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Determination of clenbuterol in human urine and serum by solid-phase microextraction coupled to liquid chromatography

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Abstract

A solid-phase microextraction (SPME)–LC–UV method for the determination of the beta-adrenergic drug clenbuterol in human urine and serum samples was developed for the first time using a polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated fiber. The procedure required very simple sample pretreatments, isocratic elution, and provided highly selective extractions. All the aspects influencing fiber adsorption (extraction time, temperature, pH, salt addition) and desorption (desorption and injection time, desorption solvent mixture composition) of the analyte have been investigated. The linear ranges investigated in urine and serum were 10–500 and 5–500 ng/ml, respectively (that covers the typical clenbuterol concentration observed in biological fluids). Within-day and between-days R.S.D.% in urine ranged between 5.0–5.3 and 8.5–8.7, respectively, while in serum ranged between 5.5–5.9 and 8.7–9.1, respectively. Estimated LOD and LOQ were 9 and 32 ng/ml (spiked urine), respectively, and 5 and 24 ng/ml (spiked serum), respectively, well below the usual clenbuterol urinary and serum level.
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1. Introduction

Clenbuterol (4-amino-3,5-dichloro-*a*-*tert*-butylaminomethylbenzyl alcohol hydrochloride) is a beta-adrenergic drug typically employed as a bronchial dilating agent for the treatment of pulmonary diseases, in particular in the case of chronic illness [1]. In addition, it was later discovered that it also possess physiological effects similar to anabolic steroids; namely it promotes [2] the growth of the muscular tissue and the reduction of body fat. As a consequence, clenbuterol has been extensively used as a growth promoter in feed for farm animals [3] (to improve the lean meat portion resulting in a conspicuous gain for the farmer). Regardless its long term or high dose use has been associated with serious side effects [4–6] or acute toxic responses [7], it has been extensively misused, leading to serious outbreaks of human poisoning in Spain, Italy and France, due to elevated level in beef liver [8–11]. The illegal use of the drug in the USA has been the subject of a federal indictment [12]. In the Netherlands and other European countries, strict control strategies have been developed in order to prevent the illegal use of beta-agonists [13]. The European union (Directive 96/22/EC) has banned the administration of clenbuterol to any animal species intended for human consumption, except for therapeutic treatments after veterinary prescription.

Moreover, the use of this drug as doping agent among athletes is well known to increase strength and performance [14]. Thus, also sport federations have felt the need to control their use in athletes. For instance, clenbuterol was banned by the Medical Commission of the International Olympic Committee (IOC) [15].

As a result of this concern, a method for the determination of clenbuterol in human urine and serum samples is highly advisable for doping purposes. Existing papers on this topic have been essentially based on gas [16–19] (after tedious derivatization of the analyte) or liquid [20–23] chromatography, after purification of the analytes by means of traditional isolation procedures such as liquid–liquid extraction (LLE) [16,20] or solid-phase extraction (SPE) [17–19,22], immunoaffinity (IAC) [21] based techniques, combination between them or, very recently, by means of three-phase solvent bar microextraction [23]. A good alternative could be represented by solid-phase microextrac-

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tion (SPME) [24], a solventless technique typically coupled [25–31] to GC. In fact, a headspace (HS)–SPME coupled to gas chromatography–mass spectrometry (GC–MS) method was recently developed for the determination of clenbuterol in urine [32]. However, the SPME step was complicated by the need of a complex heating–cooling assembly and a subsequent on-fiber derivatization. A further possibility could be using SPME coupled to LC, whose employment is progressively growing up as demonstrated by a number of recent applications [33].

In the present paper, SPME of clenbuterol, was optimized and interfaced with LC–UV using a polydimethylsiloxane/divinylbenzene (PDMS/DVB) coated fiber. The developed procedure was then applied to the determination of the drug in human urine and serum samples.

2. Experimental

2.1. Chemicals

Clenbuterol was purchased from Sigma (St. Louis, MO). Stock solutions (1 mg/ml) of clenbuterol were prepared in methanol and stored at 4 °C in the dark. Dilute solutions were prepared just before use. Organic solvents (Carlo Erba, Milan, Italy) were HPLC grade. Mobile phase was filtered through a 0.45 μm membrane (Whatman Limited, Maidstone, UK) before use.

2.2. Apparatus

The SPME interface (Supelco, Bellefonte, PA), consisted of a standard six-port Rheodyne valve equipped with a fiber desorption chamber (total volume: 60 μl), installed in place of the sample loop.

The LC system used in this study includes a Spectra System Pump, model P2000 (ThermoQuest, San Jose, CA, USA) and a Luna C18 (150 mm × 4.6 mm i.d., particle size 5 μm) chromatographic column (Phenomenex, Torrance, CA, USA). Mobile phase was degassed by an SCM 1000 Vacuum membrane degasser (Thermo Separation Products). The detector was a photodiode-array (Spectra System model UV6000LP) controlled by a ChromQuest software running on a personal computer.

2.3. Chromatographic and detection conditions

The mobile phase used for standard solutions and serum samples was an acetonitrile/methanol/phosphate buffer (20 mM, pH 10) mixture (10:50:40, v/v/v); in the case of urine analysis, the mobile phase was slightly modified as acetonitrile/methanol/phosphate buffer (20 mM, pH 10) mixture (10:60:30, v/v/v) due to the presence of matrix interferences. The flow rate was 1.0 ml min^{-1} and temperature was ambient. The detection wavelength was 244 nm (10 Hz frequency, 5 nm bandwidth). Spectra were acquired in the 220–380 nm range (2 Hz frequency, 5 nm bandwidth).

2.4. Solid-phase microextraction

Fibers coated respectively with a 50 μm thick carbowax/templated resin (CW/TPR-100) film, a 60 μm thick polydimethylsiloxane/divinylbenzene (PDMS/DVB) film and 85 μM thick polyacrylate (PA) film (Supelco) were employed for comparative studies. A manual SPME device (Supelco) was used to hold the fiber. Working solutions were prepared by spiking 15 ml of a phosphate buffer (18 mM, pH 11.7) solution with different amounts of clenbuterol (5–500 ng/ml) into 15 ml clear vials (Supelco). Then, the vials were sealed with hole caps and Teflon-faced silicone septa (Supelco). The extraction was carried out at 50 °C at pH 11.7 for 60 min under magnetic stirring. Clenbuterol desorption was performed in static desorption mode by soaking the fiber in an acetonitrile/methanol/phosphate buffer (10 mM, pH 11.7) mixture (50:25:25, v/v/v) into the desorption chamber of the interface for 10 min. Then, the valve was changed to the inject position and the fiber was exposed for 6 s to the mobile phase stream.

In order to evaluate percentages of desorption and carryover, the fiber was left in the chamber after each experiment and a second chromatographic run was performed leaving the interface valve in the inject position (dynamic desorption); this operation mode ensured a total desorption of the analyte remained on the fiber.

2.5. Sample collection and pre-treatment

All samples were stored at −20 °C. Drugs free urine samples (0–12 h) were collected from healthy donors, 80 ml of each sample were added with 20 ml of NaOH 0.18N in order to reach pH 12, centrifuged for 10 min at 5000 rpm, the surnatant separated, NaCl added and 15 ml subjected to SPME.

Drugs free serum samples were colleted from healthy donors 0.15 ml diluted 1:10 with phosphate buffer (50 mM, pH 11.7), stirred and subjected to SPME.

Quantitation was performed with the standard addition method. Calibration curves were constructed spiking drug free urine/serum samples with variable amounts of clenbuterol, in order to cover the following concentration ranges: 30–500 ng/ml (urine) and 60–500 ng/ml (serum). Six replicates for each concentration were performed.

The within-day ($n = 6$) and between-days ($n = 6$ over 10 days) coefficient of variation for clenbuterol were calculated on drug free urine/serum samples spiked with variable amounts of clenbuterol in order to obtain the following concentration levels: 60, 200 and 500 ng/ml.

3. Results and discussion

3.1. Fiber coating material

Preliminary experiments were performed in order to compare the extraction efficiency obtained using the CW/TPR-100, PA and PDMS/DVB coated fibers, respectively. CW/TPR-100 and PA were soon dissolved at the very basic working pH, both of the extraction solutions and of the mobile phase, while PDMS/DVB

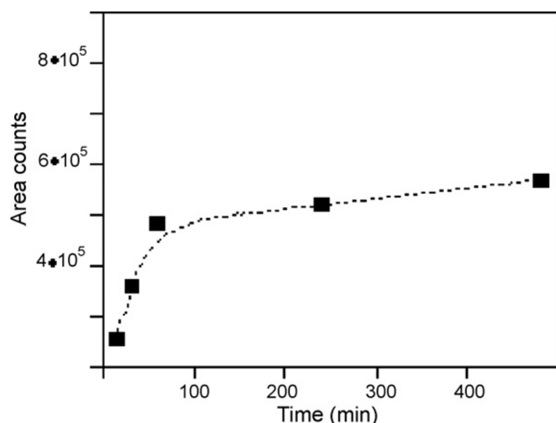

Fig. 1. Extraction time profiles obtained with the PDMS/DVB fiber at 50 °C. The concentration is 100 ng/ml.

was found to be very resistant in that conditions and was then chosen for further experiments.

3.2. Extraction time and temperature

The extraction time profiles were established by plotting the area counts vs. the extraction time, at 20 and 50 °C. Fig. 1 reports the results obtained at 50 °C. As apparent, after 60 min the equilibrium was still not reached. In any case, since it is possible to obtain good extraction yields and reliable analysis also in non-equilibrium conditions, an extraction time of 60 min was chosen for further experiments. A response decrease was observed at 20 °C (data not shown).

3.3. Ionic strength and pH

Generally speaking, salt addition improves the recovery, especially in the case of polar (hydrophilic) compounds that are difficult to extract. Thus, experiments were performed by increasing progressively the ionic strength of the extraction solutions. A progressive signal enhancement was obtained on standard solutions, while no significant effects were observed in the case of real samples. Consequently, this option was no more considered for further experiments.

Since analytes in the neutral forms are more efficiently extracted by the non-ionic polymeric coatings, the effect of the pH on the extraction efficiency was examined. Clenbuterol is in fact a basic compound and is present in its undissociated form at basic pH. As expected, a response increase was observed by increasing pH from 3 to 12; the latter value was chosen for further experiments.

3.4. Desorption conditions and “carry-over”

The dynamic mode was first employed to desorb the analyte from the fiber in the SPME–LC interface; this approach produced quantitative recoveries but very broad chromatographic peaks. Thus, the static desorption technique was used for further experiments. The fiber was soaked in mobile phase for a variable period of time before injection into the LC column. The best conditions (recovery of 78.0 ± 1.3%) were reached after 10 min of static desorption in an acetonitrile/methanol/phosphate buffer (10 mM, pH 11.7) mixture (50:25:25, v/v/v); then, the fiber was exposed for 6 s to the mobile phase stream.

Table 1
Within-day ($n = 6$) and day-to-day ($n = 6$, for 10 days) precision obtained on drug free urine samples spiked with variable amounts of clenbuterol

Clenbuterol (ng/ml)	Precision R.S.D.%	
	Within-day	Day-to-day
60	5.3	8.7
200	5.1	8.5
500	5.0	8.6

3.5. Linear range, detection limits and precision

The response of the developed SPME–LC procedure was linear in the range 10–500 ng/ml. The unweighted regression line peak area counts (arbitrary unit) vs. (clenbuterol) μg/ml was described by the following equation: $y = (3 \pm 7) + (3.53 \pm 0.04)10^3 x$; $R^2 = 0.9996$.

The estimated LOD and LOQ obtained on standard solutions were 5 and 18 ng/ml, respectively, calculated according to IUPAC as three and 10-fold the standard deviation of the intercept of the calibration curve [34].

The within-day precision of the method was investigated on standard solutions in the concentration range 10–100 ng/ml by performing daily six replicates. The same solutions were analyzed six times each day for a period of 10 days for the day-to-day precision evaluation. The within day R.S.D.% ($n = 6$) and day-to-

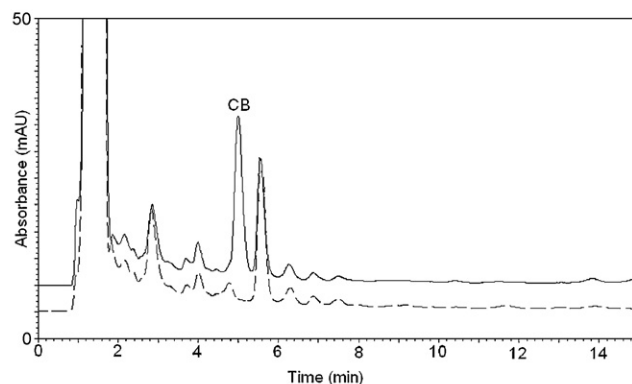

Fig. 2. SPME–LC–UV chromatograms relevant to urine samples, blank (down) and spiked with clenbuterol at 100 ng/ml (up).

Table 2
Within-day ($n = 6$) and day-to-day ($n = 6$, for 10 days) precision obtained on drug free serum samples spiked with variable amounts of clenbuterol

Clenbuterol (ng/ml)	Precision R.S.D.%	
	Within-day	Day-to-day
60	5.9	8.7
200	5.7	9.1
500	5.5	8.8

Table 3
Performance of the proposed method (urine samples) and comparison with existing methods for the determination of clenbuterol in urine samples

Parameter	Work reference							
	This work	[16]	[18]	[20]	[22]	[23]	[31]	[31]
Sample pre-treatment	SPME	LLE	SPE	SPE–IAC	SPE	TPSBME	HS-SPME	LLE
Instrumental technique	LC–UV	GC–MS	GC–MS^3	LC–ED	LC–MS	LC–MS^2	GC–MS	LC–UV
Linearity (ng ml^{-1})	10–500	0.08–100	0.5–5	n.d.	0.5–5	0.1–4	1–1000	50–3000
R^2	0.998	0.9985	0.9838	n.d.	0.997	0.9972	0.999	0.996
Average R.S.D.% within-day; day-to-day	5.1; 8.6	6.7; n.d.	n.d.	n.d.	5.0; n.d.	5.1; n.d.	3.9; n.d.	4.1; n.d.
Declared LOD (ng ml^{-1})	9.0	0.02	0.2	4.0	0.27	0.007	0.23	3.9

day ($n = 6$ over 10 days) R.S.D.% were 3.9 and 7.2, respectively, and were find to be no concentration dependent for standard solutions.

The developed procedure was then applied to urine and serum samples.

3.6. Urine samples analysis

Calibration curve resulted linear in the range 10–500 ng/ml (that covers the typical clenbuterol urinary concentration). The unweighted regression line peak area counts (arbitrary unit) vs. (clenbuterol) μg/ml was described by the following equation: $y = (3.3 \pm 11.6) + (3.46 \pm 0.04)10^3x$; $R^2 = 0.998$.

The estimated LOD and LOQ were 9 and 32 ng/ml, respectively (well below the usual clenbuterol urinary level) calculated as three and 10-fold the standard deviation of the intercept of the calibration curve [34].

Table 1 reports the obtained within-day and between-days coefficients of variation for clenbuterol in urine.

Fig. 2 reports the SPME–LC–UV chromatograms obtained from a drug-free urine (lower trace) and a spiked urine (upper trace) samples. As apparent, the analyte was clearly detected and well resolved from matrix components, even if a slight modification of the mobile phase was necessary in this case due to the presence of unresolved matrix interferences.

3.7. Serum samples analysis

Calibration curve resulted linear in the range 5–500 ng/ml (that covers the typical clenbuterol serum concentration). The unweighted regression line peak area counts (arbitrary unit) vs. (clenbuterol) μg/ml was described by the following equation: $y = (2.5 \pm 1.2) + (398 \pm 5)x$; $R^2 = 0.998$.

The estimated LOD and LOQ were 5 and 24 ng/ml, respectively (well below the usual clenbuterol serum level) calculated as three and 10-fold the standard deviation of the intercept of the calibration curve [34].

Table 2 reports the obtained within-day and between-days coefficients of variation for clenbuterol in serum.

Fig. 3 reports the SPME–LC–UV chromatograms obtained from a drug-free serum (lower trace) and a spiked serum (upper trace) samples, clearly showing that the analyte was clearly detected and well resolved from matrix components.

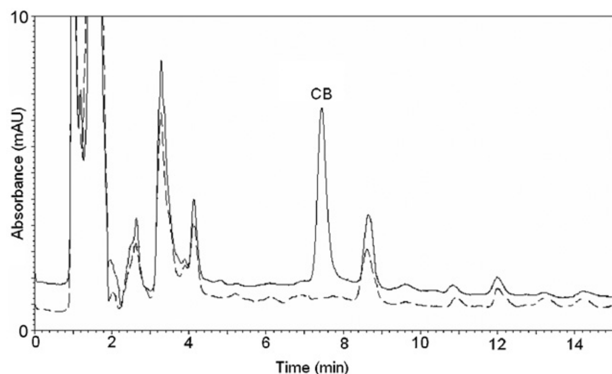

Fig. 3. SPME–LC–UV chromatograms relevant to serum samples, blank (down) and spiked with clenbuterol at 100 ng/ml (up).

4. Conclusions

An SPME (PDMS/DVB fiber)–LC–UV method for the determination of clenbuterol was developed for the first time and applied to the determination of the drug in both human urine and serum samples. As far as we know, only one work dealing with the determination of this drug in plasma samples is available in the literature [21], possessing a quite higher LOD (100 ng/ml) compared to the present approach (5 ng/ml). As far as urine analysis is concerned, even the LOD of the present method is higher than those already reported (see Table 3 for a detailed comparison) it is well below the usual clenbuterol urinary level. Furthermore, the proposed sample pre-treatment is definitely the simpler and cheaper available up to date, and allows an easy quantification of clenbuterol within its typical urinary and serum concentration.

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