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A study of the conditions of the supercritical fluid extraction in the analysis of selected anti-inflammatory drugs in plasma

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Abstract

Supercritical fluid extraction (SFE) was employed to analyze selected anti-inflammatory drugs in plasma. Evaluation of selected drugs (ibuprofen, indomethacin, and flufenamic acid) was performed using the HPLC method on columns with the reverse phase C-18 and detection in the UV region of the spectrum. A study of the conditions of SFE carried out for 30 min at 50 °C investigated the magnitude of the pressure of carbon dioxide suitable for drug extraction, the selection of the collecting solvent, and the modification of CO_2 with an organic solvent. The results of the study made it possible to determine the optimal procedure for SFE of ibuprofen, indomethacin, and flufenamic acid from plasma, which renders their HPLC quantification possible.

Keywords: Supercritical fluid extraction; HPLC; Plasma; Ibuprofen; Indomethacin; Flufenamic acid

1. Introduction

Non-steroidal anti-inflammatory drugs represent a considerably large and chemically heterogeneous group of medicaments. In therapy they are widely used primarily in the treatment of the diseases of the locomotor system (inflammatory chronic diseases of the joints, extra-articular rheumatism or osteoarthrosis).

Their analytical evaluation in biological material employs primarily HPLC [1–8], less frequently capillary electrophoresis [6,9,10] or gas chromatography [11,12], and rarely, e.g. for the analysis of a mixture of anti-inflammatory agents in a methanolic solution or dosage forms, also supercritical fluid chromatography [13]. In HPLC analysis, if the column-switching system [6] is not used, it is advantageous to extract the drug under analysis from a sample of biological material first. Of extraction methods available [14], liquid–liquid extraction [2,5,7], solid–liquid extraction [1,4] as well as supercritical fluid extraction (SFE) [3], which was used to extract several anti-inflammatory drugs from a sample of the serum prior to their HPLC analysis, have been employed for this purpose.

SFE is being employed as a method of isolation because of the advantageous behaviour of supercritical fluids [15], which in dependence on the temperature and pressure show partially the properties of liquids and gases. In the present paper, some conditions of SFE were examined in the isolation of selected anti-inflammatory drugs (ibuprofen, indomethacin, and flufenamic acid) from plasma samples prior to HPLC analysis.

The paper aims to study the optimal working conditions for the extraction of drugs with carbon dioxide, i.e. the determination of sufficient pressure of carbon dioxide for extraction, selection of a suitable collecting solvent, or modification of carbon dioxide with a suitable organic solvent in order to improve extraction efficacy. The suitability of SFE conditions was evaluated by HPLC analysis both from the viewpoint of extraction efficacy of drugs and extractability of endogenous substances. The knowledge gained in the study of the conditions of SFE was used to determine the extraction procedures usable in HPLC analysis of

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ibuprofen, indomethacin, and flufenamic acid in plasma.

2. Experimental

2.1. Chemicals, reagents and materials

Ibuprofen and flufenamic acid were supplied by Sigma (St. Louis, MO, USA) and indomethacin by Léčiva (Praha, Czech Republic). Methanol and perchloric acid were obtained from Merck (Darmstadt, Germany) and methylene chloride, potassium dihydrogenphosphate, disodium hydrogenphosphate, and H_3PO_4 were purchased from Lachema (Neratovice, Czech Republic). Methanol was HPLC grade, and all other chemicals were analytical grade. Methylene chloride was distilled and water was redistilled.

2.1.1. Preparation of phosphate buffer

Mixing 95.5 parts of a potassium dihydrogenphosphate solution (0.01 mol/l) and 4.5 parts of a sodium hydrogenphosphate solution (0.01 mol/l) yielded a buffer of pH 5.6 which was adjusted to the required pH 3.0 by adding phosphoric acid (10% w/v).

Samples of rabbit plasma were obtained from the Medical Faculty of Charles University in Hradec Králové.

2.2. Chromatographic conditions and instrumentation

SFE was performed on the apparatus manufactured by the Advance Designing Workshop of the College of Chemical Technology in Praha (Czech Republic) by modifying a high-pressure pump HPP 5001 (Laboratorní přístroje Praha, Czech Republic) and a thermostat U 7 (VEB Medingen, Germany). Extraction cartridge for SFE (50×5 mm I.D.) packed with the sorbent Separon SGX C-18, 60 μm (Tessek Praha, Czech Republic), was employed to trap the drug from the sample and pre-purify it on a vacuum SPE apparatus Visiprep (SUPELCO, Bellefonte, PA, USA). Extraction cartridge with a sample was placed into the extraction chamber of SFE. SFE-grade CO_2 was a product of Linde (Pardubice, Czech Republic).

A liquid chromatographic apparatus of the unit construction type: a gradient system SP 8700, an integrator SP 4100 (Spectra Physics, Santa Clara, USA), and a detector Spectra 100 (Thermo Separation Products, Santa Clara, USA). The analytical wavelengths were 222 nm (ibuprofen), 254 nm (indomethacin), and 294 nm (flufenamic acid), the sensitivity being 0.01 AU. The samples were applied with a feeder Rheodyne with a 3 μl loop (Rheodyne Cotati, CA, USA) to an analytical glass column Separon SGX C-18 (150×3.3 mm, I.D., 5 μm) (Tessek Praha, Czech Republic). The mobile phase was a mixture of methanol and water (3:1) adjusted to the pH 3.0 by acidification with 5% perchloric acid.

2.3. Standard solutions

Standard solutions of all selected drugs (ibuprofen, indomethacin, and flufenamic acid) were prepared in methanol of a concentration of 0.1 mg/ml.

2.4. Adjustment of plasma sample

Into a 10 ml ground glass joint test tube, 0.25 ml of plasma was pipetted, 50 μl of a solution of the drug under study was added, and the sample was diluted with 0.5 ml of water and 1 ml of phosphate buffer (pH 3). After 5 min agitation and centrifugation (5 min at $1930 \times g$), the supernatant was transferred to a pre-activated extraction cartridge for SFE (activation with 5 ml of methanol and 5 ml of water). After supernatant application, the cartridge was washed with 5 ml of buffer and 5 ml of water. After drying with vacuum (5 min), the cartridge was placed into the thermostated (50 °C) space of SFE. For extraction of the drugs under evaluation, CO_2 in the supercritical state was used alone or modified with 5% methanol (elution period 30 min under the optimal pressure). After extraction in the cartridge, carbon dioxide (flow 2 ml/min) was introduced into a test tube containing 3 ml of the collecting solvent, where the drug under study, eluted with CO_2, was trapped. The collecting solvent was blown off to dryness with a stream of nitrogen, and the evaporation residue was dissolved in 50 μl of methanol and injected onto the column.

2.5. Calibration curves of drugs

Plasma samples were prepared by adding a standard solution of the drug (5, 10, 15, 20, and 25 μg) and 100 μl of the internal standard to 0.25 ml of plasma. Samples were adjusted by the procedure described above; SFE employed carbon dioxide modified with methanol at a pressure of 35 MPa and 3 ml of the collecting solvent.

3. Results and discussion

3.1. HPLC analysis

Evaluation of three selected anti-inflammatory drugs in plasma after adjustment by SFE was performed using HPLC analysis under the described chromatographic conditions. Retention times of the agents under study raged from 8 to 10 min—ibuprofen possessed a retention time of 7.8 min, indomethacin, 7.7 min, and flufenamic acid, 10.4 min.

HPLC chromatogram served to examine not only the numbers of eluted endogenous drugs and their possible interference with the peaks of the drug being analyzed, but also the extraction efficacy of SFE from the viewpoint of the found percentage content of the drugs being analyzed. A volume of 3 μl of the dissolved evaporation residue was injected and extraction efficacy was calculated from the areas of the peak of the drug in the extract and the peak of the injected methanolic solution of the standard. For the evaluation of the residua of endogenous substances, a chromatogram of an extract of a plasma sample with an addition of the drug was compared with a chromatogram of empty plasma adjusted with the use of the identical procedure.

3.2. Studies of the conditions in SFE

SFE is an advantageous method of extraction due to the properties of supercritical fluids [15], which, under higher temperature and at higher pressure, preserve some properties of liquids (high density and high solvation capacity), and, on the other hand, whose viscosity approaches that of gases (diffusivity of the substances present is far higher than in liquids). The most frequently employed supercritical fluid is carbon dioxide, which represents a very suitable extraction medium (critical temperature, 31.3 °C, and critical pressure, 7.43 MPa). Its relatively low polarity (comparable to that of hexane [15]) can be modified by an addition of a polar organic solvent (e.g. methanol), which was also experimentally tested in the present study.

Examination of the conditions in SFE was carried out at a temperature of 50 °C, in contrast to the authors [16] who used too high a temperature (150 °C) in combination with a strong pressure (50 MPa). Also the period of the duration of extraction, in which CO_2 behaves like a supercritical fluid, was constantly 30 min. In drugs extraction from plasma, a number of parameters were examined—the value of the pressure of carbon dioxide, modification of CO_2 with methanol as well as the selection of the collecting solvent for trapping the drugs extracted with carbon dioxide.

Table 1
Effect of pressure of carbon dioxide on extraction efficacy of agents

Drugs	Collecting solvent	Recovery (mean ± SD) (%)	
		20 MPa CO_2	35 MPa CO_2
Ibuprofen	Methylene chloride	14.2 ± 4.27	73.1 ± 3.05
	Methanol	10.1 ± 3.59	54.3 ± 3.38
Indomethacin	Methylene chloride	15.3 ± 3.76	42.6 ± 3.08
	Methanol	21.7 ± 3.41	49.2 ± 2.28
Flufenamic acid	Methylene chloride	29.7 ± 3.13	61.5 ± 2.42
	Methanol	25.6 ± 3.25	55.0 ± 2.87

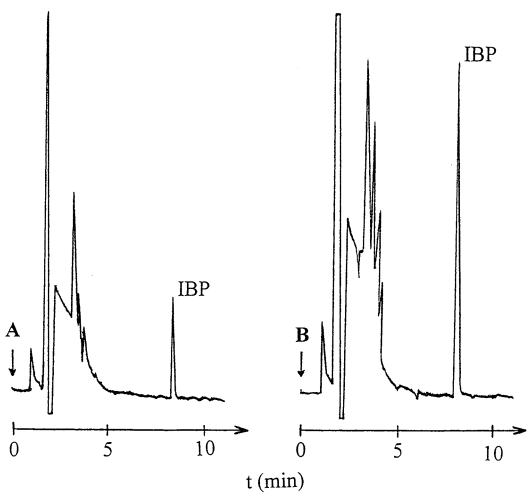

Fig. 1. HPLC chromatogram of eluates after SFE of ibuprofen (IBP) from a plasma sample (collecting solvent, methylene chloride). (A) Pressure of 20 MPa, (B) pressure of 35 MPa. Chromatographic conditions: column: C-18; mobile phase: methanol–water (3:1) pH 3.0; wavelength: 222 nm.

3.2.1. Pressure of carbon dioxide

The effect of pressure of carbon dioxide at a flow of 2 ml/min on drug extraction was of great importance as at a pressure of 10 MPa hardly any drug was extracted, and extraction efficacy was increased with increasing pressure (see Table 1).

Residues from plasma were eluted more at a higher pressure (Fig. 1); the peaks of the residues, nevertheless, did not interfere with the peaks of the drugs under study.

3.2.2. Collecting solvent

In the selection of a suitable collecting solvent to capture the extracted drugs from CO_2, various organic solvents were tested, methylene chloride and methanol being the most useful ones. When extracting ibuprofen and flufenamic acid, methylene chloride was the most suitable from the viewpoint of recovery, and methanol in the case of indomethacin, as follows from Table 1.

Extractability of endogenous drugs was different. Fewer residues are released from carbon dioxide to methanol (they are eluted maximally within 2.5 min) than to methylene chloride, as follows from Fig. 2.

3.2.3. Modification of carbon dioxide

Effect of the extraction medium in SFE can be also regulated by an increase in the polarity of carbon dioxide, which is achieved by modifying carbon dioxide with an addition of a polar solvent. In the evaluation of selected drugs, a modification of carbon dioxide with methanol (5%) was tested (at a pressure of 35 MPa), which resulted in a marked increase in the extraction

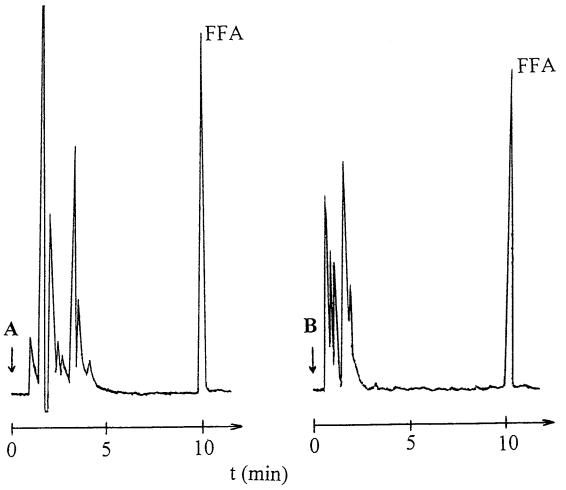

Fig. 2. HPLC chromatogram of eluates after SFE of flufenamic acid (FFA) from a plasma sample at a pressure of 35 MPa into the collecting solvent. (A) Methylene chloride, (B) methanol. Chromatographic conditions: column: C-18; mobile phase: methanol–water (3:1) pH 3.0; wavelength: 294 nm.

efficacy of indomethacin by nearly 30% (see Table 2). In the extraction of flufenamic acid there was an increase in extraction efficacy by approximately 12% and the modification of CO_2 with methanol in the extraction of ibuprofen was manifested inexpressibly. Table 2 shows a comparison of the effect of carbon dioxide alone and that of carbon dioxide modified with methanol on the extraction of anti-inflammatory drugs at a pressure of 35 MPa. At a pressure of 20 MPa, the modification of CO_2 with methanol was also manifested, but the recovery was smaller.

In the modification of carbon dioxide with methanol, the residues from plasma were not markedly changed as can be seen in Fig. 3A and B.

3.3. Application of SFE

It follows from the performed study that for SFE of selected anti-inflammatory drugs a pressure of 35 MPa is more advantageous from the viewpoint of the recovery of the drugs under study, although a larger number of residues are eluted than in lower pressures. The selection of the collecting solvent is dependent to a great extent on the character of the drug. Modification of carbon dioxide with methanol can markedly increase

Table 2
Effect of modification of carbon dioxide with methanol (5%) on extraction efficacy of selected anti-inflammatory drugs (at a pressure of 35 MPa)

Drugs	Collecting solvent	Recovery (mean ± SD) (%)	
		CO_2 alone	CO_2 modified with methanol
Ibuprofen	Methylene chloride	73.1 ± 3.05	78.4 ± 2.25
Indomethacin	Methanol	49.2 ± 2.28	78.8 ± 2.69
Flufenamic acid	Methylene chloride	61.5 ± 2.42	73.0 ± 2.53

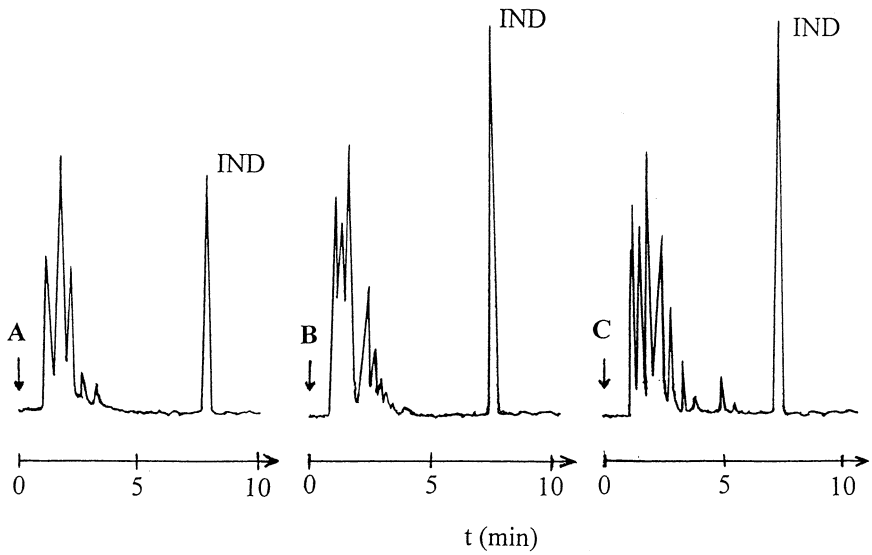

Fig. 3. HPLC chromatogram of eluates after SFE of indomethacin (IND) from a sample of plasma (collecting solvent, methanol) at a pressure of 35 MPa. (A) CO_2 alone, (B) CO_2 modified with methanol, (C) for the sake of comparison a HPLC chromatogram of an eluate after SPE of indomethacine from a plasma sample (desorption with methanol from the solid phase). Chromatographic conditions: column: C-18; mobile phase: methanol–water (3:1) pH 3.0; wavelength: 254 nm.

Table 3
Calibration curves (including internal standards and employed wavelengths for detection) of the drugs under study in plasma after SFE adjustment and HPLC analysis

Drugs	Detect. (nm)	Internal standard	Calibration curve	Correlation coefficient
Ibuprofen	222	Flufenamic acid	$y = 0.0487x + 0.0181$	$r = 0.998$
Indomethacin	254	Flufenamic acid	$y = 0.0345x + 0.0010$	$r = 0.999$
Flufenamic acid	294	Indomethacin	$y = 0.0120x + 0.0231$	$r = 0.998$

the extractability of some drugs without increasing the amount of residues on the chromatographic record too much.

On the basis of the found data, extraction procedures were worked out for the evaluation of three selected anti-inflammatory drugs in plasma. For quantification as the internal standard these drugs can be used reciprocally. Drug concentrations were determined from calibration curves (Table 3), which are linear within the range 20–100 μg in 1 ml of plasma. Plasma samples were adjusted prior to HPLC by SFE analysis under the conditions found in the present study (as an example see, in Fig. 4, a HPLC record of ibuprofen evaluation).

3.4. Comparison of SFE and SPE

Three anti-inflammatory drugs were selected for a study of the conditions of the methodology of SFE in the isolation of drugs from plasma. The results of the study were compared with the results of solid-phase extraction (SPE) carried out on extraction columns with the identical packing and at the identical adjustment of pH. For desorption of drugs from solid phases in SPE the same solvents proved useful as those selected as the most suitable collecting solvents in SFE. Plasma samples adjusted both by SFE and SPE were analyzed using HPLC under identical chromatographic conditions. The values of extraction efficacy of drugs in both methods of extraction were comparable (Table 4).

An advantage of SFE over SPE was extractability of a smaller amount of endogenous substances from plasma (see Fig. 3B and C). A great advantage is the fact that the residues of these endogenous substances were eluted in the chromatogram approximately within 3.5 min when using methylene chloride as the collecting solvent. If methanol was used as the collecting solvent, even a smaller number of residues were eluted (maximally within 2.5 min). These amounts of eluted residues were not influenced by modifying CO_2 with methanol, even though the efficacy of extraction of some drugs was markedly increased.

Elution of residues from plasma just a few minutes after injection in SFE makes it possible to evaluate also possible metabolites of these drugs without interference with the residues. From the same reason it is advantageous to use SFE also in the evaluation of other, more polar drugs in plasma.

Similar conclusions, suitability of use and comparable results, followed also from the evaluation of drugs in serum [3] by both SFE method and solid-phase extraction and liquid–liquid extraction. SFE is fully comparable with the classic methods of extraction.

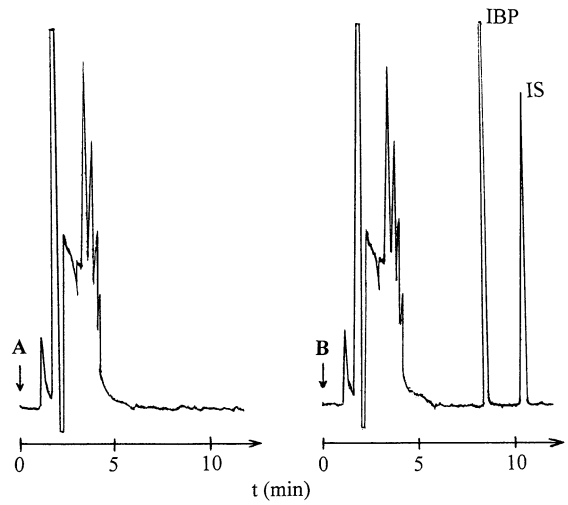

Fig. 4. HPLC chromatogram of ibuprofen analysis (IBP) in plasma. (A) Blank plasma, (B) control plasma spiked with the standard of ibuprofen (60 μg/ml) and IS (40 μg/ml). Chromatographic conditions: column: C-18; mobile phase: methanol–water (3:1) pH 3.0; wavelength: 222 nm.

Table 4
Results of extraction efficacy of selected anti-inflammatory drugs when using SFE and SPE to modify plasma samples prior to HPLC analysis

Drugs	Solvent	Recovery (mean ± SD) (%)	
		SFE	SPE
Ibuprofen	Methylene chloride	78.4 ± 2.25	83.2 ± 2.31
Indomethacin	Methanol	78.8 ± 2.69	77.4 ± 2.70
Flufenamic acid	Methylene chloride	73.0 ± 2.53	70.3 ± 2.65

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