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Short communication

Rapid and simple determination of clenbuterol residues in retina by high-performance liquid chromatography with ultraviolet detection

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Abstract

A new method for the determination of clenbuterol by reversed-phase HPLC with UV detection has been developed. Clenbuterol was eluted on a C_8 column (250 × 4.6 mm I.D.), using an isocratic eluent consisting of an acetonitrile–0.02 *M* phosphate buffer (25:75, v/v) adjusted to pH 2.8 with phosphoric acid. The method was linear from 2.5 to 50 ng injected. The detection limit was established to be 0.5 ng (signal/background ratio: 3), and the quantification limit was 2.5 ng. With the proposed method, we got a simple and rapid detection of clenbuterol in the retina, part of the animal where the biggest amount of clenbuterol is accumulated and where it remains for the longest time after any treatment.

Keywords: Clenbuterol

1. Introduction

Clenbuterol (4-amino-[(*tert.*-butylamino)methyl]-3,5-diclorobenzyl alcohol hydrochloride) (see Fig. 1) is a β-adrenergic drug used as a bronchodilating agent in human and animal medicine [1,2]. Clenbuterol is illegally used as a growth promoter in animal production [3] because it causes a considerable reduction in fat deposits (approximately 18% [4]) and favours protein synthesis (above 15%, [4]), which permits the production of fat-free carcasses with extraordinary development of the muscular masses [5,6].

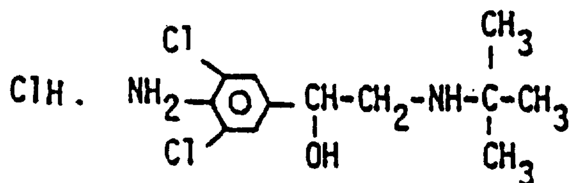

Fig. 1. The structure of clenbuterol.

Several methods for determination of clenbuterol in urine, plasma, vitreous humor and tissues (muscle, liver, heart, kidney, lung, suet, brain, spinal cord and thymus) have been published. High performance thin-layer chromatography (HPTLC) [7] and enzyme immunoassays (EIA) [8–10] have been used for screening. Quantification and confirmation have been made with liquid chromatography–mass spectrometry (LC–MS) [11], gas chromatography–mass spectrometry (CG–MS) [7,12–17] and several high performance liquid chromatographic (HPLC) meth-

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ods [9,17–31]. The use of substances which form ion-pairs is indispensable in all HPLC methods, to achieve a good chromatographic separation, except in the work of Botterblon [31]. We are not aware of any reported HPLC method applied to the determination of this residue in the retina where clenbuterol remains for longer, (up to 56 days) and in a larger amount [10]. Thus, the development of analytical HPLC methods specific for this matrix is important.

This paper describes a rapid and simple reverse-phase HPLC method using a UV detector, without derivatization, and extraction of retina samples on Sep-Pak C_{18} cartridges.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol were HPLC-grade; *tert.*-butylmethyl ether, *n*-hexane, sodium hydroxide, hydrochloric acid (37%), monopotassium phosphate, and orthophosphoric acid were analytical-reagent grade (Merck, Darmstadt, Germany). Sep-Pak C_{18} columns were from Millipore (Waters Chromatography, Bedford, MA, USA).

2.2. Apparatus

Ultracentrifuge with cooling system, 2K15 (Sigma, St. Louis, MO, USA). Nitrogen evaporation system, with thermostated heating plate (Liebisch, Bielefeld, Germany).

The chromatographic system consisted of a Model 600 pump equipped with a Model 712 WISP automatic-injector. Chromatographic separation was achieved on an C_8 Ultrabase 250 × 4.6 mm I.D., 5-μm column (Quimica Analitica, Barcelona, Spain). Detection was performed with a Model 996 diode-array detector (Waters Chromatography), scanning in the range of 190–350 nm, λ=211 nm, connected to a Nec-Image 466 computer, using Millennium v 2.00 software and a Hewlett-Packard 560 printer.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–0.02 *M* potassium phosphate (25:75, v/v) adjusted to pH 2.8 with orthophosphoric acid. The eluent was carefully degassed with helium and filtered prior its use at a flow-rate of 1 ml/min. The retention time of clenbuterol under the above-described conditions was about 8 min.

2.4. Standard solutions

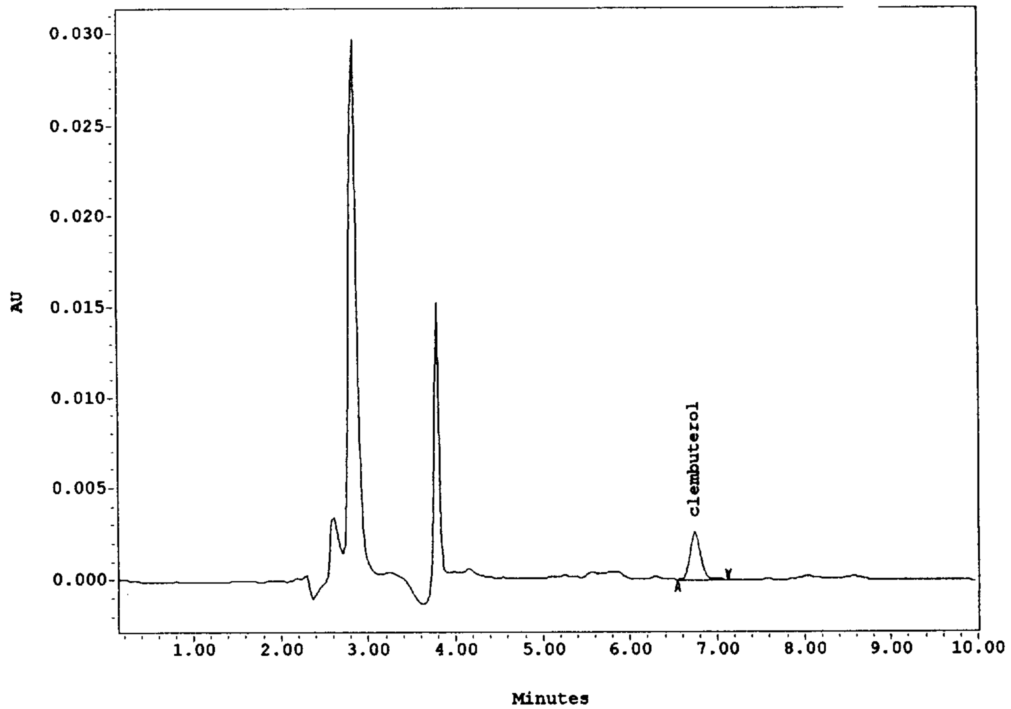
Clenbuterol was purchased from Sigma (St. Louis, MO, USA). Clenbuterol stock solutions were prepared in 0.01 *M* hydrochloric acid at a concentration of 100 μg/ml. This solution was stored at 4°C for no longer than 2 months. Standard work solutions were prepared every day, using 0.01 *M* hydrochloric acid as diluent.

2.5. Preparation of retina extracts

The whole eye (preferably previously frozen) was cut in half. Vitreous and aqueous humor were eliminated. The retina was detached and transferred to a 30-ml centrifuge tube; 1 g of retina was used. Phosphate buffer (10 ml) was added and the mixture was sonicated for 10 min. The tube was subsequently stoppered and centrifuged for 10 min at 35 000 *g* and 8°C.

After centrifugation the supernatant liquid (10 ml) was taken and adjusted to pH 9 with 2 *M* NaOH. Sep-Pak C_{18} cartridges were used for purification; 5 ml of methanol and 5 ml of demineralized water were used to activate the Sep-Pak C_{18} cartridges. Clenbuterol was eluted with 3 ml of methanol in 10-ml Pyrex tubes. The eluate was evaporated to dryness under a stream of nitrogen and the residue dissolved in 2 ml of 0.01 *M* HCl; 2 ml of *n*-hexane were added to the tubes which were shaken and centrifuged at 1500 *g* for 3 min. The organic layer was discarded and the *n*-hexane extraction repeated once. The aqueous layer was alkalinized using 100 μl of 2 *M* NaOH; after shaking, 2 ml of *tert.*-butylmethyl ether were added and the tube was centrifuged for 3 min. The organic layer was transferred into another 10-ml Pyrex tube and the *tert.*-butylmethyl ether extraction repeated [32].

The residue, after evaporation in a nitrogen stream at 35°C was redissolved in 100 ml of 0.01 *M* HCl, shaken and sonicated for 1 min.



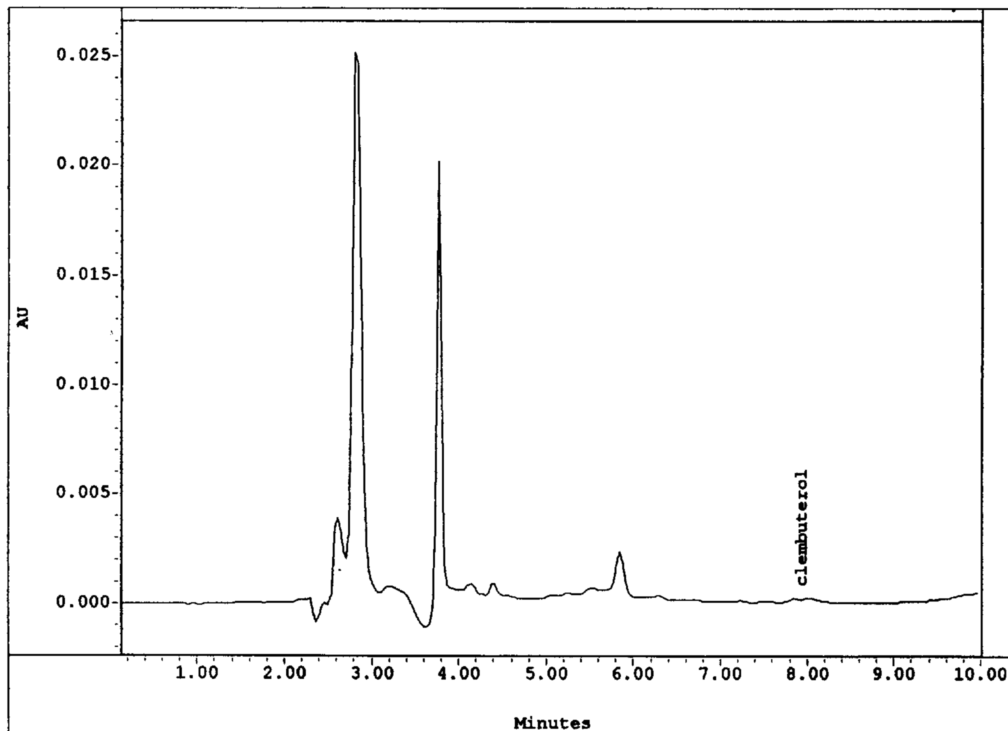


Fig. 2. Chromatogram of a retina extract from an animal treated (14.6 μg/kg of retina), or not treated, with clenbuterol (λ=211 nm).

3. Results and discussion

3.1. Extraction

The extraction method [32] includes washing the eluent from the Sep-Pak cartridge with *n*-hexane. In our laboratory, control retina samples were spiked with 100 μl of a 250 μg/l clenbuterol solution and then extracted. The recovery averaged approximately ($n=10$) 70%. In addition, ten extractions were performed without *n*-hexane washing resulting in approximately 90% recovery. Thus, retina is apparently a clean matrix that does not need to be washed with *n*-hexane, but this step is indispensable when urine or liver are used for the clenbuterol determination.

3.2. Chromatography

The method was determined to be linear for 2.5–50 ng injected on the column ($r=0.999$ and the linearity test was highly significant; $P < 0.001$). The repeatability (2.5 ng; $n=10$) seemed to be acceptable (coefficient of variation, C.V.=1.65%). A day-to-day reproducibility test was performed over 3 days. A good reproducibility of the slope of the calibration was obtained (C.V.=0.559) (Table 1). The detection limit was established to be 0.5 ng (signal/background ratio: 3) and the quantification limit 2.5 ng.

In Fig. 2, a retina extract chromatogram is shown from an animal treated, or not, with clenbuterol, which was extracted without *n*-hexane washing, to improve the extraction percentage. The concentration of clenbuterol in this sample was determined to be 14.6 μg/kg (3.3 ng injected). The spectral run of the peak corresponding to the retention time of clenbuterol confirms its identity (characteristic maxima of 209.8, 242 and 299 nm).

Table 1
Day-to-day reproductibility of the calibration graph for clenbuterol extracts of retina

Day	Calibration graph	Slope	Calibration coefficient
1	$y=6624.153x+1516.403$	6624.153	0.999
2	$y=6556.021x+4191.502$	6556.021	0.998
3	$y=6614.685x+7282.862$	6614.685	0.997
Mean		6598.286	
C.V. (%)		0.559	

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References

[1] G. Jaffé and J.J. Grimshaw, Pharmatherapeutica, 3 (7) (1983) 492.
[2] M. Giocchino, A. Mezzetti, M. Mancini, M.D. Guglielmi, E. Lo Medico, G. Proietti Franceschilli, L. Marzio and F. Cuccurullo, Respiration, 51 (1987) 205.
[3] P. Schmitz, G. Degand, G. Pelzer, P. Gaspar and G. Maghuin-Rogister, Ann. Med. Vet., 133 (1989) 427.
[4] R.L. Mossel, R.H. Dalrymple, S.G. Cornelius, J.E. Pettigrew and C.B. Allen, J. Anim. Sci., 62 (1986) 21.
[5] A. Gabriel, L. Istasse, A. Clinquart, I. Dufrasne, C. van Eenaeme, M. Gielen and J.M. Bienfait, Ann. Med. Vet., 133 (1989) 281.
[6] P. Gustin, M. Ansay and G. Maghuin-Rogister, Ann. Med. Vet., 133 (1989) 293.
[7] J. Henion, G. Maylin and B.A. Thomson, J. Chromatogr., 271 (1983) 107.
[8] I. Yamamoto, E. Matsura, M. Horiba, K. Akima, K. Nomura and T. Aizawa, J. Immunoassay, 6 (1985) 261.
[9] H.H. Meyer, L. Rinke and I. Dürsch, J. Chromatogr., 564 (1991) 551.
[10] C.T. Elliott, J.D.G. McEvoy, W.J. McCaughey, D.H. Shortt and S.R.H. Crooks, Analyst, 118 (1993) 447.
[11] W.J. Blanchflower and D.G. Kennedy, Biomed. Environ. Mass Spectrom., 18 (1989) 953.
[12] J. Girault and B. Fourtillan, J. Chromatogr., 518 (1990) 41.
[13] J. Girault, P. Gobin and B. Fourtillan, Biomed. Environ. Mass Spectrom., 19 (1990) 80.
[14] L. Leyssens, C. Driessen, A. Jacobs, J. Czech and J. Raus, J. Chromatogr., 564 (1991) 515.
[15] M.C. Dumasia and E. Houghton, J. Chromatogr., 564 (1991) 503.
[16] J.A. García Regueiro, B. Pérez and G. Casademont, J. Chromatogr. A, 655 (1993) 73.
[17] J.A. van Rhijn, W.A. Traag and H.H. Heskamp, J. Chromatogr., 619 (1993) 243.
[18] R.T. Wilson, J.M. Groneck, K.P. Holland and C. Henry, J. Assoc. Off. Anal. Chem., 4 (77) (1994) 917.
[19] B. Diquet, L. Doare and P. Simon, J. Chromatogr., 336 (1984) 415.
[20] J.A. Haman, K. Johnson and T. Jeter, J. Chromatogr. Sci., 23 (1985) 34.

[21] C. Eddins, J. Haman and K. Johnson, J. Chromatogr. Sci., 23 (1985) 308.
[22] G.A. Quereshi and A. Erksson, J. Chromatogr., 441 (1988) 197.
[23] E. Ciranni Signoretti, C. D'Arpino and F. de la Torre, J. Chromatogr., 473 (1989) 301.
[24] L. Rinke, H.H.D. Meyer and M. Rapp, Chromatogram, 11 (1990) 6.
[25] G.F. Bambrilla, S. Castelli, A. Riberzani, M. Montana and F. Manca, Ind. Aliment., 39 (1990) 674.
[26] W. Haasnoot, M.E. Ploum, R.J.A. Paulussen, R. Schilt and F.A. Huf, J. Chromatogr., 519 (1990) 323.
[27] D. Courtheyn, C. Desaever and R. Verhe, J. Chromatogr., 564 (1991) 537.
[28] P.A. Biondi, L. Guidotti, M. Montana, F. Manca, G. Bambrilla and C. Lucarelli, J. Chromatogr. Sci., 29 (1991) 190.
[29] G.F. Bambrilla, U. Agrimi and E. Pierdominci, Ital. J. Food Sci., 4 (1991) 303.
[30] H. Hooijerink, R. Schilt, W. Haasnoot and D. Courtheijn, J. Pharm. Biomed. Anal., 6 (9) (1991) 485.
[31] M.H.A. Botterblom, M.G.P. Feenstra and E.B.H.W. Erdtsieck-Ernste, J. Chromatogr., 613 (1993) 121.
[32] C. Presa, M. Ramos and Th. Reuvers, Alimentaria, 1–2 (1990) 85.