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Detection of clenbuterol in bovine retinal tissue by high-performance liquid chromatography with electrochemical detection

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Abstract

A method for the detection of the β-agonist drug clenbuterol in bovine retinal tissue has been developed. The extraction procedure involves sonication and centrifugation, followed by the addition of ethylenediaminetetraacetic acid (EDTA) to the supernatant. The pH of the supernatant is then brought to 12.2, which is then allowed to sit for 2 h. This is followed by a diethylether extraction. The diethylether extract is dried under nitrogen and the residue is dissolved in 1% formic acid. The quantitation of clenbuterol was accomplished by high-performance liquid chromatography with electrochemical detection. The electrochemical detector was an amperometric detector. The detector was set in the pulsed mode. The oxidizing potential of a carbon electrode was 1.3 V vs. a Ag/AgCl reference electrode and was pulsed to a reduction potential of −2.0 V vs. a Ag/AgCl reference electrode. The limit of detection for this method was 5 ng/ml of clenbuterol ($S/N=3$). Typical spiked recoveries are 75%.

Keywords: Clenbuterol; Beta-Agonists

1. Introduction

This laboratory was requested to develop a sensitive method for the determination of clenbuterol in bovine retinal tissue. The goal was to provide state laboratories with a method to monitor the use of clenbuterol in show livestock. Livestock fitters (handlers) have used clenbuterol to enhance the physical attributes of their animals. The effects of clenbuterol as a repartitioning agent that reduces body fat and increases muscle tissue have been documented [1]. Handlers who used clenbuterol had an unfair advantage over other show entries and have been successful in collecting large sums of prize money. Following these shows, which are referred to as terminal shows, the animals were slaughtered and meat containing clenbuterol residue entered the consumer market.

It was difficult to identify livestock that were exposed to clenbuterol as urine was used previously for testing and clenbuterol residues have been shown to persist in urine for only five days after the cessation of clenbuterol administration [2]. Clenbuterol residues have been shown to persist in the liver for 25–30 days after withdrawal. Repartitioning effects have been shown to be present for up to 70 days after withdrawal. After 140 days, clenbuterol residues can still be found in retinal tissue [2]. This is well past the time that the effects of repartitioning are evident. Therefore, retinal tissue is the tissue of choice for detecting illegal use of clenbuterol.

The method currently used by most laboratories to detect clenbuterol in bovine retinal tissue is a gas chromatography–mass spectrometry (GC–MS) method that involves a derivatization step [3]. This

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method includes an overnight protease incubation step. It is believed that clenbuterol binds by ionic bonds to mucopolysaccharides [4]. Retinal tissue is high in mucopolysaccharides. The overnight incubation step is used to digest the sample matrix and release clenbuterol.

The method described in this paper is an HPLC method with electrochemical detection. Electrochemical detection provides the sensitivity that is required to detect clenbuterol in these cases. The electrochemical detector cannot provide the same level of confirmation that the mass selective detector provides, however, it is more selective than a UV detector.

This method is faster than the GC–MS method described above because it does not require the protease incubation step. Brambilla et al. [5] report that recoveries of clenbuterol in synovial fluid can occur in 1 *M* HCl and 5 *M* NaCl due to an ionic-exchange mechanism. This method employs 1 *M* HCl as the extraction medium. Ethylenediaminetetraacetic acid (EDTA) is added later in the extraction to obtain the ionic strength necessary to achieve higher extraction efficiencies.

2. Experimental

2.1. Chemicals and standards

Clenbuterol HCl, ethylenediaminetetraacetic acid (purified grade), and formic acid (ACS reagent) were obtained from Sigma (St. Louis, MO, USA). Hydrochloric acid (trace metal grade), sodium hydroxide (ACS reagent), diethyl ether (spectranalyzed grade) and methanol (HPLC grade) were obtained from Fisher Scientific (Fairlawn, NJ, USA). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

A 250 μg/ml stock solution of clenbuterol (free base) was prepared in a 1% formic acid solution. This solution could be used for one month when refrigerated. The spiking solution was made by diluting 100 μl of the 250 μg/ml clenbuterol solution to 10 ml with 1% formic acid (2.5 μg/ml). A 25 ng/ml injection standard was made by diluting 100 μl of the 2.5 μg/ml clenbuterol solution to 10 ml with 1% formic acid. The spiking solution and the injection standard were also stored refrigerated and could be used for one week.

The mobile phase combined 34% of a 1% formic acid solution in methanol and 66% of a 1% formic acid solution in water. The mobile phase was filtered through a 0.2-μm Nylon filter (Alltech, Deerfield, IL, USA) and was continually sparged during use with He (Wright Brothers, Cincinnati, OH, USA).

2.2. Apparatus

A Hewlett-Packard (Wilmington, DE, USA) Model 1050 liquid chromatograph was employed for this study. The chromatograph was a quaternary pumping system. The flow-rate was maintained at 1 ml/min. The injection volume was 50 μl. A Hewlett-Packard Model 1049A electrochemical detector was used for quantitation of clenbuterol. The working electrode was a glassy carbon electrode and the reference electrode was a Ag/AgCl electrode. Clenbuterol is known to foul carbon electrodes [6]. The 1049A detector is an amperometric detector that has the capability of being used in a pulsed mode. By pulsing the electrode to a more negative potential than the working potential, components that have fouled the electrode will be reduced and will come off the electrode. The working potential is 1.3 V. The pulsing sequence is as follows: pulse potential 1 is −2.0 V, pulse potential 2 is −2.0 V, pulse time 1 is 200 ms, pulse time 2 is 0 ms and pulse time 3 is 999 ms. Pulse potentials 1 and 2 are the potentials that the detector applies during the pulse cycle [7]. Pulse time 1 is when the first pulse potential is applied. Pulse time 2 is when the second pulse potential is applied (since pulse potential 2 is the same as pulse potential 1 and pulse time 2 is 0 ms, the detector remains at −2.0 V). Pulse time 3 is when the detector goes back to the working potential. The current is measured just before pulse time 1. The response time of the detector is set at 4 s. The detector is equipped with a solvent thermostat that is maintained at 40°C.

The column is a LiChrospher 100 RP-18e 5 μm column obtained from Hewlett-Packard. The column dimensions are 250×4 mm I.D. A guard column with the same packing material was also employed. The column was maintained at a temperature of 40°C.

The data were collected with Hewlett-Packard's HPLC^{2D} ChemStation Software, Revision Code A.03.02. The electrochemical signal was sent to the ChemStation through a Hewlett-Packard analog-to-digital converter (HP35900).

2.3. Bovine eyeball dissection

The eyeballs were collected at slaughter and stored frozen until dissection. At the time of dissection, excess fat, muscle and other connective tissue was removed. The eyeball was placed, cornea side up, in a clean petri dish. The center of the cornea was punctured and an incision was made through the cornea to the sclera on both sides of the eyeball. The lens and vitreous humor were poured out of the eyeball into the petri dish. The eyeball was turned inside out. The retinal tissue was collected by picking it apart from the sclera with forceps. The retina was cut into two halves. Each half of the retina was placed into a separate pre-weighed scintillation vial. The mass of the retina was determined and the retina was stored frozen until the day of analysis.

2.4. Sample preparation

On the day of analysis, both frozen retina halves of samples and controls to be analyzed were thawed. The tissue was minced using a scissors. To prepare spikes for evaluating extraction efficiency, a control retina was spiked with 20 μl of 2.5 μg/ml clenbuterol standard. Preparation of the spiked control as a sample was continued. A 5-ml volume of 1 *M* HCl was added to the tissue. The tissue was vortex-mixed for 1 min. The tissue was sonicated for 10 min using a Cole-Palmer ultrasonic processor (model CP 50T) at 10 W (setting of 20% using a 50 W model). The tissue was centrifuged for 20 min at 2100 *g* using a Marathon 21 K/R centrifuge from Fisher Scientific. The supernatant was decanted and placed in another scintillation vial. A 5-ml volume of 1 *M* EDTA in a 4 *M* NaOH solution was added to the supernatant. The pH of the solution was adjusted to 12.2±0.1 with 10 *M* NaOH. The sample was left sitting at room temperature for 2 h.

After 2 h, a diethyl ether extraction was performed on the sample. A 5-ml volume of diethyl ether was added to the sample, which was vortex-mixed vigorously for 1 min. The sample was continuously shaken for 4 min and then was centrifuged for 2 min at 2100 *g*. The diethyl ether layer was removed and placed in a 4-ml vial. The diethyl ether was evaporated to dryness under nitrogen at 80°C and the vial was put to one side. To the remaining aqueous phase, another 5 ml of diethyl ether were added and the sample was vortex-mixed and centrifuged. The diethyl ether layer was removed and placed in the same 4 ml vial that contained the residue from the previous extraction. The diethyl ether was evaporated to dryness under nitrogen at 80°C. The residue for spikes was reconstituted in 1.0 ml of a 1% formic acid solution. One half of a retina weighs about 0.5 g. This corresponds to about 100 ng/g clenbuterol in the retina. The residue for samples was reconstituted in 200 μl of 1% formic acid. Typical sample concentrations were 10 ng/g. Samples were vortex-mixed for 5 min and then filtered through 0.2 μm, 13 mm PTFE filters obtained from Gelman Sciences. A 50-μl volume was injected onto the HPLC system.

3. Results and discussion

3.1. Chromatographic separation

Formic acid was chosen as the electrolyte in the mobile phase for the electrochemical detector because the standards and the final reconstitution buffer contained 1% formic acid. Clenbuterol solutions have been shown to be stable in 1% formic acid [6].

As stated earlier in this paper, clenbuterol fouls carbon electrodes. The effects of electrode fouling can be reduced with acetonitrile [6]. Clenbuterol is not easily oxidized. It requires high oxidation potentials. In this method, 1.3 V was used. At this potential, methanol is a better organic modifier to use than acetonitrile because acetonitrile may have impurities that oxidize at this potential. Therefore, methanol was chosen as the organic modifier. With a mobile phase composition of 34% of a 1% formic acid solution in methanol and 66% of a 1% formic acid solution in water, clenbuterol is separated from other eluting matrix peaks at a reasonable retention time (8 min).

Initially, one problem encountered was a continuously drifting baseline. The reference electrode was a

solid state Ag/AgCl electrode. Chloride ions had to be added to the mobile phase in order for the electrode to function properly. Mobile phase components can cause baseline problems. By installing a reference electrode with an internal electrolyte, chloride ions no longer had to be added to the mobile phase and the baseline stabilized.

Initially, the working electrode had to be polished every morning to mechanically clean it due to clenbuterol fouling. However, the responses of the detector to replicate injections of a clenbuterol standard were not reproducible. By the third injection, the signal response would be half of that for the first injection and by the fifth injection, there was no signal response for clenbuterol. Consequently, the electrode was cleaned electrochemically by holding the electrode at a reducing potential (−1.4 V) for about 15 min. This reduced the fouled material on the surface of the electrode. The results of this electrochemical cleaning were similar to those obtained with the manual polishing (i.e. decreasing clenbuterol response). The solution to this problem was to pulse the electrode throughout the chromatographic experiment to continuously clean the electrode. The pulsing experiment that was described above has prevented the constant mechanical and long electrochemical cleaning of the electrode. This reduced the amount of time spent waiting for a newly cleaned electrode to stabilize. It also reduced costs by extending the life of the glassy carbon electrode by eliminating all of the polishing that scratched the surface and degraded the response. Fig. 1 is a clenbuterol standard chromatogram using the conditions stated above.

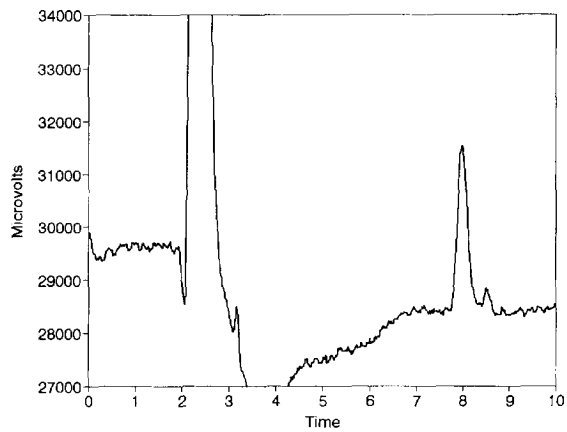

Fig. 1. Clenbuterol standard (50 ng/ml).

3.2. Clenbuterol extraction procedure

The retina was mixed with 5 ml of 1 *M* HCl. Other methods require high salt- or buffer concentrations [3,5,6,8]. Initially, several salt and buffer methods were attempted in this laboratory. Although we were often able to obtain high efficiencies for standard preparations (70–90% recovery, depending on the buffer system), when these extraction methods were attempted on retinal tissue there would be as little as 0–30% recovery. Likewise, if a buffer system yielded 40–50% recovery in retinal tissue, there was usually no recovery for standard preparations that were taken through the method.

Brambilla et al. [5] believed that the ionic strength of the sample matrix was important. We also believe that to be true. If a clenbuterol standard can be recovered using a particular method and the standard cannot be recovered when spiked into a retina, the ionic strength of the retinal extract may have been too high. It must be taken into consideration that the retina is releasing ions into the extract. If recoveries were better for retinal extract then for a standard, the ionic strength of the standard extract may have been too low.

An initial experiment was conducted in a spiked control retina to determine what recovery would be obtained if an extraction were carried out only in 1 *M* HCl. The pH of this preliminary experiment was not strictly controlled and the sonication took place in a bath sonicator, not an ultrasonicator. The extraction efficiency was 49±5.7% (n=3). For a quick reference to this extraction efficiency and those that follow, see Table 1. We believed that it was possible that, without adding any buffers, the ionic strength was already too high. In an attempt to chelate ions, 1 *M* EDTA was added to the supernatant after centrifugation. Using pH paper, the pH of the supernatant was adjusted to 12 by titration with 10 *M* NaOH. This solution was allowed to sit for 2 h to allow the EDTA to chelate ions. The extraction efficiency was 68±11% (n=15). These data were collected over nine days. The range for this data set

Table 1
Extraction efficiencies for optimization experiments of recovery of clenbuterol in spiked retinal tissue

Experiment	Efficiency	*n*
1 *M* HCl, pH 12, bath sonicator	49±5.7%	3
1 *M* HCl, 1 *M* EDTA, pH 12, sit 2 h, bath sonicator	68±11%	15[a]
1 *M* HCl, 1 *M* EDTA, pH 12, sit 2 h, bath sonicator	69±13%	3[b]
1 *M* HCl, 1 *M* EDTA, pH 12.2, sit 2 h, bath sonicator	74±5.0%	4
1.5 *M* HCl, 1 *M* EDTA, pH 12.2, sit 2 h, bath sonicator	55%	2
1 *M* HCl, 1 *M* EDTA (one day old), pH 12.2, sit 2 h, bath	36±16%	4
1 *M* HCl, 1.6 *M* EDTA, pH 12.2, sit 2 h, bath sonicator	62%	2
1 *M* HCl, 1 *M* EDTA, pH 12.2, sit 2 h, ultrasonicator, 20 W of power	54±11%	6
1 *M* HCl, 1 *M* EDTA, pH 12.2, sit 2 h, ultrasonicator, 10 W of power	76±7.5%	9[c]
1 *M* HCl, 1 *M* EDTA, pH 11.5, sit 2 h, ultrasonicator	61%	2
1 *M* HCl, 1 *M* EDTA, pH 11.0, sit 2 h, ultrasonicator	53%	2
1 *M* HCl, 1 *M* EDTA, pH 13.0, sit 2 h, ultrasonicator	cannot quantitate	2
1 *M* HCl, 1 *M* EDTA, pH 12.2, sit 1 h, ultrasonicator	67%	2
1 *M* HCl, 1 *M* EDTA, pH 12.2, sit 2.5 h, ultrasonicator	cannot quantitate	2
1 *M* HCl, 1 *M* EDTA, pH 12.2, sit 2 h, ultrasonicator, "incurred" retinas	80±6.0%	3

[a] These data were collected over a period of nine days.
[b] These data were taken from one of the days of the nine-day experiment.
[c] These data were collected over a period of four days.

was 50–87%. Although the extraction efficiency increased, the precision was poor. The precision obtained within one day was similar (69±13%, $n=3$). The exact pH was difficult to determine with pH paper. Therefore, the pH values of the extracts were monitored using a pH meter as the pH was adjusted to 12.2 with 10 *M* NaOH. The extraction efficiency improved to 74±5.0% ($n=4$). If the HCl concentration is increased from 1 to 1.5 *M*, the efficiency drops to 55%.

The EDTA solution must be made fresh each day. With one day old EDTA, the extraction efficiency drops to 36±16% ($n=4$). If the EDTA concentration is increased to 1.6 *M*, the efficiency drops to 62%.

The above experiments using spiked retinal extracts were performed using a bath sonicator. One concern was that an incurred eyeball may not work with this method due to possible strong clenbuterol–retina binding that did not have time to occur in a spiked retina. Therefore, an ultrasonic processor was used instead of the bath sonicator. The initial sonicator setting was 20 W. Although the eyeball was completely homogenized, the extraction efficiency dropped to 54±11% ($n=6$). We believe that the more complete homogenization resulted in more ions in solution and, therefore, the efficiency dropped. When the power setting was reduced to 10 W, the efficiency improved to 76±7.5% ($n=9$). These data were collected over four days.

A pH study demonstrated that the pH range that was chosen, based on initial experiments using pH paper, was correct. If the extract is taken to a pH of 11.5, the efficiency drops to 61% and at a pH of 11.0, the efficiency drops to 53%. If the pH is increased to 13.0, it becomes difficult to quantitate clenbuterol. Large interference peaks elute before and after clenbuterol. In some instances clenbuterol was a small shoulder on the tailing or fronting edge of these interference peaks.

The time that was allowed for the extract to sit was also studied. If the extract was allowed to sit for only 1 h, the efficiency dropped to 67%. If the extract was allowed to sit for 2.5 h, an interference peak formed that eluted prior to clenbuterol and made quantitation difficult. At 3 h, the interference peak was larger and was not resolved from clenbuterol. A chromatogram of a spiked bovine retina taken through the optimized extraction procedure is shown in Fig. 2 and a blank bovine retina is shown in Fig. 3. The limit of detection for this method is 5 ng/ml of clenbuterol ($S/N=3$). This corresponds to about 10 ng/g in bovine retina.

All of these experiments were spiked experiments in which the clenbuterol was placed on a minced

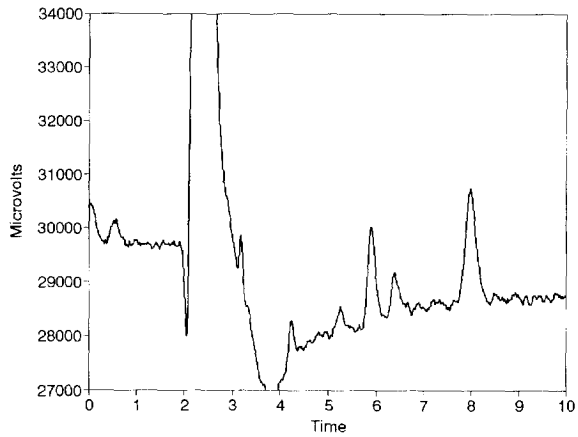

Fig. 2. Spiked (100 ng/g) bovine retinal tissue extract.

retina and 1 *M* HCl was added immediately. We were concerned that the clenbuterol was dissolving in the HCl and that the extraction efficiencies that we were obtaining would not occur in an incurred retina. Therefore, we spiked minced retinas with clenbuterol and let them sit in the refrigerator for approximately 18 h to allow clenbuterol–retina interactions to occur. After 18 h, the spike samples were processed using the outlined method. The extraction efficiency was 80±6.0% (n=3).

Retinas were spiked with 5, 10, 25, 50 and 100 ng/g of clenbuterol to determine the linearity of the method. The correlation coefficient was 0.9985. A 25 ppb standard of clenbuterol was injected ten times.

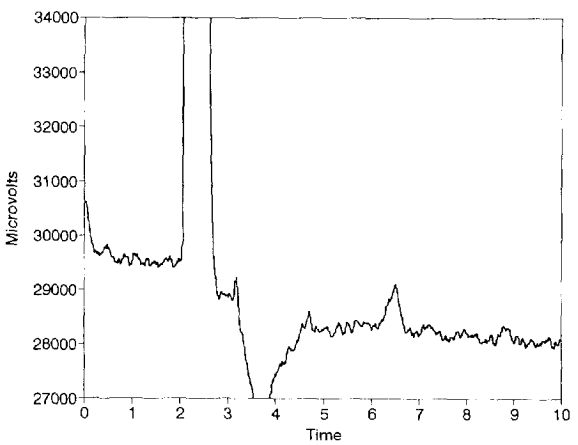

Fig. 3. Blank bovine retinal tissue extract.

The relative standard deviations for the retention time and area counts were 0.421 and 6.65%, respectively.

4. Conclusions

An extraction method and an HPLC method with electrochemical detection have been developed for detecting clenbuterol in bovine retinal tissue. In this laboratory, we have been analyzing these samples by LC–MS–MS [9]. The goal of this work was to develop a method that could be easily employed by state laboratories. This HPLC method with electrochemical detection is a good alternative method for other laboratories that may not have access to the expensive instrumentation required for LC–MS–MS. This method shows good sensitivity and is more selective than a UV detector. Often, enzyme-linked immunosorbent assays (ELISA) are used to screen for the presence of clenbuterol [5]. This HPLC method is more selective for clenbuterol than an ELISA is. The HPLC method can be used as a confirmatory method for the ELISA method. Experiments are underway to validate this method for quantitation of clenbuterol in bovine retinal tissues.

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