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# Use of high-performance liquid chromatography as an extraction procedure for analysis of triazolam in decomposed human muscle by gas chromatography–mass spectrometry

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## Abstract

A reliable and sensitive method for the determination of triazolam in human muscle using gas chromatography–mass spectrometry (GC–MS) is described. The drug was extracted from decomposed human muscle using three-step liquid–liquid extraction and HPLC which was performed isocratically on a conventional ODS column with a mobile phase of 0.01 *M* phosphate buffer (pH 6.5)–acetonitrile (7:3). Estazolam was used as an internal standard. GC–MS analysis was performed on a DB-5 capillary column. Excellent linearity was obtained over the concentration range 1–200 ng/g. The lower limit of detection was approximately 0.5 ng/g. Forensic application of the present method was also described. © 1997 Elsevier Science B.V.

*Keywords:* Triazolam

## 1. Introduction

Triazolam, one of benzodiazepines, is widely used as a short-acting hypnotic in various conditions where a rapid onset and short duration of action is required. It is also abused and often used for suicidal and criminal purposes [1–3]. Current methods for the determination of the drug available in clinical or forensic toxicology include gas chromatography (GC) [4–7], gas chromatography–mass spectrometry (GC–MS) [8,9], high-performance liquid chromatography (HPLC) [10,11] and HPLC–MS [12]. Almost all of these methods are performed with fresh and/or frozen materials and include liquid–liquid or solid-phase extraction of the drug. However, in cases of

decomposed cadavers, these extraction procedures would not sufficiently eliminate interfering substances derived from putrefaction. We describe here a reliable and sensitive method for the determination of triazolam in decomposed human muscle using GC–MS. The method features the combination of liquid–liquid extraction and subsequent HPLC fractionation as a sample pretreatment.

## 2. Experimental

### 2.1. Chemicals

The chemical structures of three benzodiazepines used in the present study are shown in Fig. 1. Triazolam was obtained from Upjohn (Kalamazoo,

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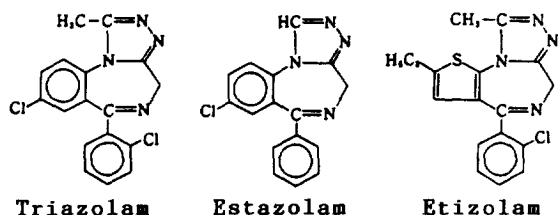


Fig. 1. Chemical structures of the three benzodiazepines used in the present study.

MI, USA), estazolam from Takeda Chemical Industries (Osaka, Japan), and etizolam from Yoshitomi Pharmaceutical Industries (Osaka, Japan). Diethyl ether (Aqunasol<sup>®</sup>), chloroform, acetonitrile, methanol (HPLC grade) and other reagents (analytical grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Water for HPLC solvents was from a Milli-Q Labo purification system (Millipore Corp., Bedford, MA, USA). Borate buffer (pH 9.0) was prepared according to Kudo et al. [4]. Triazolam, estazolam and etizolam were dissolved in methanol and the solutions were further diluted with methanol to appropriate concentrations.

## 2.2. Specimens for analyses

Human tissue samples were obtained at autopsy and were kept at  $-40^{\circ}\text{C}$  until analysis.

## 2.3. Extraction procedure

The method of Kudo et al. [4] was modified as follows: 5 g of skeletal muscle was homogenized in 25 ml of borate buffer (pH 9.0) with 10  $\mu\text{l}$  of estazolam solution (50  $\mu\text{g}/\text{ml}$ ) added as an internal standard. The homogenate was shaken three times with 50 ml of diethyl ether for 10 min and centrifuged at 2000 g for 10 min. The organic layer was evaporated to dryness in vacuo and reconstituted in 10 ml of 0.1 M citrate buffer (pH 5.0). The solution was then washed with 10 ml of *n*-hexane for 10 min and centrifuged at 2000 g for 10 min. The aqueous layer was made alkaline by adding sodium hydroxide solution to pH 9. The solution was shaken three times with 10 ml of diethyl ether and centrifuged. The organic layer was evaporated to dryness in vacuo and the residue was dissolved in a small volume of HPLC mobile phase mentioned below.

## 2.4. High-performance liquid chromatography (HPLC)

The liquid chromatographic system used was a Shimadzu LC-6A liquid chromatograph system (Kyoto, Japan) or an LKB Ultrochrom GTi system. The column used was an L-column ODS (150 $\times$ 4.6 mm I.D., Chemicals Inspection & Testing Institute, Tokyo, Japan). Solvent A was a 7:3 (v/v) mixture of 0.01 M phosphate buffer (pH 6.5) and acetonitrile, and solvent B consisted of these solutions in a 3:7 (v/v) ratio. The flow-rate was 1.0 ml/min. All solvents were degassed with helium prior to use. The column effluent was monitored at 240 nm of UV.

The column was equilibrated with solvent A and the sample was applied to the column. After fractionation of triazolam and estazolam, the column was washed thoroughly with solvent B and re-equilibrated with solvent A for the next fractionation.

HPLC fractions of triazolam and estazolam were collected, combined and concentrated in vacuo until acetonitrile in the mixture was almost evaporated. The resultant solution was made alkaline by adding borate buffer and sodium hydroxide solution to pH 9. The solution was shaken with 4 ml of chloroform for 10 min and centrifuged. The organic phase was evaporated to dryness in vacuo and the residue was dissolved in 50  $\mu\text{l}$  of methanol. The extraction procedure is summarized in Fig. 2.

## 2.5. Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses were performed using a Shimadzu QP-5000 (Kyoto, Japan) equipped with a DB-5 capillary column (15 m $\times$ 0.25 mm I.D., film thickness 0.25  $\mu\text{m}$ , J&W Scientific, Folsom, CA, USA). The oven temperature was held at 150 $^{\circ}\text{C}$  for 1 min following injection and programmed to 300 $^{\circ}\text{C}$  at a rate of 10 $^{\circ}\text{C}/\text{min}$ . The injection port and interface temperatures were 290 and 300 $^{\circ}\text{C}$ , respectively. Helium was used as a carrier gas (a head pressure of 50 kPa, a total flow-rate of 50 ml/min). The mass spectrometer was operated under electron impact mode at an ionization energy of 70 eV. The following masses were monitored by selected ion monitoring (SIM):  $m/z$  313, 342, 315, 238, 344, 203, 75, 240, 239, 279 and 259.

- Sample (muscle, 5 g)**
1. Add borate buffer (pH 9.0, 25 ml) and IS (500 ng)
  2. Homogenize
- Extraction**
1. Add diethyl ether (50 ml x 3)
  2. Shake (10 min) and centrifuge (2,000 g, 10 min)
  3. Organic layer, evaporate to dryness
- Back-extraction**
1. Add 0.1 M citrate buffer (pH 5.0, 10 ml) and *n*-hexane (10 ml)
  2. Shake and centrifuge
  3. Aqueous layer, adjust to pH 9 with NaOH aq.
- Re-extraction**
1. Add diethyl ether (10 ml x 3), shake and centrifuge
  2. Organic layer, evaporate to dryness
  3. Dissolve in a small volume of HPLC mobile phase
- HPLC fractionation**
1. Fractions of triazolam and IS, collect, combine and concentrate
  2. Adjust to pH 9
- Extraction**
1. Add chloroform (4 ml), shake and centrifuge
  2. Organic layer, evaporate to dryness
  3. Dissolve in methanol (50  $\mu$ l)
- GC-MS analysis**

Fig. 2. Extraction procedure for triazolam.

Two microliters of the samples were injected in the splitless mode at a column temperature of 150°C, and the splitter was opened after 1 min. The calibration curve for triazolam was obtained by plotting the peak area ratio of triazolam ( $m/z$  313) to estazolam ( $m/z$  259) versus the amount of triazolam.

### 3. Results and discussion

When the tissue samples were relatively fresh, only the liquid–liquid extraction procedure we used could extract effectively the objective compounds with few interfering peaks on GC–MS chromatograms. However, in cases of decomposed samples, GC–MS was distinctly unsuitable for sampling of the extracts, including certain putrefactive substances. Kudo et al. [4] overcame these difficulties by further purification of the liquid–liquid extracts from putrefied human tissues by treatment with silica gel column chromatography, for capillary GC with a nitrogen phosphorus detector to determine triazolam.

We used reversed-phase HPLC in place of silica gel column chromatography for further purification because of much higher resolution.

Fig. 3b illustrates the HPLC separation of triazolam and estazolam from a sample of decomposed human muscle after three-step extraction. It was difficult to determine triazolam directly from the chromatogram. The drugs were then extracted from their HPLC fractions with chloroform under weak-basic conditions and finally dissolved in methanol. Fig. 4 illustrates the SIM chromatogram of an extract from decomposed human muscle spiked with triazolam and estazolam after HPLC fractionation. The blank extract contained no peaks that interfered with triazolam or the internal standard on the chromatogram.

It is reported that certain benzodiazepines cannot be separated from each other under some GC conditions using a wide-bore capillary column [6–8]. Especially, Hattori et al. [8] reported that triazolam and etizolam give the same molecular mass and somewhat similar mass spectra. We investigated whether the present method separates and differentiates triazolam from etizolam. As shown in Fig. 5, the two substances were separated from each other

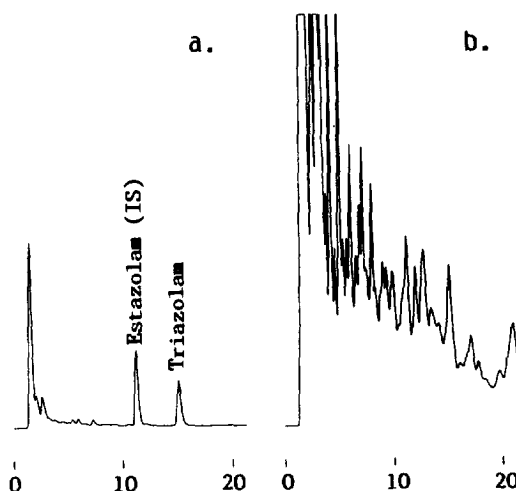


Fig. 3. HPLC chromatograms of three-step extracts from (a) water and (b) decomposed human muscle spiked with triazolam (100 ng/g) and estazolam (IS, 100 ng/g). Chromatographic conditions: column, L-column ODS, 150×4.6 mm I.D.; mobile phase, 7:3 mixture of 0.01 M phosphate buffer (pH 6.5) and acetonitrile; flow-rate, 1.0 ml/min; 0.16 a.u.f.s. at 240 nm.

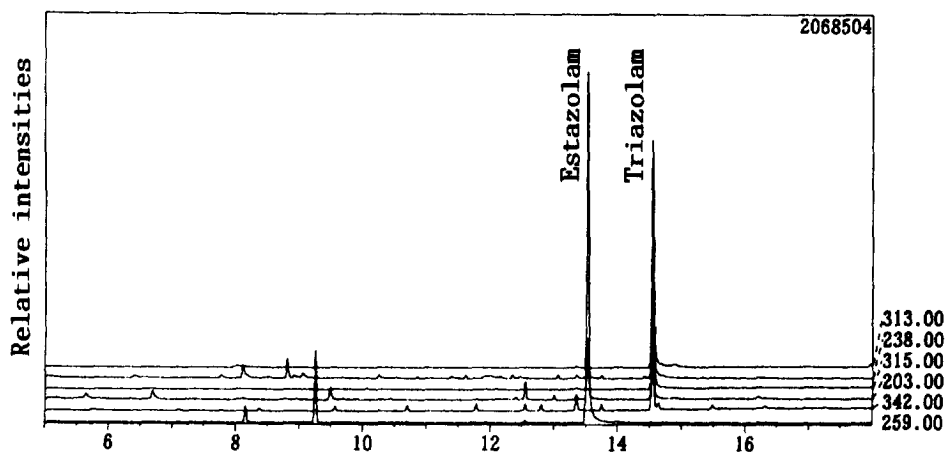


Fig. 4. SIM chromatogram of an extract from decomposed human muscle spiked with triazolam (100 ng/g) and estazolam (IS) after HPLC fractionation. Chromatographic conditions: column, DB-5 capillary column, 15 m $\times$ 0.25 mm I.D.; film thickness 0.25  $\mu$ m; column temperature, 150 (1 min) to 300°C (10°C/min); injection port, 290°C; interface, 300°C; carrier gas, He at 50 kPa; total flow-rate, 50 ml/min; electron impact mode at an ionization energy of 70 eV. Two microliters of the samples were injected in the splitless mode at a column temperature of 150°C, and the splitter was opened after 1 min.

on the SIM chromatograms, their characteristic ions being similar but with different relative intensities (Table 1). Thus, the method differentiates triazolam from etizolam.

Excellent linearity was obtained over the concentration range 1–200 ng/g with a correlation coefficient of 0.999. Experiments with spiked samples resulted in a recovery of 76% at a concentration

of 100 ng/g. The lower limit of detection was approximately 0.5 ng/g.

We used the present method for a case study. A man was found dead in a burned-out car. Most of the body was charred and burnt down, and the remaining skeletal muscle was putrefied. The postmortem time period was estimated to be from 4 days to 2 weeks. The cause of death was unknown. Fig. 6 shows the

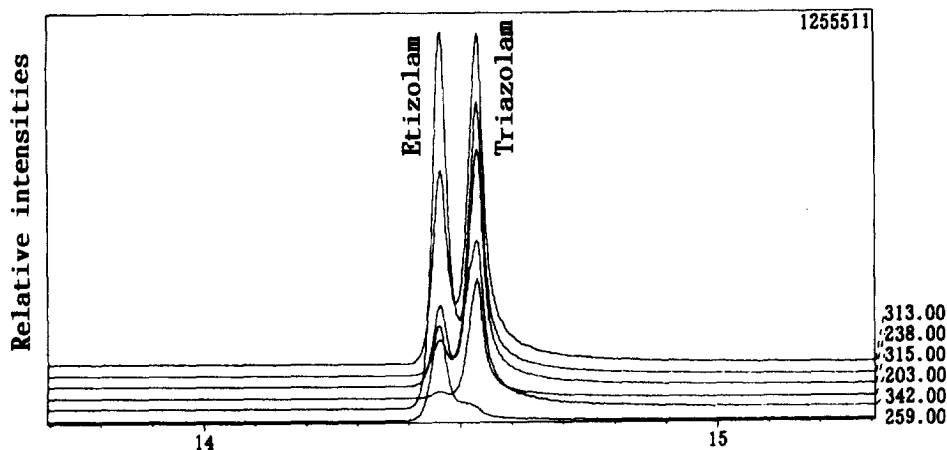


Fig. 5. SIM chromatogram of an authentic mixture of triazolam and etizolam (100  $\mu$ g/ml each). Chromatographic conditions are as in Fig. 4.

Table 1  
Relative intensities of the principal ions obtained with selected ion monitoring

<i>m/z</i>	Authentics		Sample from case study
	Triazolam	Etizolam	
313	100	100	100
238	87	19	88
315	68	43	64
203	43	4	55
342	36	203	36
239	34	52	31
279	34	21	32
240	34	15	42

SIM chromatograms of an extract from the skeletal muscle (iliopsoas) after three-step extraction and subsequent HPLC fractionation. A triazolam peak was detected at a retention time of 14.5 min. Table 1 shows the relative intensities of the principal ions of the peak corresponding to triazolam. The intensities from the sample were very similar to those from the authentic. The concentration was estimated as 11.4 ng/g.

A therapeutic hypnotic oral dose of 0.5 mg triazolam given to healthy subjects leads to a peak plasma level of  $5.0 \pm 3.9$  ng/ml at  $1.2 \pm 0.5$  h, with a peak plasma half-life of  $2.6 \pm 0.7$  h [13]. There is no literature concerning triazolam levels of skeletal muscle at therapeutic doses. Garrriott [14] suggested

the usefulness of muscle as an alternative specimen to postmortem blood for drug analysis. On the other hand, several reports have been published on the postmortem diffusion of drugs from the stomach [15–17]. According to the animal experiments with rats [15], triazolam concentrations in the thigh muscle were almost equal to those in the blood and there was no change in the concentrations during the postmortem time period, while those in the abdominal muscle remarkably increased compared to the initial levels. It is considered that the degree of postmortem diffusion of drugs from the stomach is dependent upon various factors, such as postmortem time period, the content of the drug remaining in stomach and the nature of the drug. In the present case, the triazolam level was relatively low and almost equal to that in plasma with therapeutic dosage. So, it was assumed that there was no or little influence on the level in muscle by postmortem diffusion of the drug from the stomach. Although it is still difficult to estimate drug concentrations in blood at the time of death by making use of those in alternative specimens, triazolam was considered not to be linked to the cause of death. However, the presence of triazolam in the cadaver was very important and this evidence contributed to elucidating the truth in the case.

In conclusion, the combination of three-step liquid–liquid extraction and subsequent HPLC frac-

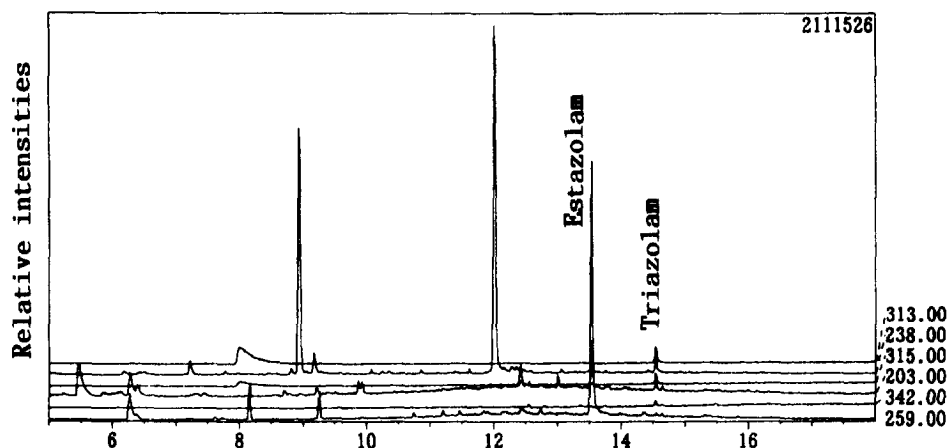


Fig. 6. SIM chromatogram of an extract from decomposed human muscle after HPLC fractionation. Chromatographic conditions are as in Fig. 4.

tionation made it possible to remove interfering substances effectively from decomposed human muscle. The method differentiates triazolam from etizolam. The method is very reliable and sensitive.

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### References

- [1] J.P. Sunter, T.S. Bal, W.K. Cowan, *Br. Med. J.* 297 (1988) 719.
- [2] A. Mori, E. Takeda, H. Suzuki, I. Ishiyama, *Acta Crim. Japon.* 59 (1993) 200.
- [3] K. Uemura, S. Komura, *Am. J. Forensic Med. Pathol.* 16 (1995) 66.
- [4] K. Kudo, T. Nagata, T. Imamura, S. Kage, Y. Hida, *Int. J. Leg. Med.* 104 (1991) 67.
- [5] T. Edeki, D.W. Robin, C. Prakash, I.A. Blair, A.J.J. Wood, *J. Chromatogr.* 577 (1992) 190.
- [6] O. Suzuki, H. Seno, T. Kumazawa, *J. Forensic Sci.* 33 (1988) 1249.
- [7] M. Terada, T. Yamamoto, Y. Kuroiwa, M.N. Islam, C. Wakasugi, in: *The Proceedings of International Symposium of Forensic Science, Tokyo, 1993*, p. 73.
- [8] H. Hattori, O. Suzuki, K. Sato, Y. Mizutani, T. Yamada, *Forensic Sci. Int.* 35 (1987) 165.
- [9] E.R. Cairns, B.R. Dent, J.C. Ouwkerk, L.J. Porter, *J. Anal. Toxicol.* 18 (1994) 1.
- [10] K. Hama, K. Matsubara, A. Akane, C. Maseda, K. Tanabe, Y. Fukui, *Jpn. J. Legal Med.* 41 (1987) 45.
- [11] F. Mußhoff, T. Daldrup, *Int. J. Leg. Med.* 105 (1992) 105.
- [12] K. Sato, C.M. Moore, Y. Mizuno, H. Hattori, Y. Katsumata, *Jpn. J. Forensic Toxicol.* 10 (1992) 9.
- [13] R. Jochemsen, J.G.J. Wesselman, C.J. Van Boxtel, J. Hermans, D.D. Breimer, *Br. J. Clin. Pharmacol.* 16(Suppl. 2) (1983) 291.
- [14] J.C. Garriott, *J. Forensic Sci.* 36 (1991) 60.
- [15] K. Kudo, T. Nagata, K. Kimura, T. Imamura, N. Urakawa, *Jpn. J. Legal Med.* 46 (1992) 293.
- [16] D.J. Pounder, C. Fuke, D.E. Cox, D. Smith, N. Kuroda, *Am. J. Forensic Med. Pathol.* 17 (1996) 1.
- [17] W.R. Sawyer, R.B. Forney, *Forensic Sci. Int.* 33 (1988) 259.