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Short communication

Stereoselective determination of clenbuterol in human urine by capillary electrophoresis

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Abstract

The effectiveness of capillary electrophoresis (CE) in the field of stereoselective determination of drugs in biological matrices is demonstrated by analyzing clenbuterol in human urine. Due to the very low therapeutical doses of 20–40 μg per day the total concentrations in urine are 1–10 ng/ml. The sample was extracted with hexane–*tert*.-butyl methyl ether (99.5:0.5). The reconstituted sample was injected electrokinetically (50 s, 10 kV). Using phosphate buffer, pH 3.3 and hydroxyethyl-β-cyclodextrin as chiral selector the total analysis time was below 15 min. The limit of determination was estimated as 0.5 ng/ml per enantiomer. *S*-(−)-Bupranolol was used as internal standard. Both precision and accuracy of the method were within the limits for biological samples. The application to human urine from patients having received therapeutical doses showed a slightly predominant excretion of the (+)-enantiomer to the (−)-enantiomer.

Keywords: Clenbuterol

1. Introduction

Clenbuterol (Fig. 1) is a highly potent β_2-sympathomimetic drug. In therapy of human respiratory diseases the racemic drug is used being administered orally at doses of 2×20 μg per day. It is also applied in veterinary medicine as a tocolytic agent. Due to lipolytic and anabolic effects of higher doses clenbuterol has been illegally applied as a growth promoter in meat-producing industry and as a doping agent in sports [1,2]. The (−)-enantiomer is described as responsible for the mimetic effect on β_2-receptors while the (+)-enantiomer reveals a blocking effect on the β_1-receptors [3]. Clenbuterol is metabolized only to a small extent. Up to 65% is excreted unchanged in the urine. Low levels of glucuronide conjugates have been reported. Total concentrations for the enantiomers in human urine after administration of therapeutical doses of the racemate have a maximum of about 10 ng/ml [4].

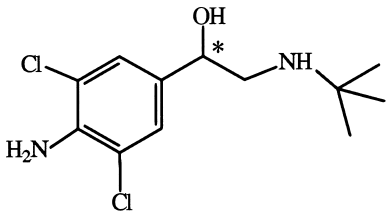

Fig. 1. Structure of clenbuterol.

Various methods for the determination of clenbuterol in biological matrices have been established, e.g., immunoassays and gas chromatography–mass

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spectrometry (GC–MS) [5]. No enantioselective application to human body fluids has been described previously.

Capillary electrophoresis (CE) is a very useful technique for the determination of drugs in body fluids because of its high resolution [6]. The addition of chiral selectors allows direct separations of enantiomers [7–10]. However, using CE with UV detection, the limit of determination is frequently insufficient for drugs in biological matrices due to low concentration sensitivity. Electrokinetic injection technique combined with sample preconcentration offers an enormous enhancement in sensitivity.

2. Experimental

2.1. Chemicals

Clenbuterol·HCl (NAB 365) and both enantiomers were kindly donated by Dr. Karl Thomae (Biberach, Germany). The internal standard bupranolol·HCl was a gift of Schwarz Pharma (Monheim, Germany).

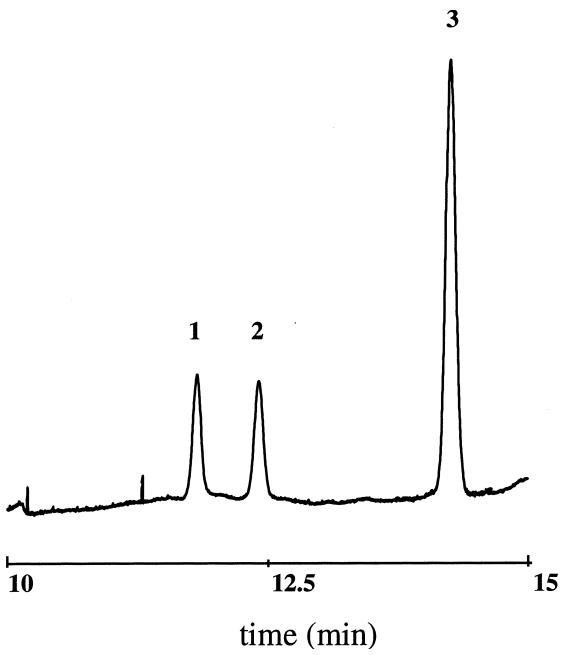

Fig. 2. Representative electropherogram of human urine spiked with racemic clenbuterol (1 ng/ml per enantiomer) and internal standard (I.S.). Peaks: 1=(−)-enantiomer; 2=(+)-enantiomer; 3: I.S.

Hydroxyethyl-β-cyclodextrin was supported by Wacker-Chemie (Munich, Germany).

Methanol, hexane, *tert*.-butyl methyl ether (HPLC grade), orthophosphoric acid and sodium hydroxide were obtained from Baker (Deventer, The Netherlands).

Stock solutions were prepared in methanol and stored at −18°C.

2.2. Sample pretreatment

To 2 ml of urine 10 μl methanolic solution of *S*-(−)-bupranolol (5.7 μg/ml) were added as internal standard. The solution was alkalized with 2 ml 0.5 *M* sodium hydroxide followed by extraction with 10 ml hexane–*tert*.-butyl methyl ether (99.5:0.5). After shaking (30 min) and centrifugation (10 min, 300 *g*) the organic layer was dried at 40°C under a stream of nitrogen. The residues were reconstituted in 100 μl methanol and analyzed by CE.

2.3. Electrophoresis equipment and operating conditions

A HP ^{3D}CE System (Hewlett-Packard, Waldbronn, Germany) with an untreated fused-silica capillary was used. For UV detection at 210 nm diode array detection (DAD) was employed. The capillary (Polymicro Technologies, Phoenix, AZ, USA) dimensions were 48.5 cm (40 cm to detector)×50 μm I.D. The outer diameter was 360 μm.

Separation was carried out in a 200 m*M* phosphoric acid, adjusted to pH 3.3 with 200 m*M* sodium hydroxide solution. Thirty mg/ml hydroxyethyl-β-cyclodextrin was used as chiral selector. The applied voltage was 20 kV and the temperature was maintained at 20°C. Electrokinetic injection was performed for 50 s at 10 kV. After each run the capillary was rinsed with 200 m*M* phosphoric acid and run buffer for 2 min, respectively. Migration order was determined by injecting the pure enantiomers separately. Injections were performed only once from the sample in order to avoid discriminating effects. The corrected peak areas were calculated using HP ChemStation software.

A representative electropherogram is presented in Fig. 2.

2.4. Calibration

In order to obtain calibration samples aliquots of a methanolic clenbuterol solution were evaporated under a gentle stream of nitrogen and diluted with blank urine to 10.0 ml. Five concentrations in the range of 1–20 ng/ml racemate, i.e., 0.5–10 ng/ml per enantiomer, were used. All samples were processed further as described in Section 2.2 beginning with the addition of the internal standard.

Calibration curves according to the internal standard method were obtained by plotting concentration vs. peak-area ratios followed by linear regression analysis.

2.5. Assay validation

Four spiked samples at concentrations of 0.5, 1.25, 2.5, 5, 10 ng/ml per enantiomer were analysed within one day to assess intra-day variability. The procedure was repeated on three days to investigate inter-day precision and accuracy. Absence of matrix interferences was confirmed by analysis of blank urine.

Recovery was found to be 65.0% (±3.0) in mean for the (−)-enantiomer and 63.2% (±4.5) for the (+)-enantiomer, respectively.

2.6. Application

The excreted enantiomers were determined in urine samples taken from three patients of a hospital, who had received therapeutical doses of 20 μg once (male, 28 years; male, 24 years; female, 23 years). Urine was collected in intervals of approximately 6 h. Samples were obtained up to 31 h after administration. After the determination of the urine volume an aliquot was stored at −18°C until analysis.

3. Results and discussion

3.1. General aspects

Both enantiomers and the internal standard were readily separated within 15 min from matrix constituents employing CE in untreated fused-silica capillaries. Interferences from matrix components were not observed.

3.1.1. Extraction procedure

The lipophilicity of the extraction mixture reduced the amount of polar endogenous components. The ionic strength of the reconstituted sample proved to be decisive for electrokinetic injection. The application of conventional extraction methods provided in literature [4] yielded higher recoveries but was unfavourable for sample introduction.

3.1.2. Electrokinetic injection

Samples of low conductivity, e.g., of a methanolic solution, allowed an electrokinetic migration of the analyte into the capillary filled with a solution of higher ion strength. By combination of both methods, i.e., extraction procedure and injection technique, an extremely high preconcentration from the biological sample could be achieved. Stacking effects of up to 100-fold have been reported [11,12]. In order to obtain a high field-strength in the capillary during sample injection a 200 m*M* buffer was employed. To avoid inaccuracies by discrimination effects during repeated sample introduction the prepared sample solutions were injected only once per sample vial.

3.2. Validation

The method was validated in a concentration range of 0.5–10 ng/ml per enantiomer. Calibration curves were obtained by plotting concentration vs. peak-area ratio. The results of one validation experiment are presented in Table 1. Intra-day and inter-day variabilities offered satisfactory results, i.e., both accuracy and precision were within a 20% interval. The limit of quantitation (LOQ) was estimated as 0.5 ng/ml per enantiomer.

3.3. Application

The method was applied to samples from a hospital. The results are presented in Table 2. The cumulative excretion reveals a slightly predominant elimination of the (+)-enantiomer. The ratios between the concentrations of both enantiomers however are much smaller compared to other β-agonists, e.g., albuterol, where ratios of 1.5–2.5 had been observed [13]. This could be explained by the absence of phenolic substituents in clenbuterol which are target sites for various metabolizing enzymes,

Table 1
Precision and accuracy of the determination of the enantiomers of clenbuterol in human urine with CE

	Nominal concentration (ng/ml) (−)-clenbuterol/(+)-clenbuterol				
	10.00	5.00	2.50	1.25	0.50
Concentration found (ng/ml) (arithm. mean value)					
Day 1 (n=4)	10.05/9.93	4.88/5.16	2.51/2.45	1.24/1.27	0.55/0.46
Day 2	9.95/10.11	5.11/4.78	2.49/2.43	1.27/1.36	0.43/0.60
Day 3	10.41/10.14	5.07/5.07	2.33/2.52	1.26/1.32	0.59/0.58
Inter-day (n=12)	10.14/10.06	5.02/5.00	2.44/2.47	1.26/1.32	0.52/0.55
Accuracy (%) (arithm. mean value)					
Day 1 (n=4)	100.52/99.31	97.69/103.23	100.58/98.10	99.55/101.39	110.80/92.88
Day 2	99.49/101.08	102.11/95.67	99.70/97.05	101.96/109.18	86.04/119.88
Day 3	104.11/101.41	101.43/101.49	93.35/101.00	100.70/105.47	118.96/109.44
Inter-day (n=12)	101.37/100.60	100.41/100.13	97.88/98.72	100.74/105.35	105.39/109.44
Precision (C.V., %) (arithm. mean value)					
Day 1 (n=4)	3.99/4.51	4.52/6.37	6.84/6.99	10.61/8.61	16.45/14.23
Day 2	4.11/5.46	4.98/8.94	8.65/6.47	10.10/9.55	15.33/16.24
Day 3	2.48/8.45	3.91/6.14	5.41/8.37	9.54/9.66	16.47/17.91
Inter-day (n=12)	3.53/6.14	4.47/7.15	7.30/6.61	10.09/9.27	16.08/16.12

e.g., catechol-*O*-methyltransferase (COMT). This enzyme is reported to be highly stereoselective [14].

4. Conclusions

Using CE, very low concentrations of drugs in body fluids can be determined. Due to the low concentration sensitivity sample preconcentration and electrokinetic injection technique have to be used. The determination of clenbuterol enantiomers in human urine was satisfactory with regard to precision and accuracy.

A slight stereoselectivity of excretion could be observed. The differences in the excretion are lower than compared to phenolic β_2-agonists.

Table 2
Ratios of excreted enantiomers in urine (cumulatively) after single administration of 20 μg racemate (arithm. mean values of three patients)

Interval (h)	Ratio (+)/(−)-enantiomer	S.D. (%)
0–6	1.03	2.52
7–12	1.02	0.93
13–18	1.01	1.16

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