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Clenbuterol Storage Stability in the Bovine Urine and Liver Samples Used for European Official Control in the Azores Islands (Portugal)

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Clenbuterol is a well-known growth promoter, illegally used in farm animals, especially in cattle. Samples collected for the screening of β_2-agonist residues in Portuguese Azores Islands must travel through all the nine islands until they reach Azores Central Laboratory. If any suspicious sample is detected, it must be further transported to the National Reference Laboratory in Lisbon for confirmation. As a consequence of these circumstances, samples are submitted to different transport and storage times, as well as different temperature conditions and in some cases successive freezing and thawing cycles. As clenbuterol is the most detected β_2-agonist growth promoter in the Portuguese Residue Monitoring Plan, studies were conducted on the stability of this compound in incurred samples (bovine liver and urine) at +4, −20 and −60 °C over time. Samples kept at −20 °C were also analyzed over time after successive freezing and thawing cycles. The analyses of clenbuterol over time were performed by gas chromatography−mass spectrometry (GC−MS) with selected ion monitoring (SIM). Clenbuterol in incurred urine and liver samples was significantly stable up to 20 weeks at −20 and −60 °C and after, at least, six consecutive freezings and thawings. At +4 °C, clenbuterol remained stable, at least until 12 weeks in urine and up to 20 weeks in liver.

KEYWORDS: Clenbuterol; urine; liver; stability; GC−MS

INTRODUCTION

Clenbuterol [4-amino-3,5-dichloro-α-(tert-butylaminomethyl)benzyl alcohol] is an orally active β_2-agonist authorized for clinical use in veterinarian medicine as bronchodilatant in bovines, equines and pets, or as a tocolitic in cows with a maximum residue level (MRL) of 0.5 μg kg^{-1} for liver/kidney and 0.1 μg kg^{-1} for muscle or 0.05 μg kg^{-1} for milk (*1*−*3*). It was observed in several animal species that β_2-agonist compounds, especially clenbuterol, could act as a repartitioning agent and was able to decrease fat deposition with enhanced protein accumulation when administered orally at high doses. This growth promoting property of β_2-agonists is still used in intensive production of cattle and other farm animals around the world (*2*). Within the European Union (EU) their use as growth promoting agents is banned since 1996. The ingestion of contaminated edible tissues containing residues of such compounds has been responsible for several human intoxications in some European countries (*4*−*8*) including Portugal (*9, 10*).

Control of the illegal use of β_2-agonists as well as other veterinary drugs and growth promoters, in intensive animal production, is regularly performed within the EU by the application of Residue Monitoring Programs. The overall strategy of control includes the surveillance of live animals with the collection of urine, feed, water and edible tissues like muscle and liver at slaughterhouses.

Under the application of the Portuguese Residue Monitoring Plan in Azores Islands, β_2-agonist immunoassay screening tests, by enzyme-linked immunosorbent assay (ELISA), are performed in Azores Central Laboratory. For this task, urine and liver samples are collected in each of the nine islands and preserved under cold storage until transportation to the laboratory. If the transportation is performed in 24 to 48 h after collection, samples are maintained refrigerated at +4 °C. If not, samples are frozen

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at −20 °C until transportation is made possible. At the laboratory, and until analysis, samples are stored under refrigeration (+4 °C) or frozen conditions (−20 °C), for periods of time that can vary from one day to several weeks, depending on the number of samples available for analysis. If any suspicious sample is detected, analytical confirmation must be conducted according to the criteria of Commission Decision 2002/657/EC (*11*). Since confirmation is performed in Lisbon at Laboratório Nacional de Investigação Veterinária (LNIV), the National Reference Laboratory, samples must be subjected, once more, to freezing, storage and transportation until further analysis.

From this particular situation involving Portuguese Azorean islands and the control of β_2-agonist residues in live animals and their products emerges the question of the stability of these compounds in urine and liver samples submitted to storage time and temperature extreme conditions with several freezing and thawing cycles.

In the literature, a study performed on the storage stability of clenbuterol in incurred urine as Certified Reference Material concludes for the stability of the compound in this biological matrix (*12*). This study was performed with incurred lyophilized samples from which conclusions cannot be extrapolated to laboratory samples. Two other studies, using fortified urine samples instead of incurred urine, were found: in the first it was found that clenbuterol recovery diminished in urine during frozen storage at −15 °C (*13*), but in the second one no clenbuterol degradations occurred during storage at −20 °C or at −60 °C (*14*).

For frozen incurred lyophilized liver samples with clenbuterol, similar studies were performed for the production of Certified Reference Material (*15*) and concluded in favor of the stability of the compound. The same conclusions were found for liver samples, when stored at −30 °C without any prehomogenization (*16*). Nevertheless, in a previous study it was also found that in prehomogenated liver clenbuterol was unstable at both −20 and −60 °C (*14*).

Other studies (*17, 18*) demonstrated an elevated stability for clenbuterol during several processes of cooking tissues.

The aim of this paper was to study the stability of clenbuterol in incurred urine and liver samples under storage at +4, −20 and −60 °C during 20 weeks, and under several freezing and thawing cycles at −20 °C, in an attempt to safeguard the analytical results of the official control.

MATERIAL AND METHODS

Reagents and Supplies. Clenbuterol was obtained from Sigma (St. Louis, MO), and the internal standard (IS) d_6-clenbuterol was provided by RIVM (Bilthoven, The Netherlands). The solid phase extraction (SPE) columns were from United Chemical Company (Bristol, PA) for urine (Clean Screen DAU 500) and for liver (Clean Screen DAU 1M6). The derivatizing reagent was composed of *N*-methyl-*N*-trimethylsililtrifluoroacetamide (MSTFA) from Macherey Nagel (Hoerdt, Germany), trimethyliodosilane (TMSI) from Sigma (Madrid, Spain) and dithioerythritol (DTI) from Merck (Darmstadt, Germany) [MSTFA/TMSI/DTI, 1000:2:2 (mL/mL/g)]. All other reagents were of analytical grade, and gases were supplied by Arliquid (Lisbon, Portugal).

Animal Treatment and Attainment of Incurred Samples. One Holstein cow (500 kg) was orally treated with clenbuterol: 7.5 μg per kg b.w. per day for two days, and 3 μg per kg b.w. per day for eight more days, in single doses. On the second day urine was collected, homogenized and separated in 10 mL aliquots in current polypropylene urine tubes (lot with 96 aliquots). After slaughter on the eleventh day, the liver (9.1 kg) was collected and separated in four strips after removal of 10 g, in duplicate, from each one of the five liver lobes (*19*): lobus hepatis dexter (hd), lobus hepatis sinister (hs), lobus quadratus (lq), lobus caudatus (lc) and processus papillaris (pp).

Clenbuterol Homogeneity and Stability Studies. Nine random 10 mL aliquots of incurred urine and ten samples of 10 g, two from each one of the five liver lobes, of incurred liver were analyzed fresh for previous homogeneity tests. Incurred urine aliquots and liver strips were stored at +4, −20 and −60 °C. Two sets of incurred urine aliquots and two sets of incurred liver strips were stored at −20 °C. Subsequently 10 mL aliquots of incurred urine and 10 g of incurred liver were analyzed in triplicate at several storage times from each one of the storage conditions. One set of incurred urine and of incurred liver samples stored at −20 °C were also analyzed after successive freezing and thawing cycles.

Extraction Procedures. The analytical procedure used for clenbuterol analysis is described elsewhere (*20, 21*). Briefly, to urine (10 mL) or liver (10 g) samples respectively 4.0 and 20.0 mL of 0.2 M acetate buffer pH 5.2 and 50 μL of β-glucoronidase (30 U mL^{-1})/arylsulfatase (60 U mL^{-1}) were added. As internal standard, d_6-clenbuterol (5 ng mL^{-1} in urine and 5 ng g^{-1} in liver) was added. Hydrolysis took place overnight at 45−50 °C. For liver samples an additional acid precipitation with 50 mL of hydrochloric acid was performed and the pH was brought to 6.0 ± 0.3 with 1 M potassium hydroxide. Samples were then homogenized with 0.1 M phosphate buffer pH 6, and extracted using SPE columns. In summary, after sample centrifugation, the supernatant was decanted onto a CleanScreenDAU cartridge, previously preconditioned with methanol, water and phosphate buffer. The column was washed with acetic acid and methanol, and elution was done with an ethyl acetate/ammonium hydroxide solution (97:3, v/v). Eluate was evaporated to dryness, under a current of dehydrated air, at 45 °C, and the dry residue was kept in a desiccator, under vacuum, for at least 4 h, until derivatization.

Derivatization. Dry eluates were derivatized with 50 μL of MSTFA/TMSI/DTI at 65 °C for 20 min. They were left to cool to room temperature and transferred to the GC−MS system.

GC−MS Equipment and Parameters. Gas chromatography−mass spectrometry (GC−MS) analysis was performed on a Hewlett-Packard HP 6890 gas chromatograph coupled to a Hewlett-Packard HP 5973 quadrupole mass analyzer (Hewlett-Packard, Palo Alto, CA). An HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm of film thickness, Agilent, Palo Alto, CA) was used. The mass spectrometer was operated in the electron impact (EI) mode and in selected ion monitoring (SIM) acquisition. Sample injection volume was 2 μL (splitless, 1 min) with injector temperature at 230 °C using helium as carrier gas (1.2 mL min^{-1}). Oven programmed temperature was as follows: 0.4 min at 100 °C, up 10 °C min^{-1} to 200 °C (3 min), up 10 °C min^{-1} to 300 °C (5 min), with the interface temperature kept at 320 °C. Diagnostic ions used for detection and identification of clenbuterol as trimethylsilyl derivative were *m*/*z* 86, 300, 335, 337 and 405. For quantitative determination *m*/*z* 86 and 92, respectively, for clenbuterol and d_6-clenbuterol were used.

Calibration Curves. For clenbuterol quantification a new calibration curve was used for each daily determination. These calibration curves were obtained with blank samples of urine and liver, free of clenbuterol, as verified by previous GC−MS analysis. The blank samples were spiked with clenbuterol at 4 to 6 concentration levels covering the range around the expected

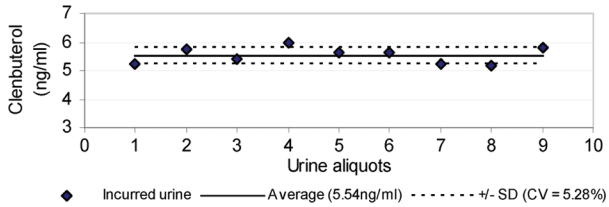

Figure 1. Homogeneity of clenbuterol concentration in the lot of incurred urine aliquots.

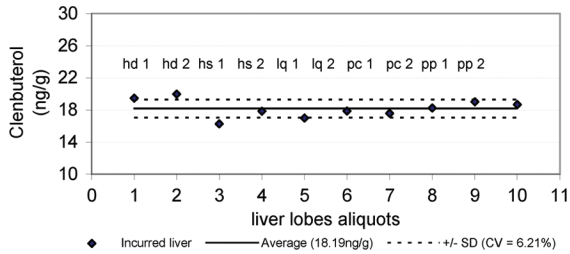

Figure 2. Homogeneity of clenbuterol distribution in five liver lobes: lobus hepatis dexter (hd), lobus hepatis sinister (hs), lobus quadratus (lq), processus caudatus (pc) and processus pappillaris (pp), with two determinations in each.

clenbuterol concentration, including one without any addition of clenbuterol (zero calibrant). These calibrants were also spiked with the same quantity of IS d_6-clenbuterol as the samples.

RESULTS AND DISCUSSION

The results of the homogeneity study of clenbuterol concentration in the urine lot and clenbuterol distribution in the liver are shown in **Figures 1** and **2**, and they demonstrate that the materials were homogeneous. The variability expressed as a coefficient of variation (CV) of mean and observed between individual aliquots of urine and between individual liver samples was very low, respectively 5.28% and 6.21%, and can be considered equivalent to the respective repeatability of the method. According to EU legislation (*11*) the typical repeatability should be between 18 and 24%, for concentrations of 5 ng mL^{-1} (similar to the clenbuterol concentration of the incurred urine), and between 15 and 20%, for concentrations of 18 ng g^{-1} (similar to the clenbuterol concentration of the incurred liver), but the repeatability of the GC−MS method for urine and for liver samples, at similar concentrations, were found to range from 0.28 to 5.36%, for the urine, and from 1.29 to 9.27%, for the liver. The prosecution of the clenbuterol stability studies in both matrices was therefore possible. Moreover, the confirmation that liver has a uniform clenbuterol distribution, as stated by Gude and co-workers (*16*) is important for all those leading with sampling and analysis for official control, since it allows the collection of any part of this organ for residue monitoring.

The precision of the analytical methodology for clenbuterol determination in each type of sample, in intralaboratorial reproducibility conditions, had been estimated from daily calibrations curves in terms of the coefficient of variation of the method (CVm). The knowledge of the CVm for clenbuterol determination in urine (7.49%, $n = 59$, in the range 0 to 10 ng/mL) and liver (9.20%, $n = 45$, in the range 0 to 24 ng/g) is of extreme value for conclusions about clenbuterol stability.

The results from the clenbuterol stability studies in incurred urine and liver are shown in **Figures 3** and **4**. The average clenbuterol concentration and standard deviation in terms of CV of mean for all the results obtained in each study are shown in **Tables 1** and **2**. Those results for urine and for liver, at each storage condition, show a small CV, each one closer of the respective CVm, except for liver under successive freezings and thawings. Here a higher CV (15.10%) was verified which could be explained by the elevated concentration obtained at 12 weeks of storage (coincident with the fourth thawing). This superior concentration could be explained by some kind of metabolic activity which was responsible eventually for increasing bioavailability of clenbuterol, from some clenbuterol conjugated forms which were not initially detectable due to aromatic and

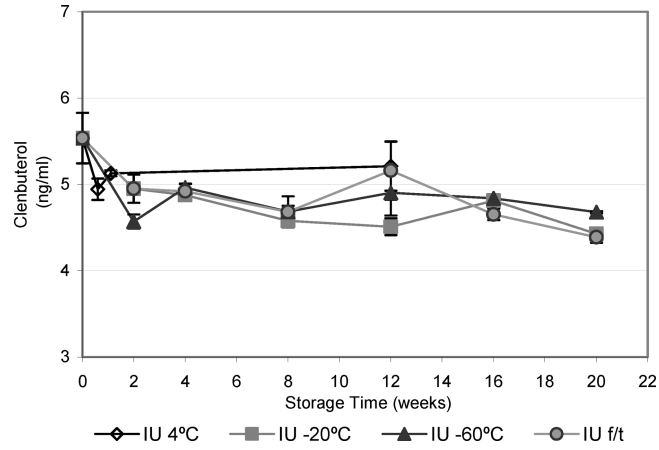

Figure 3. Stability study of clenbuterol in incurred urine (IU) stored at 4 °C, at −20 °C, at −60 °C and at successive freezing and thawing (f/t).

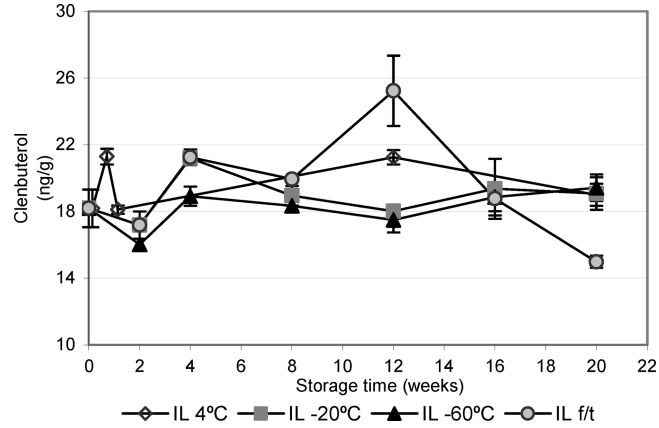

Figure 4. Stability study of clenbuterol in incurred liver (IL) stored at 4 °C, at −20 °C, at −60 °C and at successive freezing and thawing (f/t).

Table 1. Clenbuterol Concentration in Incurred Urine during Storage

storage conditions	*n*	av of clenbuterol (ng mL^{-1})	CV (%)	storage time (weeks)
4 °C	17	5.32	6.26	12
−20 °C	26	4.97	9.63	20
−60 °C	24	5.06	8.62	20
6 f/t	26	5.04	9.27	20

Table 2. Clenbuterol Concentration in Incurred Liver during Storage

storage conditions	*n*	av of clenbuterol (ng g^{-1})	CV (%)	storage time (weeks)
4 °C	22	19.13	8.30	20
−20 °C	28	18.68	7.56	20
−60 °C	27	18.13	6.63	20
6 f/t	28	19.07[a]	15.10[a]	20

[a] Rejecting data obtained at 12 weeks, the average clenbuterol concentration it will be of 18.33 ng/g with a coefficient of variation of 10.41%.

Table 3. Results of Stability Studies Performed by Regression Analyses Assuming Linearity in Incurred Urine

storage conditions	slope	standard error of slope	*P* value	storage time (weeks)
4 °C	−0.001	0. 031	0.970	12
−20 °C	−0.038	0.015	0.049	20
−60 °C	−0.019	0.017	0.316	20
6 f/t	−0.037	0.015	0.054	20

Table 4. Results of Stability Studies Performed by Linear Regression Analyses Assuming Linearity in Incurred Liver

storage conditions	slope	standard error of slope	*P* value	storage time (weeks)
4 °C	0.026	0.103	0.816	12
−20 °C	0.027	0.075	0.730	20
−60 °C	0.083	0.057	0.207	20
6 f/t	−0.057	0.194	0.782	20

secondary amines reactions (*22, 23*) or as a result of protein aggregation or denaturation during the freeze and thaw cycles (*24, 25*). Is well-known that solute concentrations could vary significantly depending upon the type of freezing employed as well as the process scale, and this hypothesis should be considered in this case since the affected samples were those subjected to several freezing and thawing processes and not those subjected to a uniform temperature. Otherwise this abnormality could be justified only by random errors during analysis. Therefore it was not problematic for clenbuterol detection, and if rejecting the data obtained at 12 weeks, the average clenbuterol concentration will be of 18.33 ng g^{-1} with a coefficient of variation of 10.41%, closer to the CVm. Comparing the average of clenbuterol results obtained at all study temperatures for each matrix it could be seen that no one differed from data obtained at the −60 °C studies, considered the best temperature for optimal results.

In addition, assuming a linear performance for clenbuterol concentration during storage, the data (average clenbuterol concentration obtained each day) from stability tests, at each set of storage conditions, was analyzed performing regression analyses. Results obtained for urine (**Table 3**) and for liver (**Table 4**) demonstrate that there was no tendency for clenbuterol degradation during storage, at any of the temperatures studied and storage times tested, since the slope obtained in each case does not deviate significantly from zero.

As it could be seen in these experiments, concerning clenbuterol determination, laboratorial samples of bovine urine or liver can be preserved at +4 °C during, at least, 12 weeks, for urine, and 20 weeks, for liver, since clenbuterol remains without significant losses. Also, those samples can be stored at −20 °C or at −60 °C during at least 20 weeks, with no differences between these temperatures, and can be subjected to 6 freezings and thawings during that time since there was not a predictable significant loss of clenbuterol.

These conclusions allow one to contradict the opinion of Gigosos and co-workers (*13*), concerning urine, and amplified in the present work, for liver, about the risk of storing laboratorial samples of urine for clenbuterol residue control, because of the instability of clenbuterol in such a matrix.

The conclusions obtained in the present work are fundamental for official residue control in animal production since it validates the procedures implemented for laboratorial sampling, transport, storage and analysis. Bovine urine or liver samples collected for clenbuterol residue control can be transported at +4 °C, at least during 24−48 h, or under freezing temperatures, and it could be possible to store samples for up to 5 months before screening or contra-analysis. Also samples can be frozen or thawed, at least 6 times, allowing defreezing during transportation without any consequence in final results, which is very important in the Azorean conditions. Moreover for the Azores the possibility of screening those samples in loco could be comparatively advantageous for meat certification and trade.

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