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A preliminary study on the stability of benzodiazepines in blood and plasma stored at 4° C

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Abstract An approach to determine the stability of benzodiazepines and some of their metabolites (n = 13) by means of a routinely applied gas chromatographic method using electron capture detection was made in this preliminary study. Validation data of the method are given. Spiked blood and plasma samples were stored at 4° C and analysed at selected times up to 240 days. The concentrations of all analytes had decreased to at least 60% of the original levels at the end of the observation period. A clear pattern of breakdown could not be established. The data obtained suggest that results from long-term stored samples should be interpreted cautiously. Further investigations concerning the stability of drugs in blood and plasma samples, additional methods of identification and determination as well as the establishment of optimal storage conditions seem necessary.

Key words Benzodiazepines · Storage condition · Long-term stability · Forensic toxicology · Gas chromatography

Introduction

The knowledge of time-dependent decreases in drug concentrations in blood or plasma samples is of considerable significance in legal cases. Frequently, there is already a delay of several days between sampling, determination of blood alcohol and initial presumptive drug screening. The subsequent confirmation may not be performed until the case goes to court for trial and may be done many days or weeks after the blood has been taken, especially when analysis for blood alcohol and drug monitoring of the same sample is done by separate institutions and at several places, a situation already present in Baden-Württemberg and Rheinland-Pfalz. Usually, additives and preservatives had not been added and sometimes, serum had been separated from blood and stored separately. Sample storage at 4° C is most favoured over freezing because it is more feasible for long-term storage of a large number of specimens. This procedure is in accordance with the official regulations. In Germany the forensic laboratories have to keep all blood samples in a cool place for a time period of at least 2 years [28] to enable reanalysis if possible.

Some data have been reported on the stability of forensically relevant drugs including cocaine [1], benzoylecgonine [19], 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid [19], phencyclidine [3] and amphetamines [21]. Recently, a comprehensive stability study has been published on drugs of abuse in authentic blood samples stored at ambient temperature [6]. Information on the influence of long-term storage on the stability of benzodiazepines in a biological matrix is rare [8, 9, 13, 16], although benzodiazepines are the most widely prescribed drug substances being heavily used and abused [12, 15, 27, 29] and play an important role in screening blood samples of conspicuous motorists.

It was the aim of this preliminary study to investigate the stability of some commonly used benzodiazepines in blood or plasma that were refrigerated at 4° C for various time periods up to 240 days by a routine gas chromatographic method.

Materials and methods

Experimental design

Analysis in the present study was performed on drug-free unpreserved fresh blood (250 mL) and frozen plasma (250 mL) samples obtained from two healthy volunteers from the blood bank of the University of Heidelberg (Germany). Blood as well as plasma were divided into three portions (60 mL), spiked with mixtures I-III (Table 1) of benzodiazepines and aliquoted. For concentration, the upper therapeutic range of the particular substance was chosen (Table 1).

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Table 1 Nominal values (ng/mL) for blood and plasma samples spiked with mixtures of benzodiazepines (mixtures I-III)

Mixture I		Mixture II		Mixture III	
Diazepam	300	Lorazepam	600	N-1-Desalkyl-flurazepam	100
Midazolam	300	Nordazepam	300	Clorazepate	700
Prazepam	100	Flunitrazepam	20	Clobazam	300
Flurazepam	100	Norflunitrazepam	50	Bromazepam	200
N-1-Hydroxyethyl-flurazepam	100				

Table 2 Concentration ranges (ng/mL) of benzodiazepine mixtures I-III used for calibration, reproducibility and repeatability

Mixture I		Mixture II		Mixture III	
Diazepam	0–400	Lorazepam	0–1400	N-1-Desalkyl-flurazepam	0– 200
Midazolam	0–400	Nordazepam	0– 400	Clorazepate	0–1000
Prazepam	0–200	Flunitrazepam	0– 80	Clobazam	0– 400
Flurazepam	0–200	Norflunitrazepam	0– 100	Bromazepam	0– 300
N-1-Hydroxyethyl-flurazepam	0–200				

Table 3 Summary of validation data. Relative analytical recovery from blood and plasma (n = 5), linearity (correlation coefficient, r), intraday and interday assays (coefficient of variation (%), replicates n = 5) of the benzodiazepines under investigation

Substance	Relative recovery from plasma	Relative covery from blood	Intraday variation (plasma)	Interday variation (plasma)	Linearity (plasma)
Diazepam	0.92	0.69	2.1	3.0	0.9896
Midazolam	0.89	0.78	2.0	1.9	0.9956
Prazepam	0.79	0.73	2.5	2.1	0.9963
Flurazepam	0.76	0.73	4.0	3.4	0.9993
Hydroxyethylflurazepam	0.73	0.88	2.9	4.3	0.9962
Lorazepam	0.71	0.60	2.5	1.1	0.9704
Nordazepam	0.73	0.69	3.8	3.7	0.9987
Flunitrazepam	0.61	0.86	2.4	5.1	0.9936
Norflunitrazepam	0.78	0.53	3.0	3.5	0.9929
Desalkylflurazepam	0.77	0.75	3.7	3.7	0.9956
Clorazepate	0.84	0.75	2.1	3.8	0.9954
Clobazam	0.77	0.99	3.5	5.0	0.9954
Bromazepam	0.59	0.67	2.5	4.1	0.9867

Aliquots of 5 mL of spiked blood and plasma were filled into headspace vials (Ziemer,GmbH, Mannheim, Germany) and sealed with a rubber stopper and an aluminium cap. At least ten specimens of both biological matrices containing one of the benzodiazepine mixtures I-III were stored at 4° C to allow duplicate measurements. In addition, ten blank plasma and blood samples were prepared and processed through the procedure for spiked samples to investigate the potential influence of storage time on the particular biological matrix. The determination for the original concentration done on day 1 and the subsequent analyses at 8-, 29-, 60- and 240-day intervals involved liquid/liquid extraction and analysis by gas chromatography, the compounds being detected by an electron-capture detector.

Chemicals

All chemicals were of analytical grade or HPLC quality. Diazepam, midazolam, prazepam, flurazepam, N-1-hydroxyethylflurazepam, lorazepam, nordazepam, flunitrazepam, norflunitrazepam, N-1-desalkylflurazepam, clorazepate, clobazam and bromazepam were purchased from Sigma (Deisenhofen, Germany), toluene and methanol from Roth (Karlsruhe, Germany), isoamylalcohol, boric acid, sodium hydroxide and potassium chloride from Merck (Darmstadt, Germany), and double distilled water from Braun (Melsungen, Germany).

Reagents

For the borate buffer 6.20 g of boric acid, 7.46 g of potassium chloride and 250 mL 0.1 M NaOH were made up to 1000 mL with double distilled water and, if necessary, adjusted to pH 8.5 with 0.1 M NaOH. Stock solutions of the particular compounds were prepared by dissolving 10 mg in 10 mL of methanol. The solutions were subsequently diluted with methanol to yield final concentrations of 0.1 and 0.01 mg/mL.

Instrumentation/chromatographic conditions

A gas chromatograph GC-9AM equipped with an ^{63}Ni-electron-capture detector (Shimadzu, Kyoto, Japan) and a SP 2490 integrator (Spectra Physics, Darmstadt, Germany) was used. The fused silica capillary column was 10 m · 0.53 mm i.d., 2 µm film thickness (CP-Sil 19, Chrompack, Middelburgh, The Netherlands). Column, injector and detector temperatures were 267° C, 290° C and 300° C respectively. The argon-methan carrier gas (9:1, v/v) flow rate was 30 mL/min.

Extraction procedure

To 500 µL of blank or spiked stored blood and plasma samples, 500 µL of borate buffer pH 8.5 and 500 µL of toluene/isoamylalcohol (9:1, v/v) were added and calibration standards were ob-

tained by spiking fresh blood and plasma. The mixture was vortexed (120 s), centrifuged (14000 rpm, 10 min, 4° C) and the upper phase was transferred to a clean test tube. The solvent mixture was evaporated under a stream of nitrogen (40° C), the residue was reconstituted with 25 µL methanol and 1 µL was injected for determination. All samples were run twice, the two results lying below a limit of 10%, and values given are mean values.

Data processing

Blank plasma and blood samples were spiked with benzodiazepine mixtures I-III covering the concentration ranges listed in Table 2. The 5-point calibration curves were established by assaying the spiked samples five times on five different days for spiked samples of group I–VI, and a blank sample was always included. The analytical recovery (Table 3) from blood and plasma was determined for the analytes at the concentrations shown in Table 1 (n = 5). The limits of detection (LOD) and of quantitation (LOQ) were established according to the guidelines proposed by Eurachem [14].

The calibrators for reanalysis of stored samples were prepared from fresh blood and plasma with two concentrations above and below the expected value. They were run twice (range < 5%), and the drug concentration was determined from the appropriate peak areas (mean) by linear regression. The particular benzodiazepine concentration in the stored sample is given as relative recovery with reference to the relative recovery of 1.0 obtained on day 1.

Results

The relative analytical recoveries (Table 3) were between 0.59 and 0.92 from plasma and between 0.53 and 0.99 from blood, but generally a higher recovery rate was obtained from plasma. For all 5-point calibration graphs strong correlation coefficients were obtained (Table 3). Due to the smooth and continuous curves from blank measurements, the LOD could not be calculated from random fluctuation of the blanks as proposed by the International Union of Pure and Applied Chemistry [17]. Therefore, the LOD was determined from a series of extracts (by 2 ng/mL steps) spiked with low concentrations close to the origin, and assigned to the lowest level that was obviously different from the blank measurement using these chromatographic conditions. The estimated values largely fitted with those reported in the literature [4, 7, 23, 24]. The LOQs for the different benzodiazepines were calculated from an approach according to Eurachem [14] and the coefficient of variation was chosen as 10% (Table 4).

The study indicated that except for lorazepam the extracts from blank blood and plasma stored under the same conditions as the spiked samples did not show peaks that interfered with the quantitative determination of the analytes. An interference from the biological matrix was apparent for lorazepam in blood after a storage of 29 days. A full scan GC/MS analysis revealed that lorazepam or its thermal decomposition product [11] had been totally degraded indicating that the observed high relative recovery calculated by gas chromatography/electron capture detection resulted from the blood matrix.

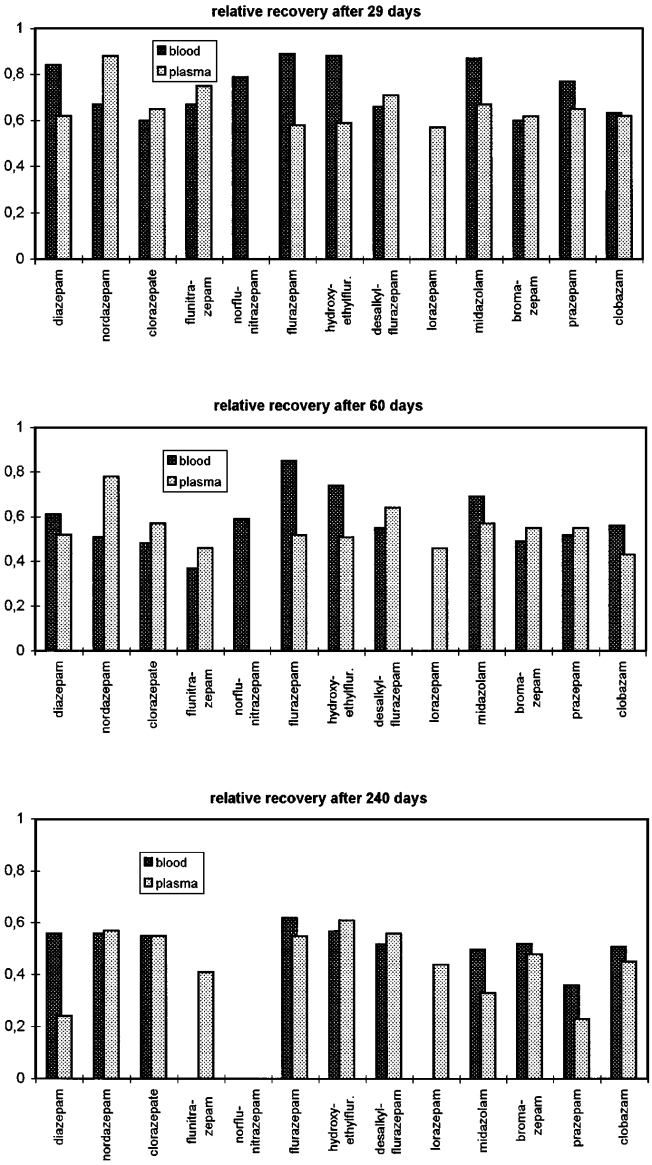

Fig. 1 Relative recoveries of benzodiazepines in blood and plasma samples after storage for 29, 60 and 240 days at 4° C referring to a relative recovery of 1.0 on day 1

Table 4 LOD (ng/mL) and LOQ (ng/mL) determined according to the Eurachem proposal

Substance	LOD	LOQ
Diazepam	2	20
Midazolam	2	8
Prazepam	4	17
Flurazepam	6	10
Hydroxyethylflurazepam	6	26
Lorazepam	10	74
Nordazepam	4	23
Flunitrazepam	2	6
Norflunitrazepam	14	16
Desalkylflurazepam	4	17
Clorazepate	2	22
Clobazam	10	55
Bromazepam	5	12

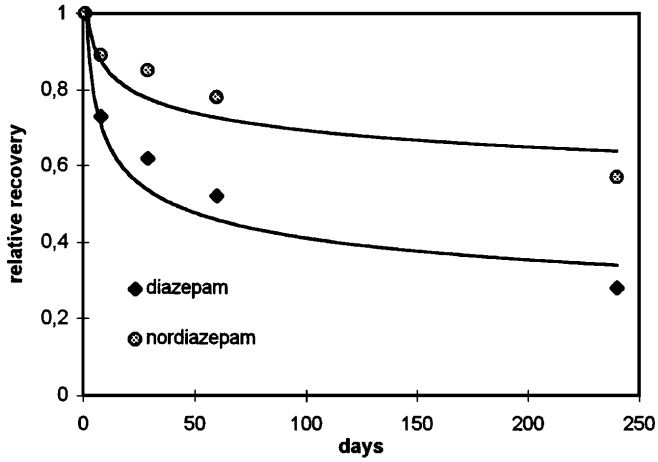

Fig. 2 Time course of diazepam and nordazepam concentrations in plasma stored at 4° C

There was a continuous decrease in concentration in both blood and plasma samples for all 13 analytes during the 240-day interval at 4° C (Fig. 1), which was more distinct at the beginning of the experiments (Fig. 2) and showed quite different tendencies depending on the biological matrix and the particular drug substance. The results upon reanalysis are summarized in Fig. 1. Flunitrazepam and norflunitrazepam were less stable, followed by prazepam and lorazepam. Norflunitrazepam could not be detected in plasma even at the shortest time interval studied and had been totally degraded in blood after 8 months. Although exhibiting poor stability, flunitrazepam could be detected in plasma on day 240 at a relative recovery of 0.4. Except for lorazepam and nordiazepam, relative recoveries from blood were comparable to or exceeded those from plasma for storage periods longer than 29 days. At the end of the storage period the previously observed differences in relative recoveries referring to the particular matrix were less obvious.

Discussion

This preliminary study on drug stability in benzodiazepine-spiked blood and plasma clearly demonstrated that a decrease in concentration must be considered to have occurred during a storage period of 8 months at 4° C. To exclude all individual differences arising in authentic samples, the experiments were performed using an identical biological matrix, either blood or plasma, from a single person. Drug concentrations chosen for spiking were at the upper limit of the therapeutic ranges [10, 22]. Although some benzodiazepines are known to bind to red blood cells resulting in plasma/blood partition ratios from approximately 1.33 (flunitrazepam) [10] to 1.9 (diazepam) [10], for direct comparison the same concentrations in spiked blood and plasma were used. Except for lorazepam, flunitrazepam and its desmethyl metabolite positive results were obtained for all other analytes up to 8 months in both blood and plasma samples, although there was a continuous decrease in drug concentration without a clear pattern of breakdown (Fig. 1). After 8 months, the concentrations of all analytes still detectable were at least 40% lower than in the corresponding initial sample. A similiar decrease in diazepam, nordazepam and bromazepam concentration has been reported by Käferstein et al. [13] in a study on authentic blood samples after a 6-month storage at 4° C.

Degradation before analysis is often a result of chemical or physical decomposition due the instability of the drug which lacks the protective effect of being bound to plasma proteins. For example, diazepam is susceptible to hydrolysis [18] and flunitrazepam was reported to significantly degrade in plasma within only 24 h when exposed to sunlight [2, 5]. Benzodiazepines show a marked protein binding with high association constants, the proportion of drug molecules bound ranging from 77–79% for flunitrazepam [10, 22] to 96–99% for diazepam [10, 22] at therapeutic levels. However, these differences in drug-protein interaction did not allow a clear relationship to be established between drug binding and the observed decrease in concentration during storage.

Degradation may also be due to enzyme activities which continue in an unpreserved sample after collection or to bacterial contamination during sampling, for example from unprotected skin. Enzyme activities in blood of healthy persons are thought to be mainly restricted to esterases, which are not known to be involved in the breakdown of benzodiazepines. Bacterial degradation of flunitrazepam was reported in post mortem blood [25]. Also, the loss of drug molecules due to adsorption to the glass vial or the rubber stopper has been observed for diazepam [26].

When deciding on the method of analysis for the present study the desirable factors of simultaneous determination of all substances under investigation, ease of extraction, and the limits of detection were considered. Besides high pressure liquid chromatography [20], gas chromatography with electron capture detection seemed to be most appropriate regarding sensitivity, but it failed to detect some important metabolites and to identify major degradation products, for example arising from 7-nitro-1,4-benzodiazepines. Moreover, it seemed important to confirm particular peaks by mass spectrometry to avoid unreliably high concentrations or false positive results arising from potential matrix interferences as demonstrated by lorazepam. Therefore, in future investigations more advanced analytical methods, preferably liquid chromatography coupled with mass spectrometry should be used to identify degradation products and to study the fate and time course of a particular drug substance under storage in detail.

Further studies seem to be urgently necessary to investigate suitable sample preparation, e.g. addition of preservatives and antibacterial agents, and to establish optimal storage conditions. Moreover, the situation often present in authentic samples, which are routinely opened several times, should be simulated to supply further aspects of changes in benzodiazepine concentrations during long-term studies.

The present study covered an observation period of 8 months which is less than half the time the blood samples of conspicuous motorists have to be stored for forensic investigations. From the preliminary findings it can already be concluded that for refrigerated samples (4° C), the time interval between sampling and analysis may strongly influence the analytical results for benzodiazepines. Pharmacokinetic calculations concerning the relationship of parent drug to metabolite concentrations in order to estimate single or repetitive dosing or the time lapse between application and collection of the sample, may be biased in stored specimens by different degradation rates as for example observed for diazepam and nordiazepam in plasma (Fig. 2). As a consequence, results from samples stored at 4° C for some time prior to analysis should be interpreted cautiously. Interlaboratory studies can determine accuracy, precision, repeatability, reproducibility, limit of detection and quantitation. However, the blood and plasma samples commonly used for interlaboratory studies do not represent authentic samples as they usually contain additives and preservatives for conservation of the drug to be analysed. The excellent results obtained from these studies hide the problems of severe changes in drug concentration in forensic samples which may occur during the pre-analytical phase. The present preliminary findings already outline that regulations for sampling, transport and storage of blood samples seem a *conditio sine qua non* for quantitative results and reliable interpretation in questions of drugs and driving.

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