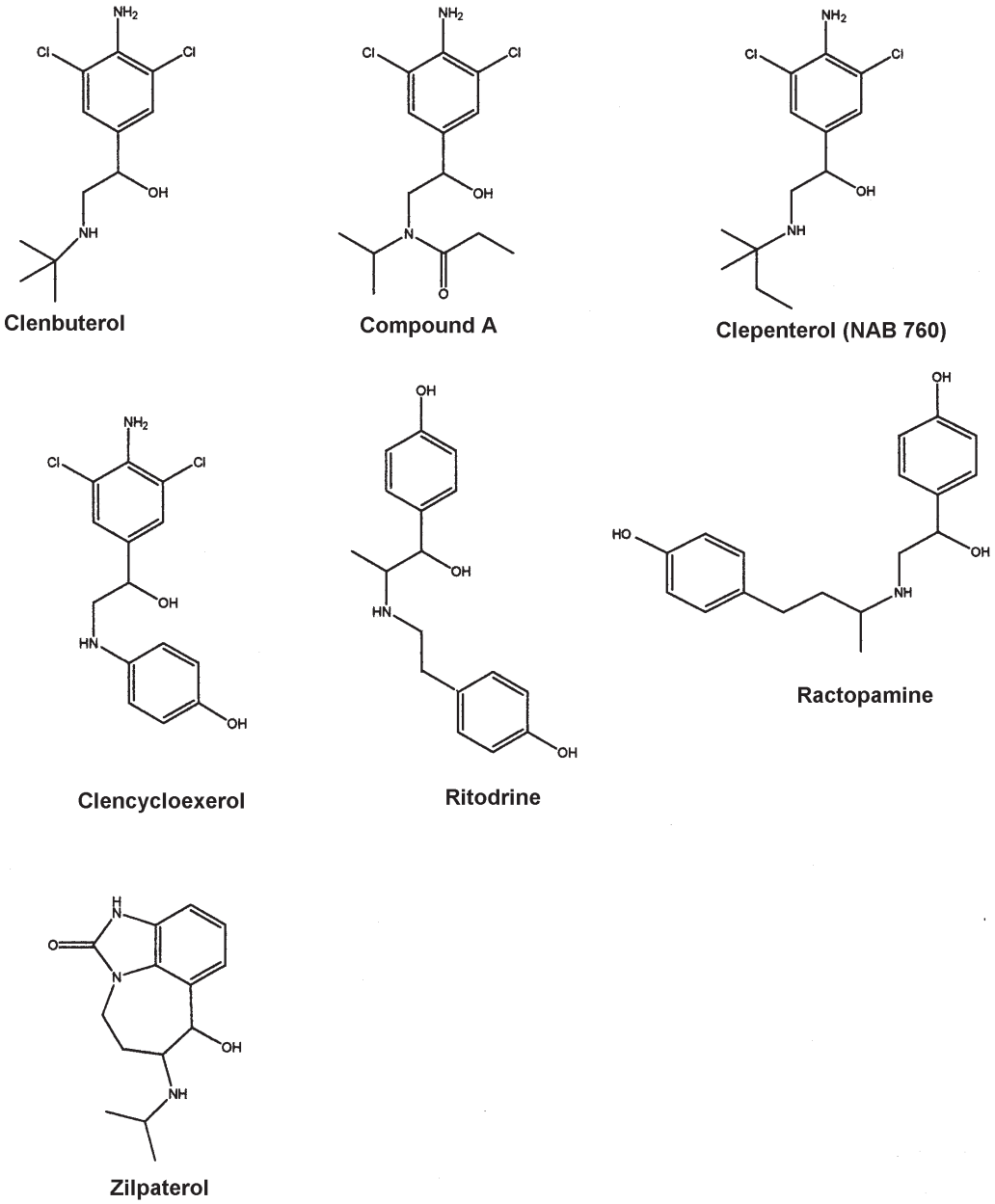

Figure 1. Structures of β-adrenergic agonists.

Table 1. Retention time (RT, min), relative RT (referred to Clenpenterol as IS), and relative abundance (%) of diagnostic ions monitored in the electron impact mode on GC–MS/MS system

Compound	RT	Relative RT	Precursor *m/z*	Product ion 1, *m/z*	Product ion 2, *m/z*
Clenbuterol	14.39	0.94	262 (38)	188 (100)	225 (59)
Clencyclohexerol	22.51	1.47	200 (10)	110 (100)	169 (17)
Clenpenterol	15.32	1.00	262 (35)	73 (100)	188 (74)
Compound A	20.53	1.34	262 (74)	73 (100)	188 (12)
Ractopamine	23.52	1.54	267 (18)	193 (100)	73 (50)
Ritodrine	22.09	1.44	236 (17)	193 (100)	219 (64)
Zilpaterol	18.30	1.19	308 (31)	293 (100)	218 (65)

and 804 mL disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), obtained by weighing 11.8 g/L.

(**f**) *Ultra-pure water*.—Obtained from MilliQ RG system (Millipore, Bedford, MA).

(**g**) *Derivatization reagent.*—Bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS) was purchased from Pierce (Rockford, IL).

(**h**) *Solid-phase extraction (SPE) cartridges*.—Light load 500 mg/3 mL non-endcapped C_{18} (C_{18}NE; Isolute IST, Stepbio SrL, Bologna, Italy).

(**i**) *Standard solutions.*—Single standard stock solutions of Clenbuterol, Clencyclohexerol, Clenpenterol, Compound A, Ractopamine, Ritodrine, and Zilpaterol at 1 mg/mL were prepared in methanol and stored at 4°C. To optimize GCQ Trap conditions, pooled solutions of the drugs with concentrations of 62.5–500.0 ng/mL were made fresh daily by dilution in methanol. To obtain spiking levels on blank matrixes, pooled solutions in concentrations of 62.5–500.0 ng/mL were made fresh daily by dilution in PBS, pH 7.4. Clenpenterol working solutions were prepared in PBS, pH 7.4, final concentration 250 ng/mL.

Instruments

(**a**) *SPE cartridge system.*—Vacuum Manifold Visiprep (Supelco, Bellefonte, PA).

(**b**) *Homogenizer*.—Ultraturrax T25 (Janke & Kunkel, Ika-Labortechnik, Staufen, Germany).

(**c**) *Ultrasonic water bath.*—Bransonic 321 (Branson Ultrasonic Corp., Danbury, CT).

(**d**) *Thermostated incubator shaker*.—Thermoshake C. Gerhardt (Fabrik und Lager Chemischer Apparate, Bonn, Germany).

(**e**) *Centrifuge.*—Sorvall Superspeed RC-2 (Kendro Laboratory Products GmbH, Hanau, Germany).

(**f**) *Evaporation and derivatization block.*—Heat Block (Pierce).

(**g**) *Capillary column.*—Fused silica 30 m × 0.25 mm id coating CP-SIL 8 CB low bleed/MS, 25 μm film thickness (Varian Chrompack, Middleburg, The Netherlands).

(**h**) *GC–MS/MS system.*—Ion trap detector GCQ (Thermoquest Italia S.p.A., Milan, Italy).

GC operating conditions: injector temperature was set at 250°C, splitless mode. The carrier gas (He) was maintained at 40 cm/s. The oven temperature program was ramped from 70 to 230°C at 20°C/min; then to 280°C at 5°C/min and kept at 280°C for 5 min.

MS operating conditions: spectrometer operated in the electron impact (EI) mode with electron energy of 70 eV. The temperatures of source and transfer line were 200 and 285°C, respectively. The multiplier voltage was set to 1300 V, and the resolution was 0.5 amu.

Analytical Procedure

(**a**) *Sample collection*.—Samples of negative bovine eye from veal calves reared in organic farms were drawn at local abattoirs and kept at –20°C until analysis. With respect to bovine spongiform encephalopathy (BSE) concern, eyes were drawn from veal calves aged 6 months and each pair was labeled with the respective animal ear tag identification number. At such age, eye and brain are not considered as specified risk material for BSE.

(**b**) *Sample preparation, extraction, and cleanup*.—After the choroid/pigmented retinal epitelium was dissected from the eye, 1.0 g was weighed into a 40 mL centrifuge tube and spiked with 1 mL of pooled solutions of the drugs at concentrations of 62.5, 125.0, 250.0, and 500.0 ng/mL. Clenpenterol, as internal standard (IS), was added at fixed concentration of 250.0 ng/g. The samples were allowed to stand overnight at room temperature, with gentle shaking to improve the interaction between melanin and drugs. Negative control samples were obtained by spiking the twin eye with 1 mL IS.

For analyses of real samples, 2 types of blanks, with and without IS, were prepared. Extraction was performed by adding 13 mL 0.1N HCl (14 mL for blank samples), followed by ho-

Table 2. Repeatability (SD_R, n = 6), within-laboratory reproducibility (SD_{w1R}, n = 18), and recovery (R) of substances at the concentrations tested (values expressed as %)

Substance	n	Clenbuterol	Clencyclohexerol	Compound A	Ractopamine	Ritodrine	Zilpaterol
SD_R at 62.5 ng/g	6	9.3	5.1	3.4	8.7	4.3	8.6
SD_R at 125.0 ng/g	6	9.2	4.8	4.9	5.1	9.6	7.8
SD_R at 250.0 ng/g	6	7.9	4.5	7.8	7.1	5.6	5.0
SD_{w1R} at 62.5 ng/g	6	10.5	9.6	10.1	8.7	10.3	9.3
SD_{w1R} at 125.0 ng/g	6	3.9	5.6	9.9	4.7	8.3	8.1
SD_{w1R} at 250.0 ng/g	6	3.2	6.1	4.5	3.5	3.4	6.2
R at 62.5 ng/g	6	85.6	90.4	91.2	92.8	85.6	86.4
R at 125.0 ng/g	6	88.8	90.5	91.8	93.0	85.3	87.4
R at 250.0 ng/g	6	90.8	92.7	88.9	87.2	94.8	87.2

Table 3. Decision limits (DL) and detection capability (CC_β) for identification of β-agonists in choroid/pigmented retinal epitelium (values expressed as ng/g)

Substance	DL CC_α	DL as 3 × S/N	CC_β
Clenbuterol	66.5	20.0	75.7
Clencyclohexerol	69.1	24.2	77.9
Compound A	70.4	39.0	79.8
Ractopamine	69.8	30.5	78.1
Ritodrine	66.3	24.0	75.3
Zilpaterol	65.7	36.6	73.9

mogenization with the Ultraturrax device (2 min, 13 500 rpm) and by sonication (10 min at room temperature). The samples were allowed to hydrolyze in an incubator shaker at 37°C, overnight, and the hydrolyzed samples were then centrifuged at 3000 × *g* for 15 min and neutralized with 5N NaOH; then, 5 mL aliquots of the supernatant were transferred into clean plastic tubes for the SPE step. Retina negative controls underwent the same procedure to assess background noise.

The SPE procedure consisted of application of 5 mL aliquots on the C_{18} NE cartridges (previously conditioned with 3 mL MeOH and 3 mL water) at a flow rate of 1 mL/min. After washing with 3 mL water–MeOH (4 + 6, v/v), the elution was performed by 2 replicates of 2 mL each of MeOH (1% diethylamine). The eluate, collected in a vial, was evaporated under a stream of nitrogen to complete dryness at 60°C and derivatized with 20 μL BSTFA (1% TMCS) for 30 min at 60°C for the further GC–MS/MS analysis.

(**c**) *Analysis.*—The MS/MS experiments were performed by using a sequence of time windows. Segment 1 (Clenbuterol and Clenpenterol), start time 14.0 min, precursor ion *m/z* 262, width 4, collision energy 1.2 V, product ions *m/z* 225–227, 188–190. Segment 2 (Zilpaterol), start time 17.0 min, precursor ion *m/z* 308, width 2, collision energy 1.0 V, product ions *m/z* 293–295, 218–220. Segment 3 (Compound A), start time 19.5 min, precursor ion *m/z* 262, width 4, collision energy 1.1 V, product ions *m/z* 188–190, 73–75. Segment 4 (Ritodrine), start time 21.2 min, parent ion *m/z* 236, width 2, collision energy 0.9 V, product ions *m/z* 219–221, 193–195. Segment 5 (Clencyclohexerol), start time 22.2 min, precursor ion *m/z* 200, width 2, collision energy 0.8 V, product ions *m/z* 169–171, 110–112. Segment 6 (Ractopamine), start time 23.2 min, precursor ion *m/z* 267, width 2, collision energy 1.1 V, product ions *m/z* 193–195, 73–75.

Validation Study

Validation parameters, such as repeatability, within-laboratory reproducibility, decision limits, and detection capability, were calculated in accordance with the criteria in the draft of SANCO/1805/2000, revision of 96/23/EC (17). For confirmatory purposes in the GC–MS/MS technique, one precursor and 2 product ions as TMS derivatives with their relative intensities (respecting maximum permitted tolerances reported in the draft) were acquired (Table 1). For quantitation of analytes, the sum of the area of the precursor ion and 2 product ions was considered. Four levels of fortification on blank retina, at concentrations of 62.5, 125.0, 250.0, and 500.0 ng/g, were considered for calibration curves. The curves were prepared daily and were constructed by plotting peak area ratios of the analytes to that of the IS vs analyte concentration. The curves were used to interpolate concentrations of analyte in the validation samples.

For the repeatability study (SD_R), 3 independent series with retina from different veals, spiked at levels of 62.5, 125.0, and 250.0 ng/g, with 6 replicates for each level were performed. The within-laboratory reproducibility (SD_{w1R}) was determined by performing the repeatability study on 3 different days by different operators and different batches of reagents and solvents. The recovery (R) was expressed as the mean recovery of the 3 independent series for each concentration level considered. The decision limit as CC_α (the lowest concentration level at which a method can discriminate whether the identified analyte is present with a statistical certainty of 99%) was calculated as the mean of measurement results of the lowest fortification level of the 3 series, plus 2.33-fold the standard deviation (SD) at the lowest concentration level. Such limit was also calculated as result of the signal-to-noise (S/N) ratio of 3:1 on 20 blank retina samples at the time window in which the analyte is expected. The decision capability expressed as CC_β (the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 95%) was calculated as the signal associated with CC_α plus 1.64-fold the SD of the mean of the 3 series at the lowest concentration level (62.5 ng/g).

Results

The repeatability (SD_R), within-laboratory reproducibility (SD_{w1R}), and recovery (R) at 62.5, 125.0, and 250.0 ng/g for each β-agonist considered are reported in Table 2. The decision limits calculated from the lowest fortification level of the calibration curve and from the S/N ratio on 20 blanks and the detection capability (CC_β) are reported in Table 3.

The GC–MS/MS chromatograms of blank retina extract (on the left) and the retina spiked at 62.5 ng/g (on the right) for Clenbuterol, Zilpaterol, Compound A, Ritodrine, Clencyclohexerol, and Ractopamine are reported in Figure 2 (a–f).

Discussion

To prevent possible human exposure to pharmacologically active residues of adrenergic agonists through animal food, there is a strong need to set up control strategies that give accurate and reliable results. Further, as new β-agonists frequently occur in feed stuffs and black market preparations, multiresidual analytic approaches are necessary.

To this purpose, we selected choroid retinal epitelium as matrix because it allows trace-back of remote illegal treat-

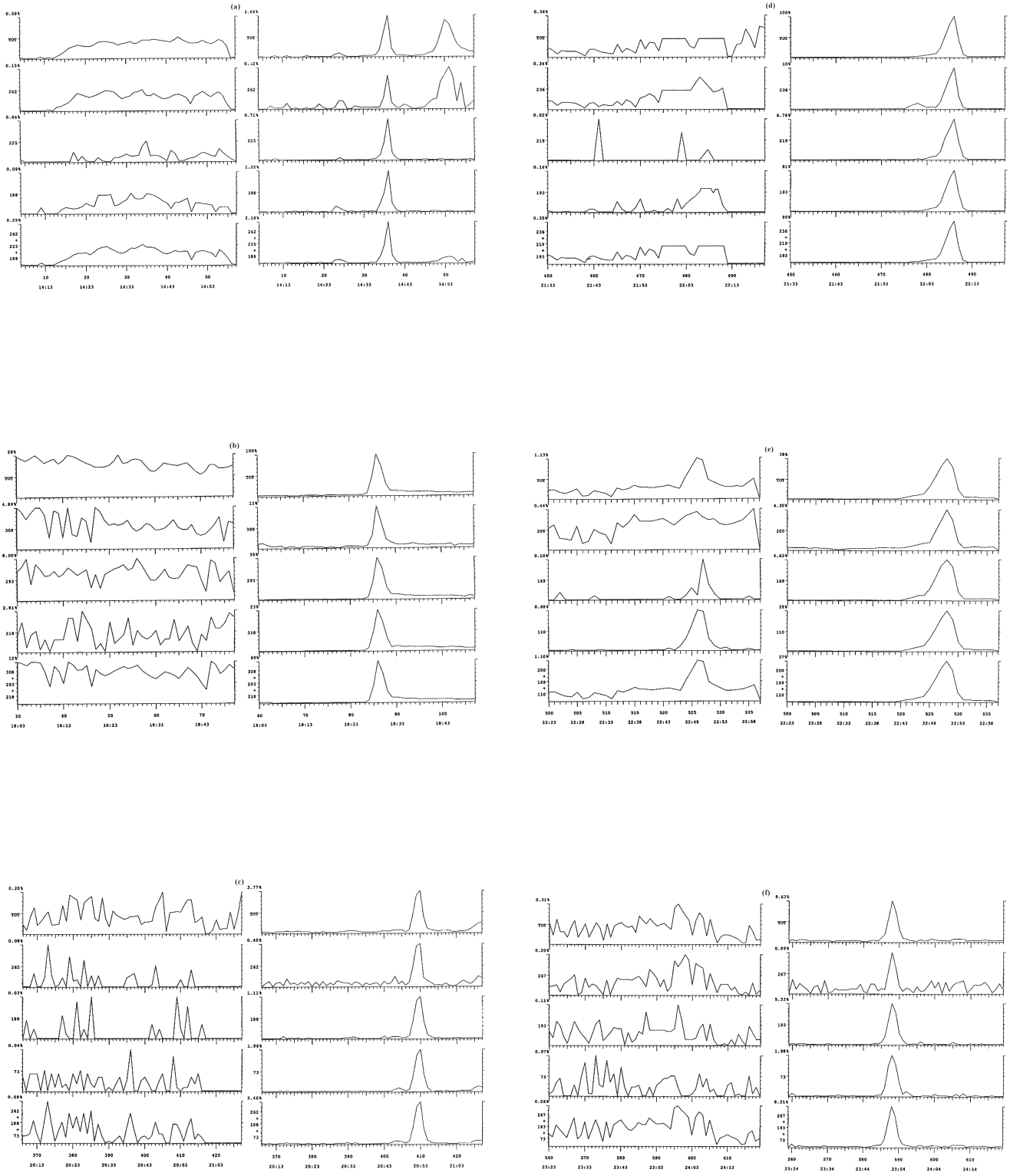

Figure 2. Time windows for GC–MS/MS analysis of blank sample (left) and sample spiked at 62.5 ng/g (right) with pool of β-agonists: (a) Clenbuterol; (b) Zilpaterol; (c) Compound A; (d) Ritodrine; (e) Clencyclohexerol; and (f) Ractopamine.

ments up to 50 days after withdrawal, with residual concentration still >100.0 ng/g, at least one order of magnitude higher than those found in liver (14, 15). However, live animals could be monitored by screening hair, but this would require investigation of the effect of pigmentation and breed on accumulation rate of the drugs (18).

The observed recoveries, from 85.3 to 94.8%, do not show significant differences among the drugs considered and the concentrations tested (Table 2). This indicates that the SPE procedure on C_{18} NE cartridges is effective for multiresidue purposes, especially compared with strong cationic ion exchange (SCX) cleanup and liquid–liquid extraction procedures already described in the literature (5, 19, 20). In fact, SCX cleanup fails to bind Compound A, due to absence of the secondary amino group in its structure, whereas the presence of phenolic groups in Ractopamine and Ritodrine structures determines the formation of a net negative charge on the molecules that compromise their repartition in the organic phase during liquid–liquid extraction (Figure 1; 19, 21). However, the hydrophobic interactions on the C_{18} columns are not sufficiently discriminatory, due to the presence of a carbon chain length ≥12 in the drugs' molecular structures (22). Thus, Clenbuterol, Clencyclohexerol, and Clenpenterol recoveries are representative of other Clenbuterol-like β-agonists able to accumulate in retina, such as Brombuterol, Cimaterol, Cimbuterol, Clenproperol, Mabuterol, Mapenterol, and Hydroxymethylclenbuterol.

Particular attention should be given to Zilpaterol, the most polar adrenergic agonist considered in this study, with relevant differences in its chemical structure. It is still retained on the SPE column after washing with methanol 60%, probably because of secondary ion-exchange interactions with free silanols groups. For this reason, a counterion such as diethylamine was inserted in the elution step for quantitative recoveries. This mechanism, already described for Clenbuterol on liver analysis, suggests that the use of NE columns could be considered for multiresidue analysis (23). The decisions limits calculated as 3 times the S/N ratio on blanks showed lower values than those calculated from the calibration curve, thus highlighting the cleanness of blank extracts (*see* chromatographic patterns in Figure 2) and suggesting a realistic limit of identification of around 25 ng/g (Table 3). In the case of Clenbuterol, for which accumulation and depletion data on bovine retinal tissue are available, the CC_α and 3:1 S/N appeared to be roughly 1/2 and 1/6, respectively, of the reasonable expected concentration in incurred samples (13, 19).

The standard deviation of analysis in triplicate showed good repeatability, with SD_R between 3.4 and 9.6%, with good interday precision of 3.2–10.5%, considering the complexity of the biological matrix (Table 2). Moreover, the coefficients of variation (CVs) recorded at each spiking level are within the precision requirements stated in the SANCO/1805/2000 (CV = 32% for analyte content of 10 ng/g; CV = 23% for analyte content of 100 ng/g) and in good agreement with those reported for Clenbuterol-like drugs on the same matrix (19).

For multiresidue purposes, the TMS derivatization for β-agonists was chosen because its efficiency is not influenced by molecular structural differences between phenolic-like and aniline-type substances. On the contrary, the procedure based on cyclic methylboronate derivatives could not be effective for the former group (24).

Analytical procedures in terms of extraction and identification for the Zilpaterol compound in matrixes of food-producing animals have not yet been published. According to our knowledge, no analytical schemes with a common cleanup for agonist drugs that have different chemical structures have been reported.

According to the evidence from the validation study and in terms of a multiresidue approach, the proposed method can guarantee reliable results in monitoring the illegal use of β-agonists as growth promoters in livestock. The method has shown good sensitivity with respect to the pharmacokinetic data available in retina and a high specificity for the class of compounds considered.

Conclusions

The application of the proposed procedure in routine laboratories could guarantee a reliable multiresidue strategy able to identify β-agonists, despite major differences in their chemical structure, that can effectively accumulate in calf eye, as in the case of Zilpaterol. Among 21 incurred samples analyzed for confirmation, we identified and quantified the presence of both Clenbuterol and Compound A in the range of 120–1300 ng/g in retina of calves at slaughter.

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