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Stability of benzodiazepines in whole blood samples stored at varying temperatures

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Abstract

Study has been undertaken to determine the stability of four benzodiazepines: clonazepam, midazolam, flunitrazepam and oxazepam in whole blood samples. Spiked blood was stored at four different temperatures (room temperature, 4°C, −20°C and −80°C) and analysed at selected times during one year. Determination was performed on the first, third and seventh day during the first week, then once a week for three weeks, once every two weeks for four weeks, then once a month for 4 months and finally, once every 2 months. Extraction was performed using liquid–liquid extraction with 1-chlorobutane, while quantification was carried out using high performance liquid chromatography equipped with a photodiode-array ultraviolet detector. At room temperature, the concentration of all benzodiazepines decreased over one year to 100 and 70% for low and high concentrations, respectively. At 4°C, the decrease was between 90 and 100% for low concentrations and between 50 and 80% for high concentrations. At −20°C, the measured decrease was between 10 and 20% for high and low concentrations, respectively. At −80°C, the measured loss was not significant at high concentration except for midazolam. However, at low concentration the determined decrease was between 5 and 12%. The data collected suggests that quantitative results concerning long-term stored samples should be interpreted with caution in forensic cases. Further investigations concerning the stability of drugs in whole blood or other biological samples, additional methods of identification and determination as well as the establishment of optimal storage conditions *should be undertaken in forensic cases*. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Benzodiazepines; Storage condition; HPLC; Long-term stability; Forensic toxicology

1. Introduction

Knowledge about time-dependent decrease of drug concentration in blood samples is of considerable significance for forensic cases. Frequently, there is a delay of a few days between sampling, drug screening and finally drug quantification in biological fluids. The subsequent confirmation may not be seeked until the case goes to court for trial and is thus performed many days or weeks after the blood has been taken. Usually, additives and preservatives are not added. Generally, foren-

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sic laboratories have to keep all blood sample frozen for a time period of one year at least to enable reanalysis if requested.

Some data has been reported concerning the stability of forensically relevant drugs including cocaine [1], benzoylecognine [2], 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid [2], phencyclidine [3] and amphetamines [4]. Recently, a comprehensive stability study has been published on drugs of abuse in authentic blood samples stored at ambient temperature [5].

Data has been reported [6–8] on the influence of long-term storage and on the stability of benzodiazepines (BZD) in biological matrix. The stability of nitrobenzodiazepines has been reported by several authors [9–11]. The stability of chlordiazepoxide has been reported by Garriott et al. [12]. Benzodiazepines are the most widely prescribed drug substances and have been heavily used and abused [13–15]. Therefore, they are important in the screening of blood samples of conspicuous motorists for example.

The aim of this study is to investigate the stability of some commonly used benzodiazepines found in blood samples refrigerated at four different temperatures and for various time periods up to one year. The BZD concentrations were measured by an isocratic high performance liquid chromatographic method [16].

2. Experimental

2.1. Chemicals

Clonazepam, flunitrazepam, midazolam and oxazepam were purchased from Promochem (Molsheim, France). Human blood was obtained from the University Hospital of Geneva (Switzerland). Monobasic and dibasic potassium phosphate phosphoric acid was purchased from Merck (Darmstadt, Germany) and acetonitrile HPLC grade was obtained from Romil (Cambridge, England).

2.2. Instrumentation and chromatographic conditions

An HPLC model 1100 (Hewlett-Packard, Palo Alto, CA) was used and consisted of a quaternary pump equipped with a diode-array detector, an automatic injector and an autosampler. The separation was achieved using a semi-micro column, C_8 reversed-phase: Lichrospher Select B 125 × 3 mm i.d. with 5 μm particle size and a guard column Nucleosil NH_2 8 × 4 mm i.d. with 5 μm particle size (Macherey-Nagel, Switzerland).The mobile phase was a mixture of phosphate buffer (20 mM, pH 2.1) and acetonitrile (65:35 v/v). The buffer solution was filtered through 0.45 μm filter (Supelco, Bellefonte, PA) before use.

The flow rate was 0.3 ml min^{-1} and the absorbance of the eluent was monitored at 220 nm. The column was thermostated at 25°C.

Three injections of a standard solution consisting of flunitrazepam and its metabolites, 7-aminoflunitrazepam, 7-acetamidoflunitrazepam and desmethylflunitrazepam, in presence of internal standard (methylclonazepam), were performed prior to each sequence in order to verify the performances of the system.

A Chemstation software G2170AA installed on a PC Vectra (model Vl4, Hewlett-Packard) was used for instrument control, data acquisition and data handling.

2.3. Sample preparation

2.3.1. Standard solutions

Stock standard solutions were prepared by dissolution of each benzodiazepine in methanol to obtain a concentration of 1 mg ml^{-1}. They were stored at −20°C and remained stable for at least 12 months.

Working solutions were prepared by dilution of stock solutions with blank blood in order to obtain the following final concentrations: 250 and 1000 ng ml^{-1} for all the benzodiazepines studied. Blood specimens, prepared with known concentrations of the four BZD, were analysed in triplicate in each experiment.

2.3.2. Phosphate buffer

The phosphate buffer was prepared by transferring 12.72 ml of 1 M KH_2PO_4 and 22.33 ml of 1 M H_3PO_4 into a 1000 ml volumetric flask, and made up to volume with distilled water. Buffer

solution is always freshly prepared and filtered just before use.

3. Quantification of benzodiazepines

Benzodiazepines were quantified by an isocratic HPLC following extraction with 1-chlorobutane (*n*-butyl chloride) [16].

4. Results

4.1. Chromatography

Under the described chromatographic conditions, the BZD studied are well separated and a summary of validation data is given in Table 1.

4.2. Effect of temperature on BZD stability

The influence of temperature on stability was studied for the four following benzodiazepines: clonazepam, flunitrazepam, midazolam and oxazepam (see Figs. 1–3).

All BZD studied were found to be stable when stored at −80°C. The concentrations measured for low concentration were 12% lower than in the corresponding initial samples, respectively for clonazepam, midazolam and oxazepam, and only about 5% lower for flunitrazepam. However, the remaining contents for high concentrations were around 100% for all BZD, except for midazolam where the remaining content was around 90%.

Contrary to what happens at −80°C, a significant decrease in concentration occurred at −20°C for all benzodiazepines at the end of study. The measured concentrations for clonazepam and flunitrazepam were at least 5% and 20% lower than in the original samples, for high and low concentrations, respectively.

At room temperature (RT) and at 4°C, the stability of all BZD was much lower. At 4°C, the decrease for low concentrations was, over six months around 80% for all the benzodiazepines mentioned above except for flunitrazepam for which the decrease was even more than 90%. For high concentrations, the decrease was around 70% for flunitrazepam, 50% for clonazepam, 40% for oxazepam and finally about 30% for midazolam.

At room temperature, we observed a dramatic decrease for all benzodiazepines. After 6 months, the remaining content of clonazepam was about 30% for high concentrations and 15% for low concentrations. As shown in Fig. 3a, for low concentrations of flunitrazepam, midazolam and oxazepam, the remaining content was respectively 0%, 6% and 15%. However, for high concentrations, we measured over 6 months the following remaining percents: around 56% for midazolam, 36% for oxazepam, 30% for clonazepam, and 13% for flunitrazepam.

After one year (Fig. 3b) all benzodiazepines were not detectable at room temperature. However, at 4°C the remaining content of clonazepam, flunitrazepam, midazolam and oxazepam was respectively 7, 0, 10 and 9.5% for low concentrations.

In these samples, it was even possible to detect the major metabolite of flunitrazepam (7-aminoflunitrazepam). The measured concentration was about 180 ng/ml.

Table 1
Summary of validation data

	Range (μg/ml)	*r*	Recovery (%)	LOD	LOQ
Clonazepam	0.03–0.5	0.998	>90	3.5	10
Flunitrazepam	0.01–0.5	0.998	>90	3.5	10
Midazolam	0.03–0.5	0.996	>90	3.5	10
Oxazepam	0.25–5.0	0.998	≅60	2.0	5

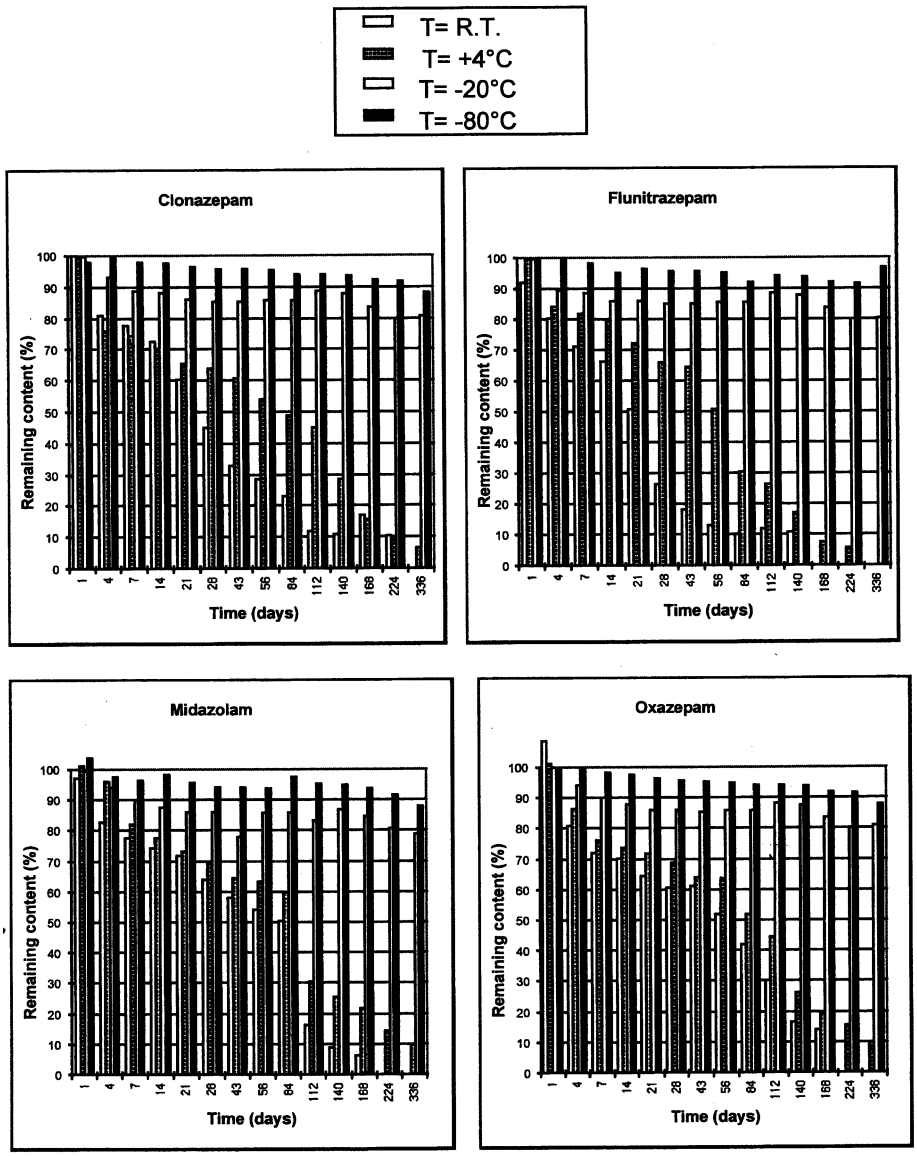

Fig. 1. Effect of time storage condition on whole blood containing 250 ng/ml of each benzodiazepine.

5. Discussion

This study on the stability of benzodiazepines in whole blood clearly demonstrated that a decrease in concentration must be considered for a storage period of 6 months or 1 year. The remaining measured concentrations were around the limit of quantification of the method for storage at room temperature. The conversion of flunitrazepam to 7-aminoflunitrazepam was already reported when incubated in bacterially contaminated post-mortem blood and tissues [21].

However, analyte degradation before analysis is often a result of chemical or physical decomposition due to the drug instability which looses its protective effect by binding to plasma proteins. For example, diazepam is susceptible to hydrolysis; flunitrazepam has been reported to signifi-

cantly degrade in a biological matrix within 1 day only when exposed to sunlight [17,18]. Benzodiazepines have been reported to bind with high association constants. For example, the range of bound flunitrazepam was reported to be between 77 and 79%, between 95 and 98% for oxazepam, of 95% for midazolam and of 85% for clonazepam at therapeutic level [19].

However, these differences in drug protein interaction did not allow to establish a clear relationship between drug binding and the concentration decrease observed during storage.

Degradation may also be due to bacterial contamination during sampling, for example because of unprotected skin. Bacterial degradation of benzodiazepines was reported in post-mortem blood and tissues [20,21]. Also, the loss of drug molecules can be due to enzyme activity in sample

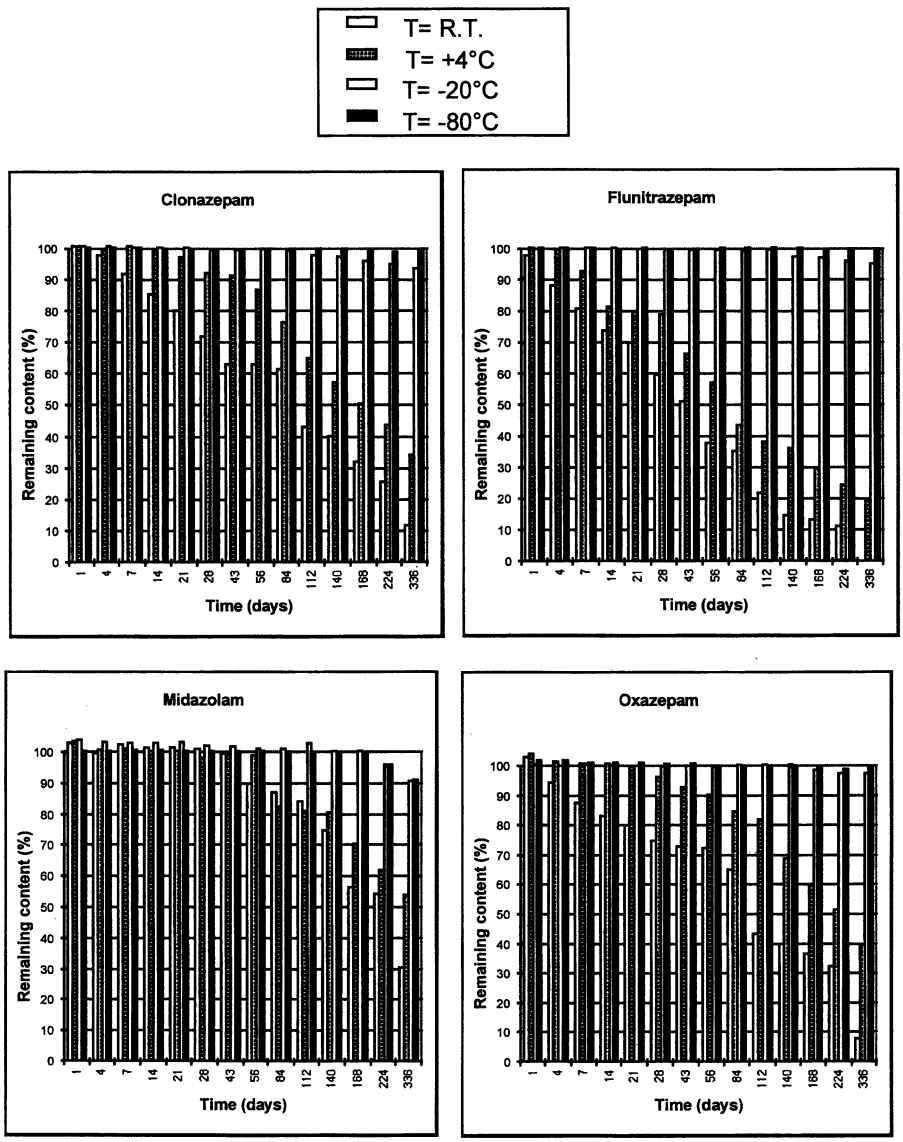

Fig. 2. Effect of time storage condition on whole blood containing 1000 ng/ml of each benzodiazepine.

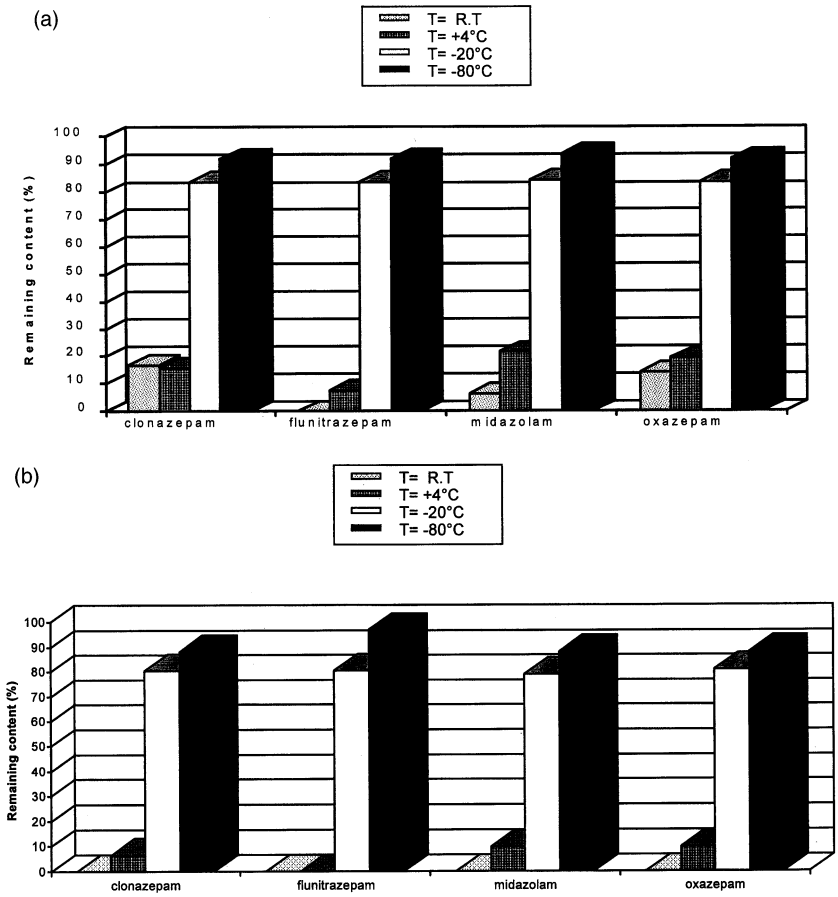

Fig. 3. Degradation of benzodiazepines in whole blood after a storage period of 6 and 12 months. Initial concentration: 250 ng/ml of each benzodiazepine.

unpreserved after collection or to adsorption in glass vials.

For further information concerning the degradation of benzodiazepines, investigations should use advanced analytical methods, such as liquid chromatography coupled to mass spectrometry, in order to better identify degradation products.

The present study clearly demonstrated that it is necessary to investigate suitable sample preparation, for example, addition of preservatives like sodium fluoride or potassium oxalate [22] and to establish optimal storage conditions.

Moreover, authentic samples should be routinely opened several times in order to simulate actual use and to study influence on benzodiazepines concentrations.

6. Conclusion

The present investigation covering an observation period of one year, shows that for ambient temperature and 4°C, the time interval between sampling and analysis strongly influences the quantitative determination of benzodiazepines. The present study also demonstrates that −80°C seems to be the best storage temperature. Even the loss observed at −20°C could influence the interpretation of toxicological results after prolonged storage. Therefore, the data obtained suggest the following:

- benzodiazepines and their metabolites should be determined as soon as possible after specimen collection;

- results from long-time stored samples should be interpreted cautiously in forensic cases in order to avoid serious consequences;
- benzodiazepines and their metabolites should be stored at −80°C or at least at −20°C. At −20°C, a decrease between 10 and 20% should be expected.

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