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Ion-pair–supercritical fluid extraction of clenbuterol from food samples

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Abstract

Simultaneous ion-pair/supercritical fluid extraction (SFE) was investigated as an alternative to liquid solvent extraction for clenbuterol determination. Clenbuterol was extracted from diatomaceous earth and food matrices (feedstuff, lyophilized milk and liver) as its 10-camphorsulfonate ion-pair using supercritical (SC) CO_2 for 30 min of dynamic extraction at 383 bar and 40°C. The ion-pair forming reagent was added to the extraction cell prior to SFE. Recoveries ranged from 12 to 87% with mean R.S.D. of 15% for triplicate extractions. The use of the ion-pair reagent as a CO_2 modifier was also studied in depth.

1. Introduction

Supercritical fluid extraction (SFE) has proved to be an expeditious technique to leach quantitatively non-polar organic compounds from a great variety of matrices [1–4]. Nevertheless, addition of a polar modifier to the extractant fluid is required for the quantitative extraction of polar or ionic analytes when the fluid is non-polar (e.g. CO_2). In situ derivatization [5–8], complexation [9–17] and ion-pairing [18,19] under supercritical conditions are recently used approaches to improve efficiency of polar- and ionic-species extraction.

Quaternary ammonium salts have been used for ion-pair–supercritical fluid extraction. Aliphatic and aromatic surfactants have been quantitatively (>90%) extracted from sewage sludges as their tetrabutylammonium ion-pairs using supercritical CO_2 [18], while trimethylphenylammonium hydroxide has been used to improve the extraction of sulphonamides [19]. Metals ions [9,11–15,17] and organometallic compounds [8,10,16] have been extracted with CO_2 after conversion to non-charged species.

The use of clenbuterol, a β-adrenergic agonist drug, as feed additive is not permitted in the European Union (EU) but it is illegally used as a growth promoter in meat-producing animals. Clenbuterol persist in liver at levels much higher than in other edible tissues. For this reason rapid methods for the determination of clenbuterol in liver samples are required. Enzyme immunoassay has been used for β-agonist screening of bovine urine [20,21], liver [21,22] and eye [21] samples, whereas liquid [23–26] and gas [27–30] chromatography have been used after laborious and time-consuming preliminary steps (liquid–

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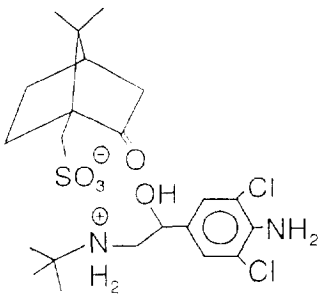

Fig. 1. Proposed interaction between camphorsulfonate ion and protonated clenbuterol.

liquid and solid-phase extractions) for the determination of clenbuterol in biological matrices. The aim of this work was to study the supercritical fluid extraction of clenbuterol from different matrices to overcome those preliminary steps.

Aminoalcohols form stable ion-pairs with camphorsulfonic acid in a non-polar medium. In addition to electrostatic attraction, hydrogen bonding between the aminoalcohol and camphorsulfonic acid takes place in a non-polar medium such as supercritical CO_2. In this paper camphorsulfonate is proposed as counterion for ion-pair–SFE of clenbuterol. The proposed interaction between camphorsulfonate ion and protonated clenbuterol is shown in Fig. 1.

2. Experimental

2.1. Instrumentation

A Hewlett-Packard HP 7680A supercritical fluid extractor equipped with a 7-ml thimble as extraction cell and a packing of small stainless-steel balls as analyte trap was used. An HP 1050 liquid chromatograph and an HP 1040A diode-array detector were used for the determination of clenbuterol in the extracts. A Fisons supercritical fluid extractor consisting of an SFC 300 double-syringe pump, an SFE 30 collector unit, an SFE 300 control system and a Haake K20 cooling circulator (ethanol-filled) and a VR 100 variable restrictor (CCS Instrument Systems, PA, USA) were used for continuous addition of the ion-pair reagent during the SFE process. An Ultraterm 6000383 P-Selecta recirculating thermostat was used to control the collector temperature.

2.2. Materials

Stock standard solution of clenbuterol hydrochloride (>95%, Sigma) of 0.4 g/l in HPLC-grade methanol (Romil Chemicals) was prepared. Diatomaceous earth (Sigma) was used as solid support.

SFE/SFC-grade CO_2 (Air Products) and methanol-modified CO_2 (nominally 10% methanol in CO_2, SEO, Spain), HPLC-grade methanol and acetonitrile (Romil Chemicals), glacial acetic acid and ammonia (Panreac, Spain) were also used.

Ion-pair reagents were prepared as 0.1 *M* methanolic solutions except where noted. The reagents tested included (1*S*)-(+)-10-camphorsulfonic acid (*S*-CamH), (1*R*)-(−)-10-camphorsulfonic acid (*R*-CamH, Sigma) and (1*R*)-(−)-10-camphorsulfonic acid ammonium salt (*R*-$CamNH_4$, Aldrich). The 0.1 *M* (1*S*)-(+)-10-camphorsulfonic acid ammonium salt (*S*-$CamNH_4$) solution was prepared from *S*-CamH and ammonia.

2.3. Coupling of the ion-pair reagent and supercritical fluid extraction modes

The ion-pair reagent was incorporated into the system in three different ways. (a) A small volume (500 μl) of reagent methanolic solution was added to the sample in the extraction cell before the extraction with supercritical CO_2 was started. (b) The reagent was dissolved in the cosolvent used as polar modifier of CO_2 and so continuously added to the fluid in the dynamic procedure. (c) A static addition followed by a dynamic addition of the ion-pair reagent was also performed.

Static addition

Extractions were performed using the HP extractor (Fig. 2A). CO_2 and methanol-modified CO_2 were delivered from a cylinder supplied with a dip tube, and they were aspirated by a

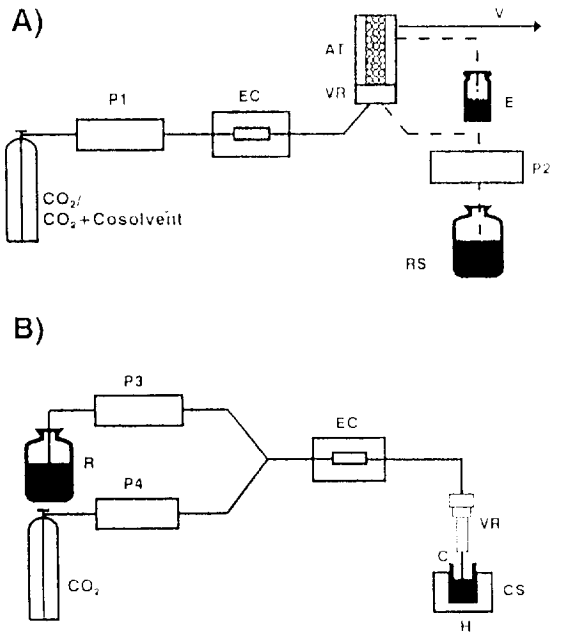

Fig. 2. Schematic diagram of the extraction systems used for ion-pair–SFE with (A) static addition and (B) dynamic addition of the ion-pair reagent. (P) pump, (EC) extraction chamber, (AT) analyte trap, (VR) variable restrictor, (E) extract, (RS) rinsing solvent, (V) vent, (R) ion-pair reagent solution, (C) collector, (CS) collection solvent and (H) collector heater with thermostat.

double-piston pump and passed through the 7-ml extraction cell which contained the sample. The extraction cell was ca. 25% filled and not-inert filler was added. The leached clenbuterol was driven to a 0.45-ml stainless-steel bead trap through an automated variable-diameter restrictor which virtually avoided plugging and provided a constant flow-rate during the extraction process. In a subsequent step (rinsing), an acetonitrile–acetic acid/ammonium acetate buffer (30:70) stream at 0.5 ml/min flow-rate was pumped through the trap by a syringe pump. In order to avoid methanol retention in the trap the temperature of this device was increased to 70°C during the extraction step when either methanol-modified CO_2 or methanolic solutions were used or added to the samples, respectively. Restrictor and trap temperatures were 60°C during the rinsing step. The extract was collected in vials which had been previously weighed in order to determine the weight of the extract as the difference. The recoveries were calculated from analyte concentrations determined by HPLC and the volume of the extract, the latter being obtained from weight/density data, which were more precise than those obtained from the values of the volume dispensed by the extractor.

Dynamic addition

Extractions were performed using the Fisons extractor (Fig. 2B). Pure CO_2 and cosolvent (pure methanol or methanolic solution of the reagent) were separately delivered by a dual-syringe pump and mixed prior to the extraction chamber. The fluid flow-rate through the 3-ml extraction cell was controlled by a manually actuated variable restrictor that was immersed in a solvent trap (4 ml of 30:70 acetonitrile–acetic acid/ammonium acetate buffer in the collector at 50°C). Larger cells were difficult to handle due to the dimensions of the extraction chamber. The collector was heated at 50°C to avoid freezing during the fluid expansion.

2.4. Chromatographic separation and detection

A liquid chromatograph with a diode-array detector equipped with an Ultrabase-C_{18} (250 × 4.6 mm, 5 μm) column was used to determine clenbuterol in the extracts. The injection volume was 20 μl. A 70:30 10 m*M* acetic acid–ammonium acetate buffer solution (pH 4.6)/acetonitrile mobile phase was used at a 1 ml/min flow-rate. The chromatogram was recorded at 244 nm, the maximum-absorbance wavelength of clenbuterol, and the peak area was used to quantify the analyte. The peak was identified by both the retention time (4.98 ± 0.08 min) and the spectrum. The calibration curve obtained by using the peak area was linear over the range 1–60 μg/ml. The equation for the linear segment obtained by the least-squares method was: $A = 1.77 + 25.65c$ (where A and c denote peak area and concentration of clenbuterol expressed in μg/ml, respectively), and the regression coefficient was 0.99989. The precision (expressed as the relative standard deviation, R.S.D.) of the HPLC method was 3.62% ($n = 11$, 10 μg/ml of clenbuterol).

2.5. Support and sample preparation

Diatomaceous earth (0.5 g) and samples (0.3–0.5 g) were directly weighed in the extraction cell following addition of 50–100 μl of clenbuterol stock solution and 0.5 ml of ion-pair reagent solution. No drying stage was performed. Support and samples were extracted 15 min after spiking.

3. Results and discussion

3.1. Static addition of the ion-pair reagent

Preliminary experiments were carried out to determine the extraction efficiency of clenbuterol with supercritical CO_2 and to search for alternatives to improve the extraction efficiency.

Ion-pair reagent survey

The two enantiomers of both camphorsulfonic acid and its ammonium salt were tested as ion-pair reagents. An amount of 0.5 ml of methanolic solution of the reagent (0.1 *M*) was added to 0.5 g of diatomaceous earth in the extraction cell that had previously been spiked with 20 μg of clenbuterol prior to SFE with pure CO_2. Recoveries were greatly improved by using the ammonium salt instead of the acid form, while there was no difference using the *R*- or *S*-enantiomers. Clenbuterol recoveries from diatomaceous earth with SC-CO_2 were 30% and 70% using camphorsulfonic acid and the ammonium salt, respectively. The (1*R*)-(−)-10-camphorsulfonic acid ammonium salt was chosen as ion-pair reagent for further experiments. This behaviour is in accordance with the features of clenbuterol, a polar compound with two–NH_2 groups which could be protonated under the extraction conditions used. A charged analyte can not be extracted with pure CO_2 unless it is converted into a neutral form, i.e. by ion-pair formation. Camphorsulfonic acid hardly would form the ion-pair, so a prior deprotonation would be mandatory, which in turn could give rise to protonation of the second amino group of clenbuterol. This latter effect, highly undesirable, does not appear when the ammonium salt is used.

Methanol and dichloromethane were studied as solvents to prepare the ion-pair reagent solution. Methanol gave higher recoveries than dichloromethane. In addition, the latter gave rise to extract turbidity, probably due to retention of dichloromethane somewhere between the trap and the vent, where the temperature was not controlled. The presence of dichloromethane caused drawbacks in the chromatographic determination, such as baseline noise.

The trap temperature was increased to 70°C during the extraction step to avoid solvent retention. The addition of volumes of reagent solution larger than 0.5 ml (1.5 ml) resulted in irreproducible results. Reproducibility was improved by decreasing the amount of liquid added to the extraction cell and increasing the trap temperature.

Solutions of ion-pair reagent with a concentration higher than 0.1 *M* were used to optimize the amount of reagent used. There was no improvement using 0.3 *M* (close to saturation) solution compared to 0.1 *M* solution.

Influence of the extraction fluid

Some experiments were performed with the aim of choosing the optimal extraction fluid. Extractions of clenbuterol from diatomaceous earth were carried out using SC-CO_2 (40°C, 281 bar, $d \simeq 0.9$ g/ml) and supercritical methanol-modified CO_2 premixed, (nominally 10%, 70°C, 314 bar, $d \simeq 0.8$ g/ml), the latter as the only procedure to use cosolvent in the HP extractor employed. Working conditions were as follows: 30 min at a flow-rate of 0.5 ml/min with and without addition of 0.5 ml of *R*-CamNH_4 methanolic solution or pure methanol.

Clenbuterol could not be extracted (0% recovery) into pure CO_2 without prior ion-pair formation. Recoveries increased (15–30%) when methanol was added to the extraction cell or when methanol-modified CO_2 was used. Regardless of the fact that the CO_2–MeOH premixed fluid was a better extractant than pure CO_2 in the absence of camphorsulfonate, the best result (80% recovery) was obtained with pure CO_2 and prior addition of *R*-CamNH_4 to the extraction

cell; this procedure was selected for further experiments. Only 60% of clenbuterol was recovered with methanol-modified CO_2 and *R*-CamNH_4 addition.

Influence of SFE variables

The experimental variables as density, temperature, equilibration, extraction time and CO_2 flow-rate were optimized in order to maximize extraction in a short time. The univariate method was used for this purpose. CO_2 density and extraction time had the largest influence on extraction efficiency.

CO_2 density. Increasing the density (or pressure) at 40°C resulted in increased recovery throughout the range studied (0.70–0.95 g/ml). A density of 0.95 g/ml was chosen for further experiments.

Temperature. The effect of this variable was studied at a constant CO_2 density of 0.8 g/ml, which allowed a wider range of temperatures than 0.9 g/ml (because of instrumental pressure limitations). The temperature range over which the effect was investigated was 40–80°C. Increasing the temperature resulted in increased recoveries, but the recovery at the highest temperature was lower than 80%, the value achieved at 0.95 g/ml and 40°C. Thus a temperature of 40°C was selected as optimum.

Dynamic variables. CO_2 flow-rate and equilibration time were studied in the range of 0.5–4.0 ml/min and 1–30 min, respectively; both variables had no influence on the recovery. Extraction times from 5 to 60 min were studied; increasing extraction time up to 30 min resulted in increased recovery, above this value recovery remained almost constant.

Reproducibility

The precision, expressed as relative standard deviation ($n = 7$), obtained for the overall process, i.e. ion-pair–SFE (static addition)–HPLC) under optimum conditions was 8.9%.

3.2. Dynamic addition of the reagent

In the dynamic addition mode, the ion-pair reagent is continuously introduced in the extractor by dissolving it in the cosolvent used as CO_2 modifier. The amount of reagent that can be used in the dynamic mode is limited by the solubility of the reagent in the supercritical CO_2–cosolvent mixture, which is lower than that in the cosolvent. The use of amounts of reagent larger than the saturation concentration could give rise to precipitation in the extraction system and restrictor plugging.

Extractions of clenbuterol (50 μl stock solution added to 0.5 g diatomaceous earth) were carried out using concentrations of 1 and 5% 10 m*M* *R*-CamNH_4 in the methanolic solution modified CO_2 with and without previous addition of this reagent to the extraction cell. Extraction time was 30 min and the flow-rate was kept between 0.8 and 1.2 ml/min. The recoveries obtained are listed in Table 1. Neither dynamic nor combined static and dynamic addition modes improved extraction compared to reagent addition to the extraction cell before SFE with CO_2. The low extraction recovery obtained in the dynamic addition mode should be expected due to the presence of methanol as cosolvent. The fact that a different extraction system was used must also be taken into account. The extraction system became plugged and CO_2 almost stopped flowing in the combined static and dynamic addition mode with 5% cosolvent, and it was unfeasible to reset the flow-rate at 1 ml/min by opening the variable restrictor. This

Table 1
Recovery of clenbuterol from diatomaceous earth

Addition procedure	Recovery ± R.S.D.[a] (%)
Static addition[b]	85 ± 8
Dynamic addition[c]	
1% cosolvent	14 ± 5
5% cosolvent	67 ± 7
Static + dynamic addition[c]	
1% cosolvent	38 ± 7
5% cosolvent	39 ± 6

[a] Here $n = 3$ except that for static addition ($n = 7$).
[b] Conditions: HP extractor, CO_2 at 40°C and 383 bar (≈0.95 g/ml).
[c] Conditions: Fisons extractor, CO_2 modified with 1% and 5% of methanolic solution of *R*-CamNH_4 (10 m*M*) at 40°C and 281 bar (≈0.90 g/ml) and 50°C and 314 bar (≈0.88 g/ml), respectively.

Table 2
Recovery of spiked clenbuterol by ion-pair–SFE

Sample	Recovery ± R.S.D.[a] (%)	
	20 μg	40 μg
Diatomaceous earth	73 ± 10	82 ± 6
Feedstuff	47 ± 12	63 ± 10
Lyofilized milk	59 ± 7	86 ± 11
Lyofilized liver	12 ± 2	37 ± 7

[a] $n = 3$.

could be the reason of the decreased recovery in this mode.

The stability of the ion pair increases in a non-polar medium such as pure CO_2, so increasing the fluid polarity by adding methanol is unfavourable. Moreover, competition of the methanol dissolved in CO_2 for the groups of clenbuterol and camphorsulfonate involved in the hydrogen bonding decreases the ion-pair stability.

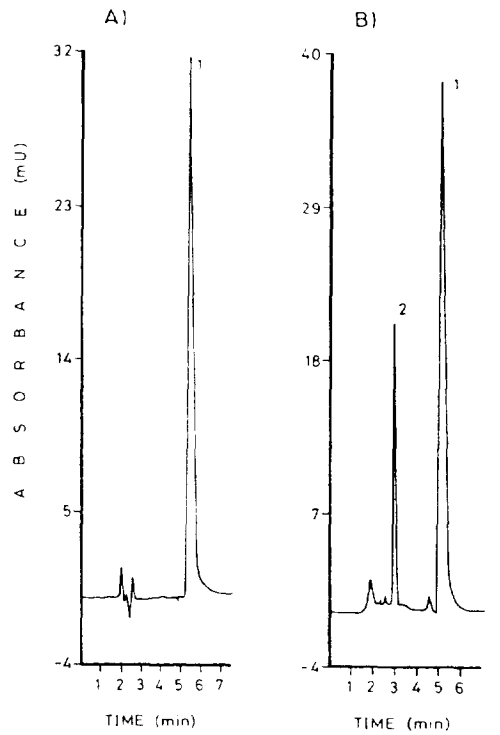

Fig. 3. HPLC chromatogram for the 20 μg/ml clenbuterol standard solution (A) and for the ion-pair–SFE extract of spiked diatomaceous earth (B). Peak identification: clenbuterol (1) and camphorsulfonate (2). For HPLC conditions see text. SFE conditions: 0.5 ml/min pure CO_2 at 40°C and 383 bar for 30 min and addition of 0.5 ml of 0.1 *M* *R*-$CamNH_4$ prior to extraction.

3.3. Application of ion-pair–SFE to spiked samples

The performance of the proposed ion-pair–SFE was tested by applying it to the recovery of clenbuterol at two different concentration levels from several food matrices (lyophilized milk, feedstuff and liver). Spiked samples were used because no real samples containing native clenbuterol were available. In addition, the HPLC–UV method used to determine clenbuterol in the extracts is not sensitive enough for the residue levels occurring in real samples. Extractions were performed in triplicate and provided good results. Table 2 shows the mean recovery and R.S.D. ($n = 3$) for each sample and spiked level (20 and 40 μg). The recoveries from food matrices were generally lower than those from diatomaceous earth. The sample amounts were 0.5 g for diatomaceous earth, feedstuff and lyophilized liver and 0.35 g for lyophilized milk.

The lowest recovery was obtained for lyophilized liver (12% for 20 μg). Recoveries from lyophilized milk ranged from 59 to 86% and were higher than that from milk mixed with diatomaceous earth as water sorbent.

Additional and successive rinsings of the analyte trap with acetone, hexane and acetone (this sequence was used in order to avoid miscibility problems with the aqueous rinsing solvent) were necessary after the extraction of milk samples to remove extracted matrix components that were not soluble in the rinsing solvent and remained in the trap after the rinsing step. In the case of feedstuff and lyophilized liver only one rinsing step with acetone was sufficient to clean the analyte trap. The extracts were very clear and did not need clean-up before HPLC analysis. Chromatograms of the extracts of diatomaceous earth and food samples are shown in Figs. 3 and 4, respectively.

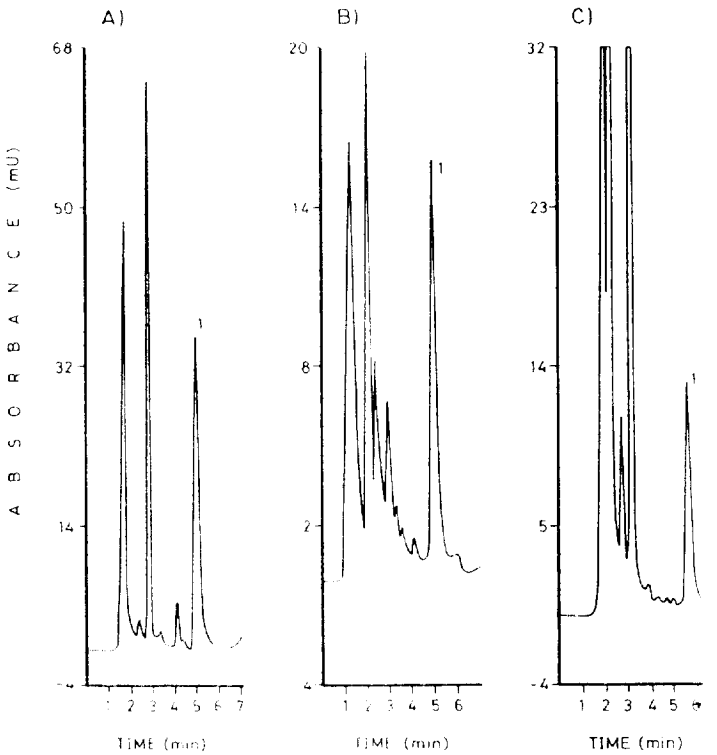

Fig. 4. HPLC chromatogram for the ion-pair SFE extracts of lyophilized milk (A), feedstuff (B) and lyophilized liver (C). Peak identification and conditions as in Fig. 3.

4. Conclusions

The extraction of clenbuterol into supercritical CO_2 is enhanced by using camphorsulfonate as counterion to form an ion-pair. The clenbuterol–camphorsulfonate ion-pair formed is less polar than clenbuterol and so more soluble in SC-CO_2.

Extraction efficiency is not enhanced by a previous static extraction. Although the SFE efficiency of clenbuterol is improved by methanol modification of CO_2, the opposite occurs for ion-pair–SFE of the target analyte since the ion-pairing extraction is favoured in a non-polar medium.

The recovery study performed on food matrices (milk, liver and feedstuff) demonstrates that ion-pair–SFE with camphorsulfonate and CO_2 reduces preliminary steps in the determination of clenbuterol by a sensitive technique, although it did not provide good recoveries for some kinds of samples, such as liver.

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