DIRECT EXTRACTION OF BENZODIAZEPINE METABOLITE WITH SUPERCRITICAL FLUID FROM WHOLE BLOOD

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ABSTRACT: Our target is to develop a simple and a speedy extraction method of drug from biological sample. Supercritical fluid extraction (SFE) is well known as a speedy and contaminant-free extraction method. So we applied, the advantages of the SFE which has replaced a conventional extraction method, that is, liquid-liquid extraction (LLE) and solid phase extraction (SPE). In previous papers, we reported that SFE methods could be combined with freeze-drying or packing materials to reduce the problems of the LLE and the SPE. The SFE method with the packing material can directly extract drugs from biological fluid samples without the need for deproteinization. However, the recovery of the drug metabolite has not yet been considered. In this report, an evaluation has been made of whether or not the drug metabolite can be extracted from whole blood with the SFE method combined with the the packing material. The metabolite of the drug is generally a polar compound having a strong polar group such as hydroxyl (OH), desalkylamino (NH), and carboxyl (COOH) group. Therefore, the metabolite of the drug might be easily adsorbed by packing material and might not be extracted. Oxazepam was chosen as a typical metabolite of the drug (Diazepam). Quantitative analysis of the extract was carried out with gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS). The metabolite of the diazepam can be extracted as long as conditions are satisfactory in the SFE using molecular sieves 5A as a filler.

KEY WORDS: Supercritical fluid extraction (SFE); Benzodiazepines; Biological samples.

Problems of Forensic Sciences, vol. XLIII, 2000, 263–274
Received 9 September 1999; accepted 16 May 2000

INTRODUCTION

When drug in the biological sample is identified, promptness is extremely important with scientific accurate. Conventional LLE methods are time-consuming, troublesome and are often replaced by SPE. However, adjustment of pH and deproteinization of the sample are still required, and problems of chemical pollution and cost effectiveness arise.

SFE has many excellent points for the replacement of LLE and SPE [3, 5]. The advantages of SFE are shorter extraction times and contaminant-free extraction.

However, the SFE method could not apply to the sample with high water contents [2, 3].

Previously, we reported modified SFE methods combined with freeze-drying [7, 9] and with packing materials [8] to solve the problems. The SFE method could easily extract the drugs from biological samples without considering the need for deproteinization or the liquid-liquid distribution coefficient.

In this report, recovery of the drug metabolite from whole blood using the SFE combined with packing material was evaluated. In outline, this is a method of filtering blood with the packing material by using the supercritical fluid as in pressurized liquid extraction (PLE) method. As a result, a colourless and transparent extract was easily obtained without a deproteinization processing. The quantitative analyses of the extracts were carried out by GC/MS and LC/MS.

The results showed that the recovery rate of the drug metabolite from the blood sample with this method was high.

MATERIALS AND METHODS

Drug analytes

Reagents

Benzodiazepine drugs were obtained by courtesy of pharmaceutical companies. Methanol, ethanol, chloroform, and tetraphenylethylene (TPE) were purchased from Wako Pure Chemicals (Osaka, Japan). Blood was for the transfusion and putrefied blood. Liquid carbon dioxide was of food additive quality.

Packing materials

Packing materials used were cellulose powder (Whatman CF11, UK), molecular sieves 5A (MS-5A) (Wako Pure Chemicals), and absorbent cotton (Kinsuzu Men brand Suzuran Co., Nagoya, Japan).

The MS-5A was mainly used as packing material, because the MS-5A was the most excellent in the recovery of diazepam in blood in a previous report. Especially, the effect of the deproteinization on the blood was excellent.

Preparation of analyte samples

Packing method to the sample vessel

Packing materials packed in the extraction vessel (10 ml of cylindrical extraction vessel, made of stainless steel) was as follows.

A 0.20 g of the cellulose powder was packed to the extraction vessel first. The vessel was tapped several times on a laboratory table to improve a packing density of the cellulose. Then a 2.5 g of the pulverized MS-5A was packed in the same way.

Finally, a 0.20 g of absorbent cotton was packed by using glass rod. A 1 ml putrefied blood sample containing each 20 µl of oxazepam (100 ng/µl) and diazepam (100 ng/µl) was added on the cotton.

Extraction with SFE

The extraction vessel that the drug sample and the packing material were loaded was installed in the instrument. The extraction was carried out for 30 min under the following conditions (Figure 1).

TABLE I. ANALYTICAL CONDITIONS FOR SFE

Fig. 1. UV chromatogram of the SFE.

Instrument: Jasco Super-200 SFE/SFC model 3 (Tokyo, Japan)
Sample vessel: Stainless steel, 10 ml (128 mm x 10 mm i.d.)
Supercritical fluid: 2.5 ml CO_2 with 0.5 ml MeOH as modifier
Flow-rate: 3 ml/min
Pressure: 200 kg/cm^2
Oven temperature: 40°C
Detectors: MD-910 UV diode array detector + UV-975 detector

Extracts were collected in two 10-ml test tubes. About 10 ml of a colorless transparent liquid was obtained.

Quantitative analyses with GC/MS and LC/MS

Concentration of the SFE extracts was conducted with a centrifugal evaporator (Taitec model VC-96N, Tokyo, Japan). The concentrate was moved to a 0.3 ml reaction vial (Pierce Reacti-Thermo, IL., USA) and evaporated to dryness under a stream of nitrogen gas at 60°C using a heating module (Pierce Reacti-Thermo, IL., USA).

The residue was a sticky and a milk-white creamy. The residue was dissolved completely with 100 µl of 30% chloroform ethanol solution containing lorazepam (20 ng/µl) as internal standard. This solution was vortex mixed for 1 min. A 1 l aliquot of the analyte was injected into the GC/MS. A 5 µl of the same solution was similarly used to analyze the LC/MS.

Quantitative GC/MS analysis

Quantitative analysis of the drug was carried out by using the selected ion monitoring (SIM) mode of the GC/MS by measuring ranges 8.7 to 9.5 min as shown in Table II.

Quantitation of the oxazepam and the diazepam recoveries used calibration graphs obtained by the method of least squares regression using an internal standard compound.

TABLE II. ANALYTICAL CONDITIONS FOR GC/MS

Instrument: Shimadzu QP-5000 GC/MS (Kyoto, Japan)
Column: J & W Scientific DB MS (30 m x 0.25 mm i.d., 0.25 µm film thickness)
Injector temperature 280°C, Split injector: Split ratio of 1:10
Oven temperature 200°C (hold 3 min), 10°C/min (program 8 min), 280°C (hold 9 min)
Carrier gas: He, Flow rate: 1.5 ml/min
Ionization mode: EI
Scan range: 40–500 (m/z)
Electron multiplier voltage: 1.5 kV
Sample volume: 1 µl

Qualitative LC/MS analysis

The quantitative analysis of the drug was similarly conducted with the LC/MS by measuring ranges 10 to 20 min as shown in Table III.

TABLE III. ANALYTICAL CONDITIONS FOR LC/MS

Instrument: Hitachi M-8000 LC/3DQ MS (Tokyo, Japan)
Ionization mode: APCI (positive)
Column: Develosil ODS HG (150 mm x 2 mm i.d.)
Mobile phase: MeOH:H_2O (20:80)
Flow rate: 0.2 ml/min
Column oven temp.: 40°C
Scan range: 40–500 (m/z)
Sample volume: 5 µl

Calibration curve for recovery

Sample preparations

Standards containing 5, 10, 15, 20 and 25 μl of the oxazepam and the diazepam (each 100 ng/μl containing) solutions were prepared in separate 0.3 μl reaction vials, respectively. The solvent was evaporated under a stream of nitrogen gas at 60°C with the above on entioned heating module.

A 100 μl of a 30% chloroforme thanol solution containing 20 ng/μl of the lorazepam as

Fig. 2. Total in chromatogram of the SFE extracts with GC/MS (upper) TIC of the background from packing material (lower).

internal standard was added to each sample. This solution was vortex mixed for 1 min. A 1 μl and 5 μl aliquot of the analyte were injected into the GC/MS and the LC/MS, respectively.

Quantitation of oxazepam and diazepam (GC/MS)

Quantitative analysis of the oxazepam and the diazepam for the measurement of recoveries from blood sample was carried out by SIM for the target drugs. The oxazepam and the diazepam were quantified using the peak area ratio of the compound to the internal standard. Examples: lorazepam m/z 239; oxazepam m/z 267, 233; diazepam m/z 283. A calibration curve was made every day.

Fig. 3. Total in chromatogram of the SFE extracts with LC/MS (upper).
Mass spectrum of the peak no. 1 (middle).
Mass spectrum of the peak no. 2 (lower).

Quanti

Fig. 4. 1,4-benzodiazepine.