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Forensic analysis of triazolam in human tissues using capillary gas chromatography

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Summary. A reliable and sensitive method has been developed to assess the concentrations of the hypnotic drug triazolam in human tissues, including putrefied tissues. The method involves a 3-step solvent extraction, clean-up on a silica gel column and gas chromatography using a nitrogen phosphorus detector and a capillary column. Estazolam was used as an internal standard. The calibration curve was linear over the concentration range 1 ng/g–1 µg/g and the lower limit of detection was 0.5 ng/g. A forensic study was performed on the toxicological effects of triazolam using putrefied tissues.

Key words: Triazolam – Estazolam – Capillary GC/NPD – Human tissue – Forensic toxicology

Zusammenfassung. Eine zuverlässige und empfindliche Methode wurde entwickelt, um die Konzentrationen des hypnotischen Medikaments Triazolam in menschlichen Geweben, einschließlich fauler Gewebe bestimmen zu können. Die Methode besteht aus einer dreistufigen Flüssig-Flüssig-Extraktion, einem Clean up an Kieselgel und Gaschromatographie mit einem Stickstoff-Phosphor-Detektor und einer Kapillarsäule. Als interner Standard wurde Estazolam benutzt. Die Eichkurve war im Konzentrationsbereich zwischen 1 ng/g und 1 µg/g linear, und die untere Nachweisgrenze betrug 0.5 ng/g. Es wurde eine forensische Untersuchung über die toxikologischen Effekte von Triazolam an faulem Gewebe durchgeführt.

Schlüsselwörter: Triazolam – Estazolam – Kapillar-Gaschromatographie/NPD – Menschliches Gewebe – Forensische Toxikologie

Introduction

Triazolam, a triazolobenzodiazepine derivative, is widely prescribed as a short-acting hypnotic. Toxicological analysis of concentrations of this drug in tissues have been made as it is often used for suicidal and criminal purposes.

A number of methods have been published for the analysis of triazolam in biological fluids, including gas chromatography (GC) with electron capture detection [1–5], high performance liquid chromatography (HPLC) [6, 7], gas chromatography/mass spectrometry (GC/MS) [3, 8] and enzyme immunoassay [9]. However, only one paper is available on the analysis of triazolam in human solid tissue [10].

A sensitive and reliable method has therefore been developed to determine levels of triazolam in human tissues using capillary GC with a nitrogen phosphorus detector (NPD). Using this method, putrefied human tissues were examined for the presence of this drug.

Materials and methods

Reagents. Triazolam was provided by Upjohn, Kalamazoo, MI, USA and estazolam was from Takeda Chemical Industries, Osaka, Japan. A buffer solution of pH 9 was prepared by mixing 21.3 ml of 0.1 *M* sodium hydroxide solution with 50 ml of 0.1 *M* each of boric acid and potassium chloride and diluting to 100 ml with distilled water. Methylene chloride, methanol and tert.-butyl methyl ether were of analytical grade and were purified by distillation. Methanol (0.5%) was added to the distilled methylene chloride as a stabilizer. A Shimadzu CBP1 capillary column was purchased from Shimadzu Co., Kyoto Japan. Other chemicals used were of analytical grade.

Biological samples. Human tissue samples were obtained at autopsy and were kept at −20°C until analysis. Outdated whole human blood was obtained from a blood bank.

Standard solution of triazolam and internal standard (IS). Triazolam (1 mg) was dissolved in 10 ml methanol to give a final concentration of 100 ng/µl. This solution was further diluted to concentrations of 10 and 1 ng/µl. Estazolam (IS) was dissolved in methanol and prepared in the same manner.

Extraction procedure. The method of extracting triazolam reported by Coassolo et al. [3] was modified as follows: 1 g of whole blood or tissue was homogenized in a mixture of 5 ml borate buffer (pH 9) and 1 µl IS solution (100 ng of estazolam) in a 30 ml centrifuge tube. To the mixture was added 10 ml of tert.-butyl methyl ether, the preparation was shaken for 10 min and centrifuged at 850 g for 20 min. The organic phase was evaporated to dryness under a stream

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Sample(1g)
  1) Add borate buffer(5 ml, pH 9.0) and IS(100 ng)
  2) Homogenize
  3) Add tert.-butyl methyl ether(10 ml)
  4) Shake(10 min) and centrifuge( 850 g, 20 min)

Organic layer
  1) Evaporate to dryness
  2) Add 0.1M disodium citrate(3 ml, pH 5.0)
     and hexane(3 ml)
  3) Shake and centrifuge

Aqueous layer
  1) Add bromothymol blue indicator(2 drops)
  2) Add 0.2M sodium hydroxide until the indicator
     turns blue(pH ~9.0)
  3) Add tert.-butyl methyl ether(2 ml)
  4) Shake and centrifuge

Organic layer
  1) Evaporate to dryness and dissolve in
     methylene chloride(1 ml)
  2) Apply to a silica gel column
  3) Elute with methylene chloride(10 ml) and
     methanol-methylene chloride(1:9,10 ml)
  4) Evaporate and dissolve in tert.-butyl methyl
     ether(20 µl)

tert.-butyl methyl ether(2 µl)
  |
GC/NPD
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Fig. 1. Extraction procedure for triazolam

of nitrogen and reconstituted in 3 ml 0.1 *M* disodium citrate solution (pH 5). This solution was then washed with 3 ml hexane for 10 min and centrifuged at 850 g for 20 min. The aqueous layer was transferred to a 10 ml centrifuge tube containing 2 drops of bromothymol blue solution (0.04%) as an indicator and the mixture was made alkaline by adding 0.2 *M* sodium hydroxide until the indicator turned blue (pH ~ 9). To the solution was added 2 ml of tert.-butyl methyl ether and the preparation shaken for 10 min. After centrifugation, the solvent layer was evaporated and the residue dissolved in 20 µl tert.-butyl methyl ether. A 2 µl aliquot of the solution was injected into a gas chromatograph.

For putrefied materials, the residue obtained at the final step was dissolved in 1 ml methylene chloride, applied to a 0.5 × 5 cm silica gel column and then eluted with 10 ml methylene chloride and 10 ml methanol-methylene chloride (1:9). After evaporating the latter eluent, the residue was dissolved in 20 µl tert.-butyl methyl ether. A 2 µl aliquot of the solution was injected into a gas chromatograph. The extraction procedure is summarized in Fig. 1.

Preparation of calibration curve. Whole blood samples were prepared to contain triazolam at concentrations of 1, 5, 10, 50, 100, 500 and 1000 ng/g, each containing 100 ng/g estazolam. These samples were extracted in the same manner as described above. The standard curve was obtained by plotting the peak area ratio of triazolam to estazolam versus the amount of triazolam.

Conditions of GC. The apparatus used was a Shimadzu GC-9AM gas chromatograph with a nitrogen phosphorus detector (Shimadzu FTD-9). The fused silica capillary column (10 m × 0.53 mm I.D., 1 µm film thickness) was coated with Shimadzu CBP1 bonded methylsilicone stationary phase. The operating temperatures were as follows: column, 220–260°C (5°C/min), injection port 270°C, detector 270°C. The flow rate of carrier gas (helium) was 20 ml/min and the make-up gas (helium) was 20 ml/min.

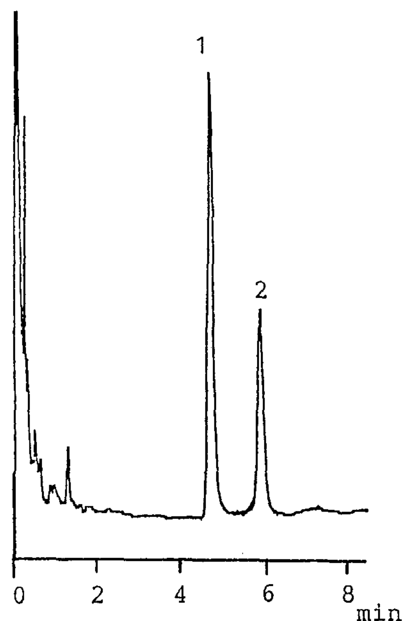

Fig. 2. Gas chromatogram of an extract from human blood containing 100 ng/g each of triazolam (2) and IS (1)

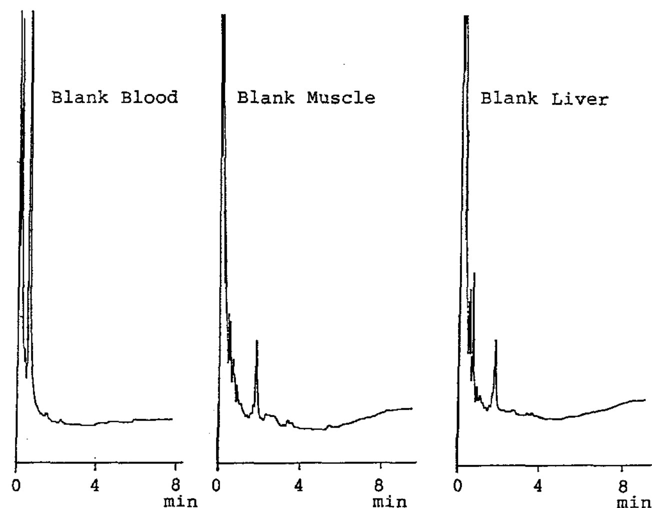

Fig. 3. Gas chromatograms of an extract from drug-free whole human blood, skeletal muscle and liver

Results and discussion

Extraction procedure

A single extraction yielded interfering peaks, therefore to eliminate these interfering substances, the 3-step extraction procedure reported by Coassolo et al. was used [3]. Back-extraction with acid caused a decomposition of the triazolam, hence the yield was unsatisfactory. This problem was overcome by using 0.1 *M* disodium citrate (pH 5) as an aqueous phase replacing 0.1 *N* sulphuric acid. Triazolam was thus stabilized and contaminants from the tissue extracts were efficiently removed in the hexane layer. The established 3-step extraction method was suitable for analysis of any non-putrefied biological fluid and tissues. Further purification on a silica gel column was required for putrefied tissues.

Determination of triazolam by GC/NPD

The gas chromatogram of the extract from the blood sample containing 100 ng each of triazolam and IS is

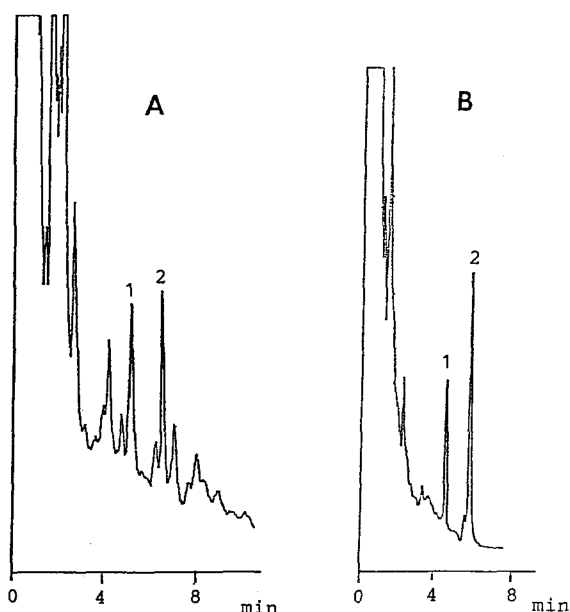

Fig. 4A, B. Gas chromatograms of an extract from putrefied liver containing 300 ng/g of triazolam (2) and 100 ng/g of IS (1), **A** before silica gel column, **B** after silica gel column

shown in Fig. 2. No, or few, interfering peaks appeared in the gas chromatogram of the triazolam-free human tissues and fluids (Fig. 3).

The gas chromatograms of the extract from a putrefied liver sample containing 300 ng of triazolam and 100 ng of IS are shown in Fig. 4. Interfering peaks on the gas chromatogram (Fig. 4A) were removed by passing the preparation through a silica gel column (Fig. 4B).

The calibration graph for triazolam was linear in the concentration range 1 ng/g to at least 1 μg/g, with a correlation coefficient of 0.999. The lowest limit of detection was 0.5 ng/g. The calculated recovery at concentrations of 10, 50 and 100 ng/g ranged from 60 to 85%.

Within-day precision was obtained using 3 different concentrations (10, 50 and 100 ng/g) of triazolam in control blood samples. The coefficients of variation (C.V.) were 10.0, 8.1 and 2.8%, respectively.

Case study

A 39-year-old man was found dead in his bed at home. Empty packages of 40 Halcion(triazolam) tablets (each 0.25 mg) were found in a trash can in his room.

A legal autopsy and toxicological examination were carried out to confirm the cause of death. He had apparently been dead for 4–5 days. Although the direct cause of death was found to be asphyxia, as determined by gastric contents in the airway, and a routine analysis by GC/MS did not reveal any drugs except ethenzamide at a therapeutic level in the muscle sample, poisoning was suspected based on findings at the scene. A further examination was performed using the method described and triazolam was detected at concentrations of 104.4, 153.1 and 398.8 ng/g in skeletal muscle, kidney and liver, respectively.

These values are extremely high in comparison to 4.4 ng/ml in plasma after oral administration of a therapeutic dose of 0.5 mg triazolam to 54 healthy men [11] and of 38 ng/g in the liver of a victim who drowned after taking at least 770 μg of the drug [10]. Gross calculations suggested that the deceased had ingested almost 10 mg of triazolam, namely all 40 tablets in the packages. The toxic influence of triazolam on the brain was considered to be directly linked to the cause of death.

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