Development of a Supercritical Fluid Extraction Method for the Determination of Temazepam in Whole Blood

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Abstract

A supercritical fluid extraction (SFE) procedure for the analysis of temazepam from whole blood was developed. Quantitative recoveries were obtained by high-performance liquid chromatography using prazepam as an internal standard and carefully monitoring the extraction temperature and pressure. The results were found to compare well with those obtained by solid-phase extraction techniques, but they also had the advantages of reduced solvent consumption and minimal sample handling. The application of this method to authentic forensic blood specimens showed the SFE method to be useful as an alternative procedure for the extraction of temazepam in the toxicology laboratory.

Introduction

In western Scotland in 1994, some 141 deaths in which temazepam was a contributory factor were reported. Between 1993 and 1995, an 80% increase in the number of drivers found with considerable levels of temazepam was observed (1). These figures reflect the increasing trend in temazepam abuse in Scotland.

At present, temazepam analysis from biological matrices involves a solid-phase extraction (SPE) method. The procedure used is a modification of that reported by Zweipfenning et al. (2). Disadvantages of this procedure include the need for high-purity organic solvents and the resultant generation of substantial quantities of waste solvent. In addition, the sample preparation can be tedious and time consuming with increased potential for contamination (3). As an alternative method of sample preparation, the use of supercritical CO2 has been investigated.

In addition to speed and convenience, the main advantages of supercritical fluid extraction (SFE) are the improved efficiency, the nontoxicity, the cost effectiveness of the extraction fluid, and the possibility for direct analysis of complex matrices, thus reducing the risk of sample contamination (4).

In order to develop a method for SFE, the analyst must have an understanding of the properties of the analyte and the composition of the matrix (5). As the benzodiazepines were relatively polar, it was necessary to modify the CO2 by the addition of an entrainer (ethyl acetate) in order for the extraction to be feasible. The solvating power of the supercritical fluid was optimized by varying the pressure and temperature to obtain the maximum recovery of temazepam.

Quantitation by the SFE method was achieved by the addition of prazepam as an internal standard. The optimum conditions for temazepam gave peak-area ratios comparable with those of unextracted standards as long as the temperature and pressure were carefully controlled.

The developed method was first used to assess SFE in comparison with SPE for the extraction of temazepam and prazepam standards from methanol and blood. It was then used to investigate the analysis of authentic forensic blood specimens, again by comparison with SPE.

Experimental

Materials
Temazepam and prazepam were supplied by Wyeth Laboratory (Hants, U.K.). Methanol, ethyl acetate, diethyl ether, and dichloromethane were high-performance liquid chromatographic HPLC/grade (Lab Scan Analytical Sciences, Dublin,

![Figure 1. Peak-height ratio versus pressure.](image_url)
Ireland), and ammonia was analytical grade (Merck, Poole, U.K.). KH2PO4 was HiPerSolv grade, and Na2HPO4 was GPR grade (Merck). The CO2 was supplied by Air Products (Walton-on-Thames, U.K.) in 25-kg cylinders fitted with a dip tube. Plastic syringes for SPE were 10-mL sterile Plastipak syringes (Becton Dickinson, Oxford, U.K.). Glass wool for SPE was silanized (Jones Chromatography, Mid Glamorgan, U.K.). Extrelut® (Merck) was prewashed using dichloromethane. The vials used for SPE were Anchor and Trident (FBG, London, U.K.) with screw caps (Merck). The vials used for SFE were 6 mL HypoVial® (Pierce, Oud-Beijerland, The Netherlands) with butyl rubber septa (Pierce and Warriner, Chester, U.K.). Columns for the SFE were made using 3 cm x 4.6-mm internal diameter stainless steel tubing. Water for the HPLC mobile phase was deionized using a Milli-Q® water purification system (Millipore, Watford, U.K.).

**Apparatus**

The SFE system consisted of two Gilson (Middleton, WI) 306 pumps, a PYE Unicam (Cambridge, U.K.) series 104 GC oven with pressure restrictor, and a Spectroflow (Kratos, Manchester, U.K.) 757 UV detector. All components were modified to suit the supercritical extractant. The CO2 pumphead was refrigerated with a Gilson model SFC3 refrigeration unit to maintain the flow of liquid CO2. The extraction cell was placed over the loop position of a Rheodyne (Cotati, CA) 7161. The extract was collected by expansion into methanol in a HypoVial at the flow outlet. The extractant was CO2-ethyl acetate (95:5) at 2 mL/min. The temperature and pressure were 65°C and 3000 psi, respectively. The extraction was monitored at a wavelength of 254 nm.

The HPLC system consisted of a Gilson 305 pump and a Gilson 115 UV detector. The column (25 cm x 4.6 mm) and guard column (2 cm x 4.6 mm) used were prepacked with Hypersil ODS (5 μm) (Capital HPLC Specialists, Bathgate, U.K.). The injector valve was a Rheodyne 7161 with a 20-μL sample loop. The mobile phase used was Na2HPO4-methanol (30:70, v/v). The flow rate was 1 mL/min, and the eluent was monitored at 254 nm.

**Preparation of standards for comparison**

A series of standards ranging from 0 to 12.8 mg/L temazepam with 11.24 mg/L prazepam were prepared in both methanol and blood. Both sets were prepared by adding the appropriate volumes of each drug stock standard (12.8 mg temazepam per liter methanol, 56.2 mg prazepam per liter methanol) to a vial, evaporating at 65°C under N2, and reconstituting in 5 mL of either methanol or blood.

**SPE procedure**

The extraction columns were prepared by plugging the end of a 10-mL plastic syringe with glass wool, filling the syringe...
with approximately 10 g of Extrelut, and pipetting 0.25 mL of a 5% NH₃ solution onto the top. Either 1 mL of standard blood, 0.9 mL of blank blood, and 100 µL prazepam standard (5 mg/100 mL in methanol) or 0.9 mL of sample blood and 100 µL prazepam (5 mg/100 mL) were pipetted into a clean glass vial. To this mixture, 1 mL of phosphate buffer (A, 13.6 g KH₂PO₄ in 500 mL H₂O; B, 4 g NaOH in 500 mL H₂O, 450 mL A and 355 mL B, pH adjusted to 4 using A or B) and 0.25 mL of a 5% NH₃ solution were added. The contents of the vial were mixed thoroughly, then pipetted onto the extraction column. After 5 min, the temazepam and prazepam were eluted under the influence of gravity using diethyl ether. Once 8 mL was collected, the eluent was evaporated to dryness at 65°C under nitrogen and reconstituted in 180 µL of HPLC mobile phase.

**SFE procedure**

Approximately 0.2 g of Extrelut was placed in a plastic weighing boat. To this solution, 0.5 mL of standard blood-methanol, 0.4 mL blank blood, and 100 µL prazepam (5 mg/100 mL) or 0.4 mL sample blood and 100 µL prazepam (5 mg/100 mL) were added. After mixing, the contents of the boat were allowed to dry at room temperature, until a friable consistency was achieved (approximately 1–2 h), before being transferred to an extraction column. It was not necessary to completely dry the samples but, when possible, the samples were left overnight. The column was sealed and placed over the loop position of the Rheodyne in the equilibrated SFE system. The extraction was started by switching the Rheodyne from load to inject and was carried out for 10 min. The collected extract was dried at 65°C under nitrogen and reconstituted in 50 µL of HPLC mobile phase.

**Results and Discussion**

Reproducibility problems were encountered when the extraction conditions were not carefully controlled; in particular, care was required in the control of pressure. Figure 1 shows how small fluctuations in pressure can affect the peak-height ratios. The pressure chosen for the extractions was 3000 psi. The rate of change in peak-height ratio with pressure for the extracted standards compared with unextracted standards was less pronounced.

Extractions were tried at various temperatures between 50 and 100°C. The temperature for the optimum recovery of temazepam was found to be around 60°C. Figure 2 shows the variation in peak-height ratio around this value, and, from this, an extraction temperature of 65°C was chosen.

In the absence of matrix interferences, the peak-area ratios obtained by SFE were found to be linear over the range 0–12.8 mg/L temazepam (r² = 0.999). Figure 3 shows that the peak-area ratios for both SFE and SPE compare well with those obtained for unextracted standards (mex = 0.15, mSPE = 0.134, mSPE = 0.158).

As SFE exhibits its best advantages when extracting analytes from solids or semi-solids (6), all blood samples were dried before analysis. This also avoids problems that may occur because of Joule-Thomson cooling as the supercritical fluid expands at the outlet. No matrix interferences from the blood were noted other than a slight decrease in the recovery of both drugs (80–100% compared with 90–100% from methanol). Again, the SFE was found to be linear over the range 0–12.8 mg/L temazepam as shown in Figure 4 (r² = 0.995). Comparison with unextracted standards and SFE was favorable (mex = 0.11, mSPE = 0.117, mSPE = 0.131). A comparative chromatogram of SPE and SFE extracted blood standards is given in Figure 5.

Investigation of the SFE and SPE methods on authentic forensic specimens showed that the methods correlate well. Table I and Figure 6 show the results obtained. The SFE method was also found to extract other benzodiazepines and their metabolites of forensic interest, including diazepam, nordiazepam, oxazepam, and chlordiazepoxide.
Conclusion

With careful control of temperature and pressure, both temazepam and prazepam can be extracted to the same extent from blood with recoveries of 80–100%. Reproducible results can also be achieved as long as the pressure fluctuations are kept to a minimum (± 150 psi).

The results obtained show that the SFE method can be used as an alternative procedure for the extraction of temazepam from forensic blood samples, with the potential for use as an extraction procedure for other benzodiazepines of forensic significance. The method offers a rapid, environmentally friendly approach that requires minimal sample handling.

References


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