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Note

Determination of clenbuterol in the high nanogram range in plasma of mice by high-performance liquid chromatography with amperometric detection

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Clenbuterol is a selective beta-2-adrenoceptor agonist in animal and man and has been suggested for the treatment of asthma [1]. Recently, an antidepressive-like activity related to beta-stimulation has been demonstrated in animals [2]. Thus, clenbuterol has been suggested for human endogenous depression [3].

Various methods for the evaluation of beta-2-agonists, such as salbutamol, terbutaline and fenoterol [4—7] have been reported. No method for the measurement of clenbuterol in biological samples has yet been described. This is probably because of the low plasma levels (< 1 ng/ml) obtained after an oral dose in humans. Experimentally the efficacy of clenbuterol leads to dosing schedules in the order of 10—40 μg for asthma and about 100 μg for depression.

The recent observation of its antidepressive-like activity in animals necessitated the determination of plasma clenbuterol concentrations for a better understanding of its effect. This has led us to develop a method of measuring clenbuterol in biological fluids. This paper proposes a method sufficiently sensitive for the analysis of clenbuterol in plasma of mice using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED).

EXPERIMENTAL

Animals and blood-sampling procedure

Male Swiss NMRI mice weighing 20—25 g were used. Blood was drawn from

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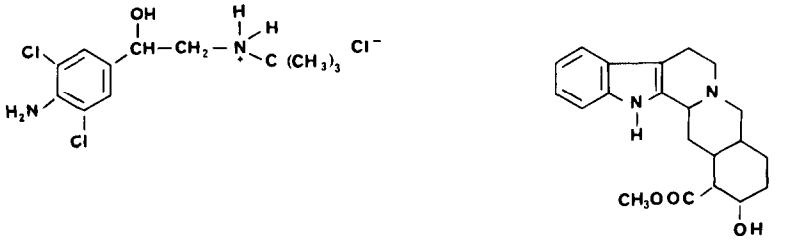
the orbital sinus and collected in heparinized tubes. After centrifugation plasma was frozen in polypropylene tubes and kept at −20°C until assay.

Chemicals and drugs

Clenbuterol hydrochloride (NAB 365) (Fig. 1) (Boehringer, Ingelheim, France) and the internal standard, yohimbine hydrochloride (Fig. 1), (Sigma, St. Louis, MO, U.S.A.) were used.

Standard solutions of clenbuterol and internal standard were prepared by dissolution in methanol at concentrations of 1 and 10 μg/ml.

All reagents used were of analytical grade: methanol (Prolabo, France), chloroform (U.C.B., Belgique), monobasic ammonium phosphate (Sigma), sodium hydroxide (Prolabo) and orthophosphoric acid (Prolabo).



CLENBUTEROL INTERNAL STANDARD

Fig. 1. Chemical structures of clenbuterol and internal standard.

Apparatus

The chromatographic system consisted of a Model A 802 solvent delivery pump (Lirec, France), and a Rheodyne sample valve fitted with a 50-μl loop. The column was a Resolve RP18, 5 μm particle size (15 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.). A guard column packed with C_{18} material was used to protect the analytical column.

A Metrohm ED system composed of a 641 VA detector, a 656 electrochemical detector equipped with a glassy carbon electrode and an Ag/AgCl reference electrode was used to oxidize the compounds at a potential of 1.15 V. The sensitivity was set at 10 nA full scale. All chromatograms were recorded on a Servotrace recorder (Sefram, France) at a chart speed of 5 mm/min.

Mobile phase

The mobile phase consisted of 1 m*M* ammonium phosphate—methanol (10:90, v/v). The pH was adjusted to 5.0 by adding orthophosphoric acid. The flow-rate was kept constant at 1 ml/min. The mobile phase was thoroughly degassed and filtered through a 0.2-μm filter disc (Millipore, Bedford, MA, U.S.A.).

Extraction

To 500 μl of plasma of mice were added 80 μl of a solution of 1 μg/ml of internal standard, 0.5 ml of 0.1 *M* sodium hydroxide and 6 ml of chloroform. The mixture was shaken for 20 min using an alternating agitator (Realis type

44-40, France) or for 1.5 min using a Vortex mixer (Bioblock, France). The solution was then centrifuged for 10 min at 900 *g* at −2°C and the supernatant discarded. The lower organic phase was transferred to a clean tube and then evaporated to dryness using a Vortex evaporator (Buchler, NJ, U.S.A.). The residue was dissolved in 100 μl of the mobile phase; a 25-μl aliquot was injected into the chromatograph.

Calibration curves

The calibration curves were obtained by adding clenbuterol to mouse control plasma to obtain concentrations of 10, 20, 40, 80, 120, 160 and 200 ng/ml. These standards were extracted under the experimental conditions as described above.

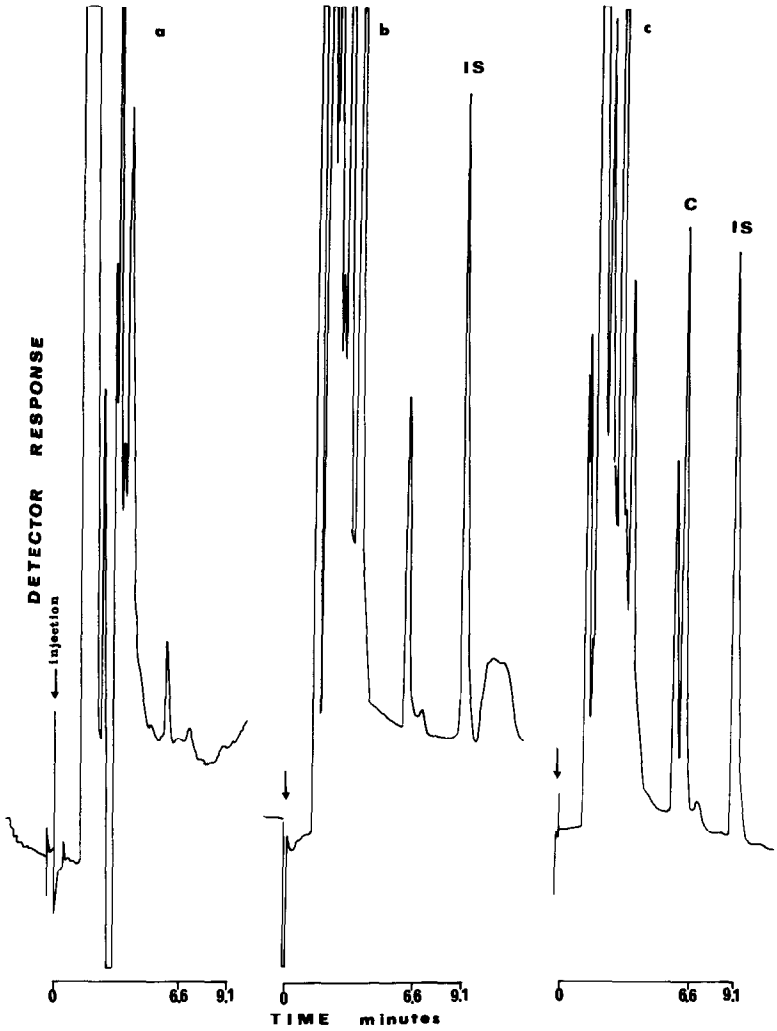


Fig. 2. Typical chromatograms obtained from mouse plasma after injection of (a) blank plasma control, (b) plasma control spiked with internal standard (IS), and (c) plasma control spiked with internal standard and 140 ng/ml clenbuterol (C).

The peak heights were measured and the peak height ratios of clenbuterol over internal standard were plotted against concentration.

RESULTS

Fig. 2 illustrates typical chromatograms obtained after extraction of blank and spiked plasma of mice. The retention times for clenbuterol and internal standard are 6.6 and 9.1 min, respectively (capacity coefficients k' = 4.0 and 6.0, respectively). Fig. 3 illustrates chromatograms obtained from plasma of mice collected 30 min after intraperitoneal administration of 0.5 mg kg^{-1} clenbuterol.

Calibration curve shows good linearity (correlation coefficient 0.9969 ± 0.0026) in the range 0—200 ng/ml. The equation of the curve is $Y = 0.013072\ (\pm\ 0.0007)X - 0.0122255\ (\pm\ 0.0212)$.

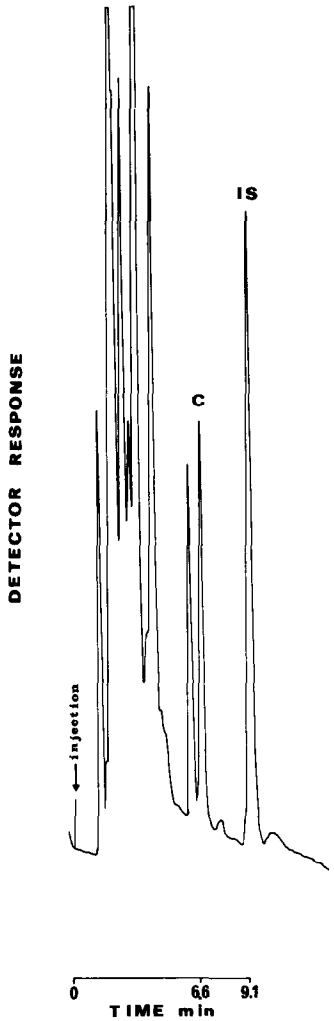


Fig. 3. Chromatogram obtained from mouse plasma 30 min after a 0.5 mg kg^{-1} intraperitoneal administration of clenbuterol.

Precision

The reproducibility of the method was checked for three plasma concentrations: 40, 120, and 200 ng/ml. Ten determinations were made on the same day.

The coefficients of variation are shown in Table I. The day-to-day reproducibility was assessed at 40, 120 and 200 ng/ml over a period of five days. The coefficients of variation are shown in Table I.

TABLE I

REPRODUCIBILITY AND ACCURACY OF HPLC—ED ASSAY FOR CLENBUTEROL

Clenbuterol (ng/ml)	Coefficient of variation (%)	
	Reproducibility	Accuracy (day-to-day)
40	4.41	5.96
120	8.24	7.56
200	3.57	6.69

Recovery

Recovery of clenbuterol was assessed by comparing the peak height after an injection of a pure solution of clenbuterol with that obtained after an injection of extracted plasma containing the same amount of clenbuterol. The percentage recovery of the extraction procedure is shown in Table II.

TABLE II

RECOVERY OF CLENBUTEROL FROM MOUSE PLASMA

Clenbuterol (ng/ml)	Recovery (%)
40	46.25 ± 0.85
120	46.51 ± 1.20
200	43.6 ± 0.99
$\bar{X}$	45.45 ± 1.13

Sensitivity

Under the experimental conditions described above the minimum detectable concentration is 3 ng/ml. But either by increasing the volume injected into the chromatograph or by setting the sensitivity of the detector at 5 nA full scale the limit of detection is 1 ng/ml.

DISCUSSION

This method, with a limit of detection of 3 ng/ml, is sufficiently sensitive for the determination of clenbuterol levels in plasma of mice. It requires a small volume of sample and so allows measurement in an individual mouse, avoiding the need to pool several plasmas. In our laboratory the method has been applied

to study the pharmacokinetics of clenbuterol after intraperitoneal administration in mice (0.5 mg kg^{-1}).

A good resolution of the chromatogram is obtained with a 5-μm Resolve™ column. Compared to a standard one, such a column gives a better sensitivity. However, it is tremendously sensitive to very small modifications in the mobile phase (which then involves significant changes in resolution and retention times).

This procedure shows a good reproducibility; however, before obtaining reproducible results some care must be taken. For example, the sensitivity of the working electrode gradually declines and cleaning of this electrode is necessary to restore it. If a rapid decrease in the response occurs immediately after cleaning, it is essential to stabilize the electrode until the response of the detector is constant. For better results, a new column must be conditioned with the mobile phase before use for 24 h; if this is not done, modifications in the retention times occur.

Many chromatographic problems arose from the extraction and purification of mouse samples. Clenbuterol is not extracted by non-polar solvents but its extraction by polar solvents leads to chromatograms containing endogenous contaminants. The solvent chosen (chloroform) yielded chromatograms containing fewer contaminants and was used for extraction although the percentage recovery of clenbuterol was low. However, the recovery of clenbuterol proved to be constant and unaffected by the concentration of the drug.

The choice of yohimbine as internal standard seems unusual at first sight since its chemical structure is different from that of clenbuterol and more related compounds can be obtained. But according to the chromatographic conditions which were chosen to obtain the best response of clenbuterol (consistent with a sufficiently short retention time), yohimbine has the advantage of being well separated from clenbuterol and from other peaks. Other beta-agonists, such as salbutamol and isoproterenol, tested as internal standard cannot be separated from endogenous peaks. Terbutaline showed a retention time consistent with a good separation, but it was not well extracted in chloroform whatever the pH. Ethyl acetate, which can be used for the extraction of terbutaline [5], was also tried. But such a solvent cannot be conveniently used with electrochemical detection; many chromatographic problems occur, endogenous plasma components appearing in the chromatogram and interfering with terbutaline and clenbuterol.

Thus, our procedure performed as described above can only be used for the determination of clenbuterol. With a few modifications to the mobile phase, it can be applied for determinations of other beta-2-agonists. Thus, this method does not allow the simultaneous measurement of different beta agonists.

In summary, the technique developed using HPLC—ED is reproducible, and is sensitive enough for the determination of clenbuterol in mouse plasma. It is easy to perform and is inexpensive.

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