Chiang Mai J. Sci. 2010; 37(3) : 451-463
www.science.cmu.ac.th/journal-science/josci.html
Contributed Paper

Stability Study of Six 1,4-Benzodiazepines in Bio-fluids Stored at -20°C

Mohammad N. Uddin*[a], Victoria F. Samanidou [b], and Ioannis N. Papadoyannis [b]
[a] Department of Chemistry, University of Chittagong, Chittagong-4331, Bangladesh.
[b] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, GR-541 24, Greece.
*Author for correspondence; e-mail: nasiru_cu@yahoo.com

Received: 22 July 2009
Accepted: 3 May 2010

ABSTRACT

An approach has been undertaken to study the stability of six 1,4-benzodiazepines; alprazolam, bromazepam, clonazepam, diazepam, flunitrazepam and lorazepam in spiked plasma, urine and saliva samples at defined time intervals. Quantification was carried out using high-performance liquid chromatography equipped with a photodiode-array ultraviolet detector while extraction was performed using SPE. Spiked samples (0.5, 2.0 ng μL^{-1}) were stored at -20°C and analysed at selected times during 180 days for both plasma and urine, and 120 days for saliva. At -20°C, at the end of the observation period decrease was measured around 40% for flunitraepam and clonazepam and around 20% for remaining four benzodiazepines of the original levels. At end of study period for low concentrations (0.5 ng μL^{-1}) all benzodiazepines were though detectable but a clear pattern of breakdown could not be established. Results of stability study through freeze-thaw cycle during seven successive days for spiked samples stored at -20°C were also reported. All analytes were seemed to be completely stable up to five cycles in plasma, urine, and up to three cycles in saliva. Moreover, stability study of all analytes over a period of one year revealed that standard solution in methanol stored at 4°C can be used without any remarkable degradation. The data collected suggest that quantitative results concerning long-term stored samples should be interpreted with caution in forensic cases.

Keywords: 1,4-benzodiazepines, bio-fluids, plasma, urine and saliva, solid-phase extraction.

1. INTRODUCTION

Benzodiazepines are the most widely prescribed drugs being heavily used and abused [1-3] and they play an important role in screening blood samples of conspicuous motorists. Information on the influence of long-term storage on the stability of benzodiazepines in biological matrices is rare [4]. Knowledge, about decrease of drug concentration in biological samples depending upon time, is of considerable significance for forensic cases. Usually, there used to be a delay of several days between sampling, drug screening and finally drug quantification in biological fluids. 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 drug monitoring of the same sample is done by separate institutions and at several places. Usually, additives and preservatives had not been added and sometimes, plasma had been separated from blood and stored separately. Generally, forensic laboratories have to store samples for a time period of one year at least to enable reanalysis if requested.

Recently, a comprehensive stability study has been published on drugs of abuse in authentic blood samples stored at ambient temperature [5]. The stability of forensically relevant drugs cocaine, benzoylecognine, 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid, phencyclidine, amphetamines along with benzodiazepines has also been reported [6-8]. The influence of long-term storage on the stability of benzodiazepines (BZD) in blood, plasma has been studied [4, 9-10].

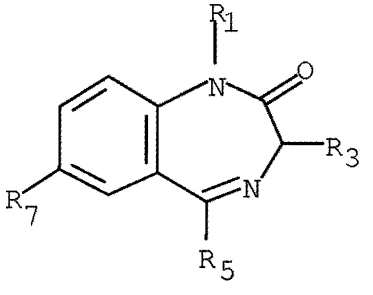
The aim of this study is to investigate the stability of commonly prescribed six 1,4 benzodiazepines (Figure 1) in biological samples (plasma, urine, saliva), refrigerated at -20°C for various time periods up to six months, by a routine HPLC method developed previously after solid-phase extraction (SPE). In addition stability of the methanolic solution of the analytes, stored at 4°C for a period of one year, has been investigated.

2. EXPERIMENTAL

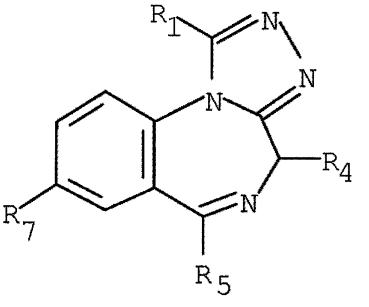
2.1 Chemicals

Colchicine used as the internal standard was supplied by Sigma (Sigma-Aldrich Chemie BV, The Netherlands). HPLC-grade organic solvents (methanol, ACN) were supplied by Carlo Erba (Milano, Italy). Water used throughout the study was purified as required by the reverse osmosis method to gain high-purity water with a Milli-Q water

Figure 1. Chemical structure of benzodiazepines under study.



I



II

Benzodiazepines	Group	R_1	R_3/R_4	R_5	R_7
Bromazepam (BRZ)	I	H	H	2'-pyridyl	Br
Clonazepam (CLZ)		H	H	2-Cl-Phenyl	NO_2
Diazepam (DZP)		CH_3	H	Phenyl	Cl
Flunitrazepam (FNZ)		CH_3	H	2-F-phenyl	NO_2
Lorazepam (LRZ)		H	OH	2-Cl-phenyl	Cl
Alprazolam (APZ)	II	CH_3	H	phenyl	Cl

purification system from Millipore (Millipore, Bedford, MA, USA). Ammonium acetate p.a. was supplied from Riedel-de Haen (Buchs, SG, Switzerland). Six drug standards were from Sigma Chemical Company (St. Louis, MO, USA). Purity of reference compounds was not less than 98%.

2.2 Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low-pressure gradient system was used for the chromatographic determination of the examined analytes. The solvent lines were mixed in an FCV-10ALVP mixer. An LC-10ADVP pump equipped with a Shimadzu SCL-10ALVP System Controller, permitting fully automated operation, was used to deliver the mobile phase to the analytical column. Sample injection was performed via a Rheodyne 7725i injection valve (Rheodyne, Cotati, California, USA) equipped with a 20 μL loop. Detection was achieved by an SPDM10AVP Photodiode Array Detector, complied with data acquisition Lab Solutions-LC-Solutions by Shimadzu. Degassing of the mobile phase was performed by helium sparging in the solvent reservoirs by a DGU-10B degassing unit.

The analytical column, a Kromasil C_8 (250×4 mm, 5 μm) was purchased from MZ-Analysentechnik (Mainz, Germany). A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using 0.2 μm cellulose nitrate 0.2 μm-WCN Type (47 mm DIA) membrane filters (Whatman Laboratory Division, Maidstone, England). The SPE study was carried out on a 12-port vacuum manifold from Supelco (Bellefonte, PA, USA). LC-18 cartridge 500 mg/3 mL was supplied from Supelco. All evaporations were performed with a Supelco 6-port Mini Vap concentrator/evaporator.

2.3 Collection of Specimens

Drug free plasma samples were kindly provided from the Blood Donation Unity of a State Hospital, (Thessaloniki, Greece) while urine and saliva samples were provided by one male healthy volunteer. Pooled samples were prepared. Both urine and saliva samples were collected in polypropylene tube. Saliva samples was collected over 180 min by spitting at fixed time intervals, without any stimulation of the saliva production, to obtain approximately 3 mL of sample. By the 30 min before sampling no food or drink was taken by saliva donor and mouth was rinsed with fresh water before spitting [11]. Supplied drug-free plasma, as soon as possible after collection without any preservative the urine and supernatant of saliva obtained after centrifugation at 2,500 g for 10 min to remove potential food debris, were separated into 1.5 mL aliquots in eppendof and stored at -20°C until analysis.

2.4 Chromatographic Conditions

Chromatography was performed under gradient conditions at ambient temperature (about 25°C). The mobile phase consisted of a mixture of methanol, acetonitrile and ammonium acetate buffer (0.05 M) delivered in gradient mode as shown Table 1 at a flow-rate of 1.0 mL min^{-1}. It generated an operating back pressure of initial 230 km/cm^2 which was finally reduced to 200 km/cm^2 at 10 min. Before use, the mobile phase was degassed for 10 min with helium. Absorbance of the eluent was monitored with a variable wavelength UV-vis detector set at 240 nm. After an equilibration time of about 30 min the first injection can be applied. Three injections of a standard solution consisting of all analytes in presence of internal standard were performed prior to each sequence in order to verify the performances of the system.

Buffer: 0.05 M aqueous solution of

ammonium acetate buffer was always freshly prepared by mixing appropriate weight in MQ water and filtered just before use.

Table 1. Gradient program for the proposed method.

Program	Time (min)	Solvent Percentage			Flow rate mL/min	Elution time(min)
		CH_3OH	CH_3CN	0.05 M CH_3COONH_4		
A*	0.01	30.00	15.00	55.00	1.0	9.94±0.07
	2.00	50.00	18.30	31.70		
	3.00-10.50	50.00	20.00	30.00		

2.5 Standard Solutions

Stock solutions (100 ng μL^{-1}) of all benzodiazepines and colchicine (IS) were prepared by dissolving an appropriate amount of each compound in methanol and were stored at 4°C, protected from light and remained stable for at least 12 months. The mixture solution of drugs at concentration level 2 ng μL^{-1} were prepared containing internal standard at a concentration of 4 ng μL^{-1}. Mixture solutions were prepared without internal standard for spiking biological samples at concentration levels 0.5, 2 ng μL^{-1}.

2.6 Preparation of Spiked Samples and Storage

200 µL standard solutions at experimental levels 0.5, 2 ng μL^{-1} for spiked sample or 200 µL of methanol for blank samples was added to aliquots of 50 µL of pooled plasma or 100 µL of urine or 500 µL saliva samples. All spiked samples were stored in freezer at -20°C before analysis for a period up to 6 months.

2.7 Sample Pre-treatment

Prior to extraction, spiked biological samples were pre-treated for the removal of proteins by precipitation with the addition of 200 µL of acetonitrile. After centrifugation for 15 min at 3,000 rpm, the clear supernatant was applied to solid-phase extraction protocol optimized previously using *Nexus Varian* cartridge for the isolation of 1,4-benzodiazepines [12].

2.8 Solid-phase Extraction

With the addition of 2 mL distilled water to decrease the percentage content of ACN, supernatant was applied to SPE cartridge preconditioned with 2 mL of methanol and 2 mL of water. After washing with 1 mL water retained drugs were eluted with (2×1) mL MeOH:CH_3CN (50:50 v/v) followed by the evaporation to dryness under a gentle flow of N_2 at 30°C. The residue was reconstituted with 200 µL of methanolic internal standard solution at concentration 4 ng μL^{-1} prior to their injection into the liquid chromatographic system. Aliquots of 20 µL were injected onto column.

2.9 Stability Study

2.9.1 Standard solution

The stability of standard solutions was tested by the proposed HPLC method over a period of 1 year. The freshly prepared solutions at room temperature and seven different days over a period of 1 year stored samples in a refrigerator at 4°C, were analyzed, chromatogram and recoveries were compared.

2.9.2 Spiked Solution

Freeze-thaw cycle of spiked samples: Biological plasma, urine and saliva samples, spiked with 2 ng μL^{-1} of analytes, were stored in freezer at -20°C. For short-time stability assay each sample was analyzed once daily for seven successive days after freeze-thaw cycle for investigation of stability when spiked samples were allowed to thaw at room temperature. Recovery of analytes for the stored samples were calculated and compared to that of freshly prepared spiked samples.

Long-term Stability: Biological samples spiked at 0.5, 2 ng μL^{-1} concentration levels were stored in freezer at -20°C, and analyzed over a period of 180 days for plasma, urine and 120 days for saliva. Each sample was subjected to deprotenization before analysis at definite days during tested period. The comparison of recoveries in spiked samples over study period was performed to that obtained for freshly prepared spiked samples at room temperature.

3. RESULTS

3.1 Chromatography

The influences on stability of six 1,4-benzodiazepines: alprazolam, bromazepam, clonazepam, flunitrazepam, lorazepam and diazepam in spiked biological samples, stored at -20°C for long-term storage of 6 months and short-term through freeze-thaw cycle, were studied. Under the described chromatographic conditions, the BZDs were well separated and summary of linearity and sensitivity, and validation data are given in Table 2 and Table 3, respectively. Relative standard deviation (RSD) was calculated for standard at three concentration levels (1, 5, 10 ng μL^{-1}) taking six measurement each for both *within-* and *between-day* analyses. Precision of retention times was examined to evaluate system suitability from these measurements (n=36) which revealed RSD values of 0.4–0.7%. The limit of detection, LOD was obtained by use of the slope (*a*) and the standard error of the intercept (S_{xy}) of the regression line; $LOD=3\cdot(S_{xy}/a)$ [13]. Column performance was evaluated by calculating resolution factor (R_s) and number of theoretical plate (N) for a standard representative chromatogram (Figure 2). Retention times of the examined benzodiazepines are 5.51±0.05 for the internal standard, 6.63±0.02 for BRZ, 7.23±0.04 for CLZ, 7.45±0.04 for FNZ, 7.96±0.04 for LRZ, 8.22±0.05 for ALP, and 9.94±0.07 min for DZP. The study indicated that the extracts from blank plasma, urine and saliva did not show peaks that interfered with the quantitative determination of the analytes. Therefore, high precision and sensitivity were observed from the biological matrices calculated by proposed HPLC-SPE method.

Table 2. Sensitivity data for the proposed method.

Analytes	Standard		Blood		Urine		Saliva	
	r^2	LOD ng μL^{-1}	r^2	LOD ng μL^{-1}	r^2	LOD ng μL^{-1}	r^2	LOD ng μL^{-1}
Bromazepam	0.999	0.07	0.999	0.13	0.997	0.35	0.999	0.20
Clonazepam	0.999	0.10	0.998	0.50	0.999	0.17	0.999	0.20
Flunitrazepam	0.999	0.11	0.999	0.36	0.998	0.19	0.999	0.13
Lorazepam	1.000	0.02	0.998	0.38	0.998	0.29	0.998	0.25
Alprazolam	0.999	0.15	0.998	0.51	0.998	0.20	0.999	0.17
Diazepam	0.999	0.01	0.999	0.34	0.999	0.13	0.999	0.15

Table 3. Validation data for the proposed method.

Analytes	Relative standard deviations (RSD)						RSD of R_t	R_s	N
	Within-day (ng μL^{-1})			Between-day (ng μL^{-1})					
	1	5	10	1	5	10			
BRZ	9.7	3.4	2.6	3.1	3.2	5.1	0.3	3.64	3885.4
CLZ	10.1	0.6	1.6	2.2	5.6	4.0	0.6	2.75	4776.3
FNZ	9.6	2.0	1.8	5.0	6.6	3.2	0.5	0.80	2855.8
LRZ	3.2	2.8	4.9	3.0	5.2	7.3	0.5	1.76	4157.7
ALP	4.0	5.1	3.0	9.2	9.0	9.3	0.6	1.10	6355.4
DZP	1.2	2.5	8.8	7.2	10.1	10.1	0.5	4.51	12494.3

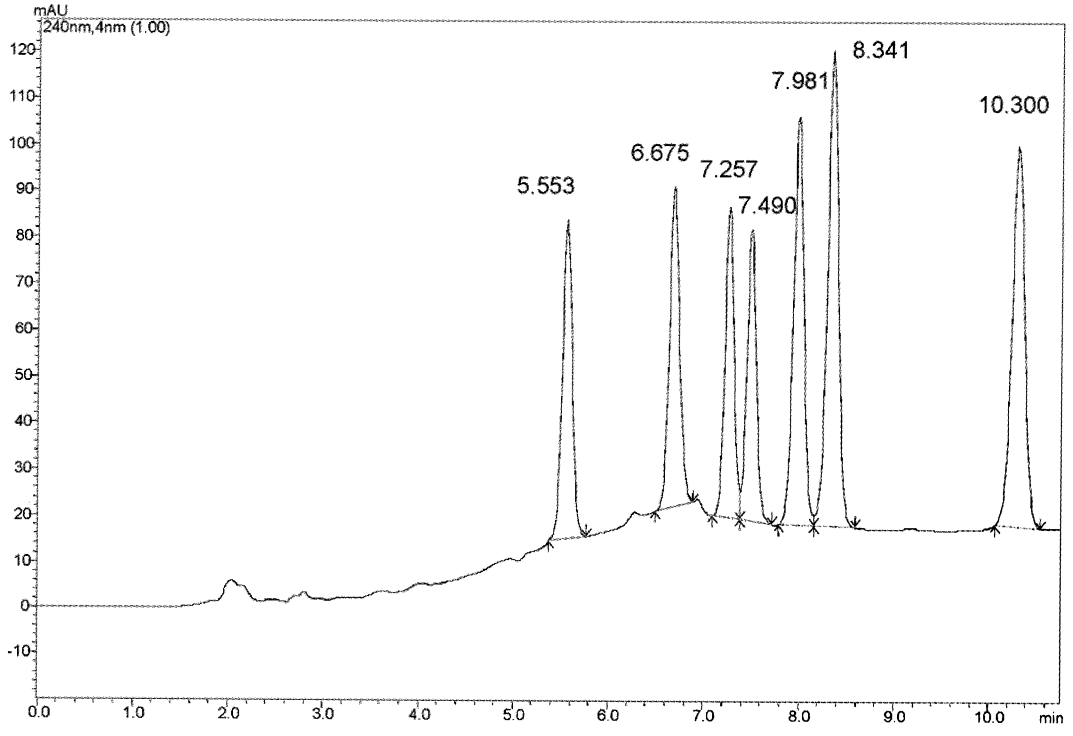


Figure 2. Typical HPLC chromatogram of the six examined benzodiazepines (10 ng μL^{-1}) in the presence of colchicine (4 ng μL^{-1}) as internal standard. Chromatographic conditions are described in text. Peaks: 5.553 min (IS), 6.675 min (BRZ), 7.257 min (CLZ), 7.490 min (FNZ), 7.981 min (LRZ), 8.341 min (ALP), and 10.300 min (DZP).

3.2 Stability

3.2.1 Standard Solution

The freshly prepared solutions in methanol at room temperature and seven different days over a period of 1 year stored samples in a refrigerator at 4°C, were analyzed and recoveries were compared. Comparison concludes that there were no degradation drugs and the drug is stable at 4°C for at least 1 year, indicating the possibility of using all

studied drugs over a period of 1 year stored in refrigerator without degradation. Analytical data are presented in Figure 3.

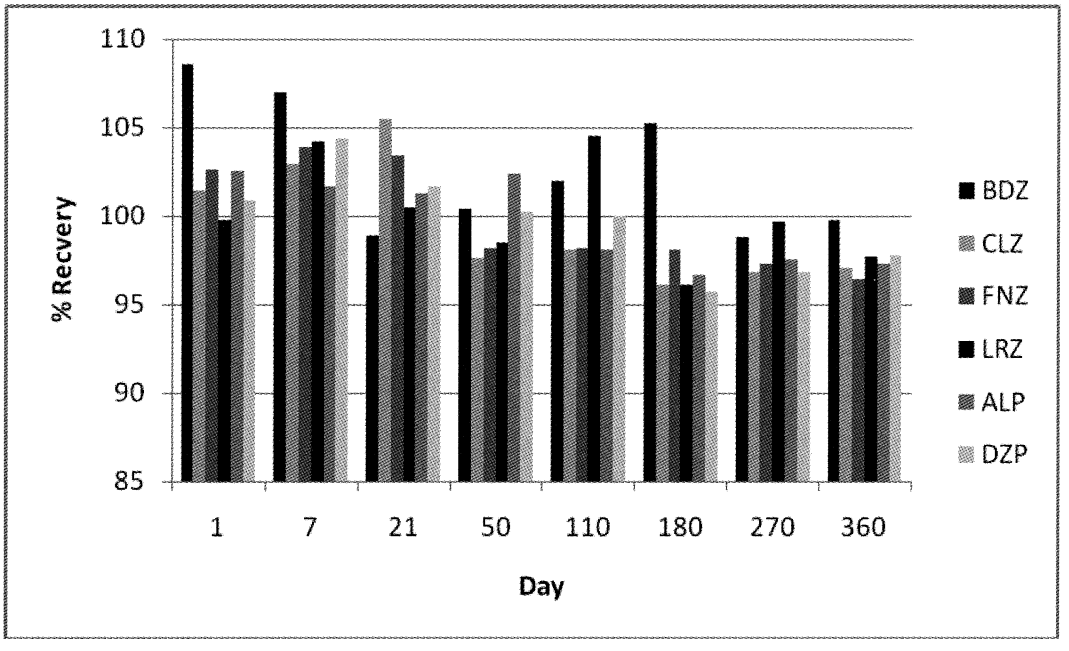


Figure 3. Long-term stability of benzodiazepines in standard solution in methanol.

3.2.2 Spiked samples

Short-term stability through freeze-thaw cycle

Spiked plasma, urine or saliva samples (2 ng μL^{-1}), were analyzed once daily for seven successive days after freeze-thaw cycle for investigation of stability when spiked samples were allowed to thaw at room temperature. Recovery of analytes for the stored samples was compared to that of freshly prepared spiked samples. Short-term stability results of plasma have already been reported in previous experiments and it was found to be consistence with three experiments conducted before [12,14,15]. Short-term stability results for remaining two samples, urine and saliva are reported and these are graphically presented in Figure 4, 5, respectively. All analytes seemed to be completely stable up to five freeze-thaw cycles in plasma and urine, and up to three freeze-thaw cycles in saliva while recovery was decreased by less than 10%.

Long-term Stability

Spiked samples, stored in freezer at -20°C at two different concentrations of 0.5 ng μL^{-1} and 2 ng μL^{-1}, were analysed by the proposed HPLC method over a period of 180 days for plasma, urine and 120 days for saliva. There was a continuous decrease in high concentration at -20°C, which was more distinct at the beginning of the experiments and showed quite different tendencies depending on the biological matrices and the particular drug substance. The comparison of recoveries to that obtained for freshly spiked samples at room temperature concludes that decrease was remarkable for saliva than plasma and urine. A significant decrease in concentration occurred at - 20°C in both plasma and urine samples for all analytes during the 180-day interval and in saliva at the end of study period of 120 days. The estimated values largely fitted with those

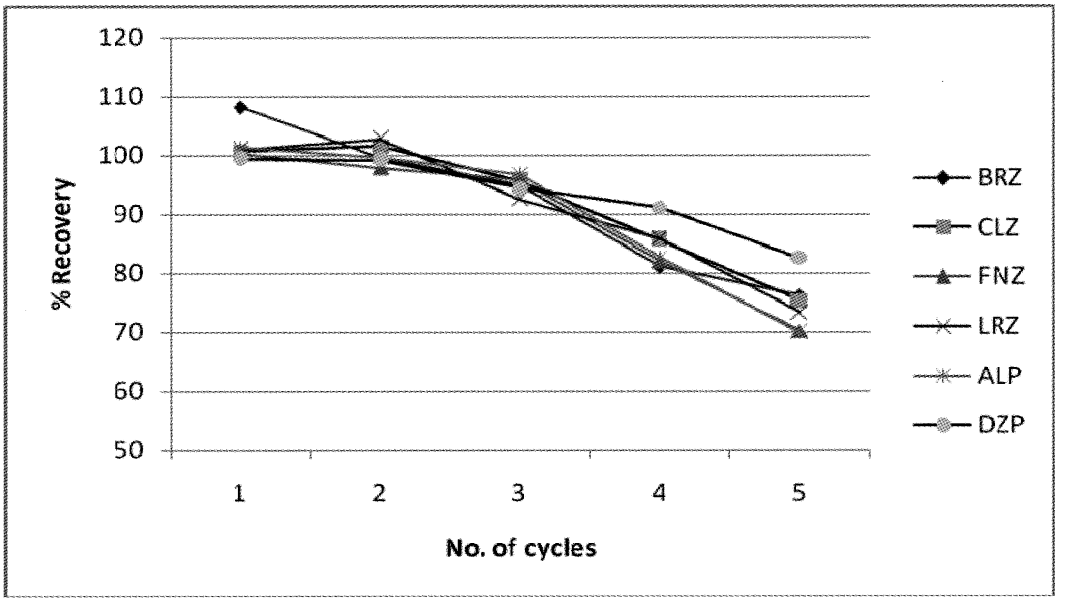


Figure 4. Short-term stability of spiked saliva through freeze-thaw cycles.

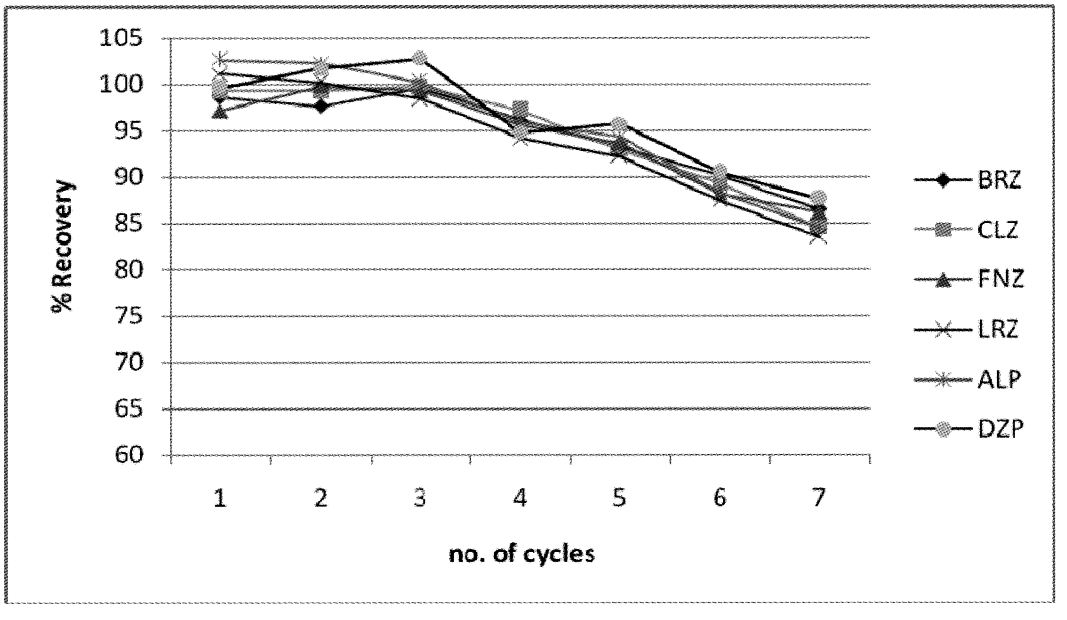


Figure 5. Short-term stability of spiked urine through freeze-thaw cycles.

reported in the literature [9,10]. The results upon analysis are summarized in Figure 6-8. Concentrations of four benzodiazepines for storage periods 60 days were comparable to freshly spiked samples, for remaining two nitro compounds it was comparable for the period of 30 days only. At -20°C, the decrease for low concentrations (0.5 ng μL^{-1}) at end of study period was around 60%, for all the benzodiazepines mentioned above, except

for clonazepam and flunitrazepam for which the decrease was even more than 80% but a clear pattern of breakdown could not be established.

4. DISCUSSION

When deciding on the method of analysis for the present study the desirable factors of simultaneous determination of all substances

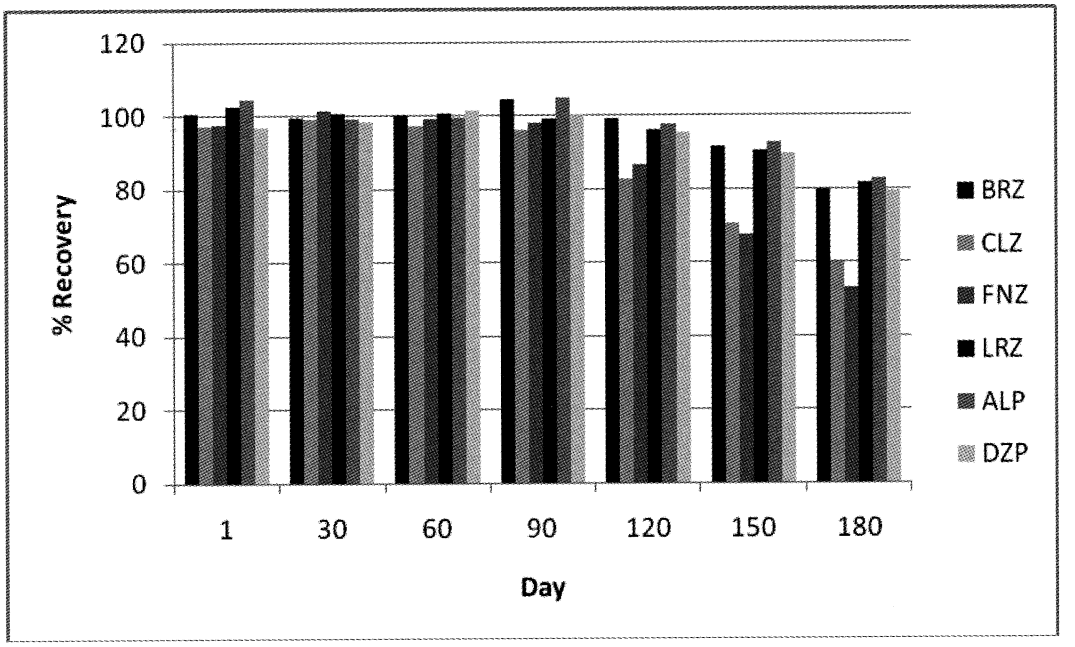


Figure 6. Long-term stability of benzodiazepines in spiked plasma sample.

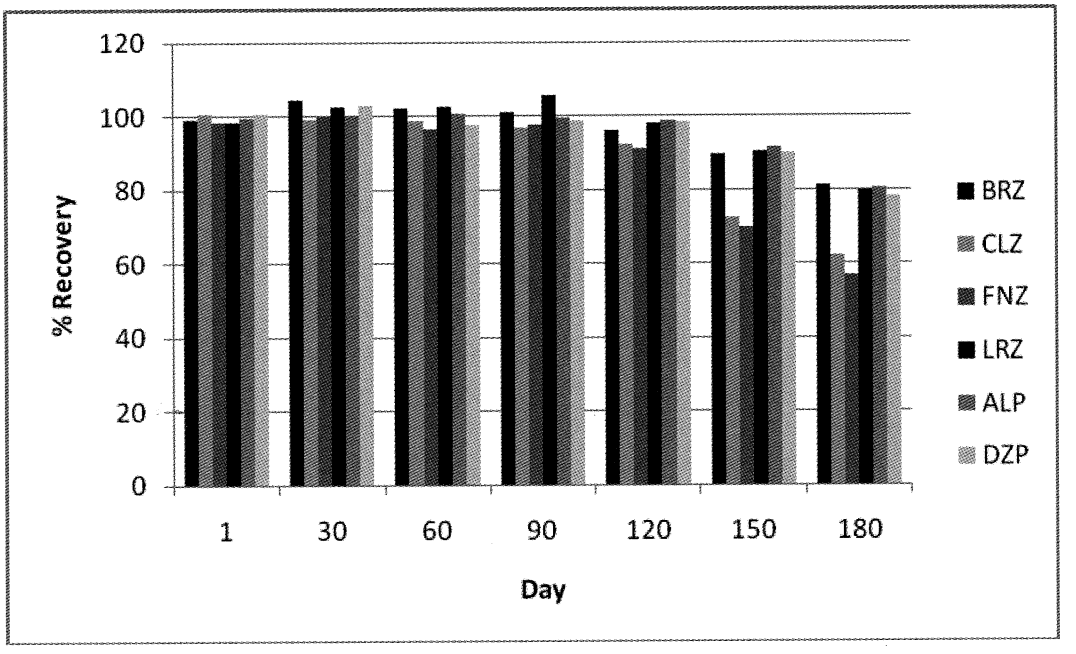


Figure 7. Long-term stability of benzodiazepines in spiked urine sample.

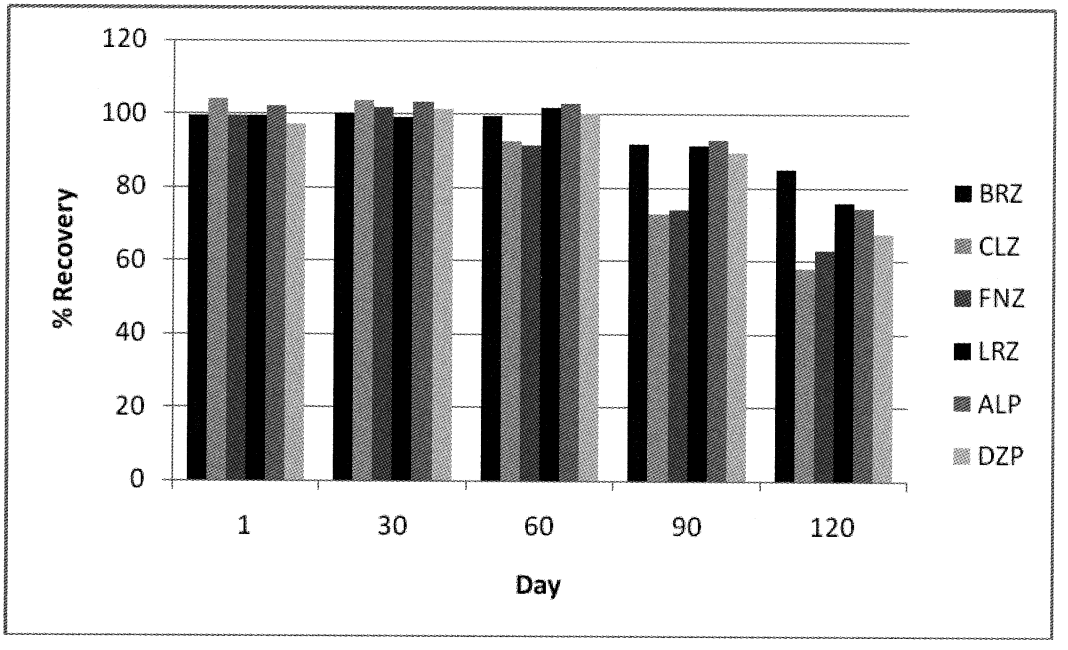


Figure 8. Long-term stability of benzodiazepines in spiked saliva sample.

under investigation, ease of extraction, and the limits of detection were considered. This study on the stability of benzodiazepines in plasma, urine, saliva clearly demonstrated that a decrease in concentration must be considered for a storage period of 6 months at -20°C. The experiments were performed using identical biological matrices. Drug concentrations chosen for spiking were at the upper limit of the therapeutic ranges [16]. There was a continuous decrease in drug concentration without a clear pattern of degradation. After 6 months, the concentrations of all analytes still detectable were at least 40% lower than in the corresponding initial sample. A similar decrease in diazepam, nordazepam and bromazepam concentration has been reported by Skopp *et al.* [10] in a study on authentic blood, plasma samples after 8 months storage at 4°C. Present study shows that after four months storage at -20°C detection level was less than 70% for saliva sample. Both flunitrazepam and clonazepam was detected less than 60%. The stability was found to be less in saliva than plasma or urine samples which was consisted with the previous reports [17].

However, before analysis analyte degradation is often a result of chemical or physical decomposition because of the drug instability which loses the protective effect by binding to plasma proteins. For example, bromazepam is susceptible to hydrolysis [18] and flunitrazepam was reported to significantly degrade in plasma within only 24 h when exposed to sunlight [19,20]. The water present in blood samples plays a very important role in hydrolysis reactions as an active reagent that cleaves drug molecules [21]. Benzodiazepines show a marked protein binding with high association constants, the proportion of drug molecules bound ranging from 77-79% for flunitrazepam to 96-99% for diazepam at therapeutic levels [18]. However, these differences in drug protein interaction did not allow establishing a clear relationship 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. The conversion of flunitrazepam to 7-aminoflunitrazepam was already reported when incubated in bacterially contaminated post-mortem blood and tissues [22]. The conversion rate of nitrazepam was strongly dependent on the composition of the subject's saliva [17]. Also, the loss of drug molecules due to adsorption to the glass vial or the rubber stopper has been observed for diazepam [23].

Kratzsch *et al.* [24] evaluated the long-term stability over 1 month at "20°C, freeze/thaw stability over three cycles of a number of benzodiazepines and their metabolites, and it might be concluded that benzodiazepines are rather stable and un-degradable analytes. However, benzodiazepines show considerable degradation to complete degradation if not stored at "20°C or lower [25-28]. Benzodiazepines and their metabolites are relatively stable species under short-term storage conditions. However, it is strongly recommended to store specimens under frozen conditions (–20°C or lower) even if blood is collected in tubes containing fluoride/oxalate [9,10, 29-32]. Freeze-thawing was found not to affect the concentration of nitrobenzodiazepine and 7-amino metabolites [22]. Both flunitrazepam and clonazepam showed less stability than remaining four as the possibility of conversion of nitro group to corresponding amino group. It is reported previously that 7-nitro benzodiazepines were unstable in blood, particularly post-mortem blood (and other tissues) due to conversion to the respective 7-amino form [33]. This can be reduced by using fluoride/oxalate but in most post-mortem cases this conversion has already occurred prior to specimen collection [34].

Further studies seem to be urgently necessary to investigate suitable sample preparation, e.g. addition of preservatives like potassium/sodium fluoride or oxalate and antibacterial agents, 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 concentration [16]. High-pressure liquid chromatography [35] 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. 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.

5. CONCLUSION

For refrigerated samples at -20°C over a period of six months, it can be concluded from the present investigations that the time interval between sampling and analysis may strongly influence the analytical results for benzodiazepines. As a consequence, results from samples stored at -20°C for some time prior to analysis should be interpreted cautiously. The present study also demonstrates that -20°C could influence the interpretation of toxicological results after prolonged storage. In case of saliva degradation is faster compared to plasma and urine. Therefore, the data obtained suggest the following: benzodiazepines 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; plasma collected for the purpose of nitro-benzodiazepine determinations should be preserved with oxalate or

fluoride, stored at -20°C and assayed soon preferably within a month of collection; a decrease between 10 and 20% should be expected when benzodiazepines should be stored at least at -20°C.

REFERENCES

[1] Kaa E. and Teige B., Drug-related deaths during the 1980s. A comparative study of drug addict deaths examined at the institutes of forensic medicine in Aarhus, Denmark and Oslo, Norway. *Int. J. Legal. Med.,* 1993; **106**: 5-9.

[2] Laux G., *Aktueller Stand der Behandlung mit Benzodiazepinen. Nervenarzt,* 1995; **66**: 311-322.

[3] Steentoft A., Worm K., Pedersen C.B., Sprehn M., Mogensen T., Soerensen M.B. and Nielsen E., Drugs in blood samples from unconscious addicts after the intake of an overdose. *Int. J. Legal. Med.,* 1996; **108**: 248-251.

[4] Le Solleu H., Demotes-Mainard F., Vincon G. and Bannwarth B., The determination of bromazepam in plasma by reversed phase high-performance liquid chromatography. *J. Pharm. Biomed. Anal.,* 1993; **11**: 771-775.

[5] Giorgi S.N. and Meeker J.E., A 5-year stability study of common illicit drugs in blood. *J. Anal. Toxicol.* 1995; **19**: 392-398.

[6] Baselt R.C., Yoshikawa D., Chang J. and Li J., Improved long-term stability of blood cocaine in evacuated collection tubes. *J. Forensic Sci.,* 1993; **38**: 935-937.

[7] Nagata T., Kimura K., Kara K. and Kudo K., Methamphetamine and amphetamine concentrations in postmortem rabbit tissues. *Forensic Sci. Int.*, 1990; **48**: 39-47.

[8] McCurdy H.H., Callahan L.S. and Williams R.D., Studies on the stability and detection of cocaine, benzoylecgonine and 11- nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid in whole blood using Abuscreen radioimmunoassay. *J Forensic Sci.,* 1989; **34**: 858-870.

[9] El Mahjoub A. and Staub C., Stability of benzodiazepines in whole blood samples stored at varying temperatures. *J. Pharm. Biomed. Anal.*, 2000; **23**: 1057-1063.

[10] Skopp G., PöWFK/ □ QL□DQG Mattern R., A preliminary study on the stability of benzodiazepines in blood and plasma stored at 4°C. *Int. J. Legal. Med.,* 1998; **111**: 1-5.

[11] Link B., Haschke M., Wenk M. and Krahenbuh S., Determination of midazolam and its hydroxyl metabolites in human plasma and oral fluid by liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. *Rapid Commun. Mass Spectrom.,* 2007; **21**: 1531-1540.

[12] Uddin M.N., Samanidou V.F. and Papadoyannis I.N., Development and validation of an HPLC method for the determination of six 1,4-benzodiazepines in pharmaceuticals and human biological fluids. *J. Liq. Chromatogr. Rel. Technol.,* 2008; **31**: 1258-1282.

[13] Miller J.C. and Miller J.N., (Eds.) *Statistics for analytical chemistry,* vol. IV, Horwood E, Chichester, 1986, p. 96.

[14] Uddin M.N., Samanidou V.F. and Papadoyannis I.N., Development and validation of an HPLC method for the determination of benzodiazepines and tricyclic antidepressants in biological fluids after sequential SPE. *J. Sep. Sci.,* 2008; **31**: 2358-2370.

[15] Uddin M.N., Samanidou V.F. and Papadoyannis I.N., Validation of SPE-HPLC determination of 1,4-benzodiazepines and metabolites in blood plasma, urine, and saliva. *J. Sep. Sci.,* 2008; **31**: 3704-3717.

[16] Reynolds J.E.F., *Martindale. The extra*

pharmacopeia. Royal Pharmaceutical Society, London, 1996.

[17] Hart B.J., Wilting J. and de Gier J.J., *Methods Find Exp Clin Pharmacol.,* 1988; **10**(1): 21-26.

[18] Damjanović T., Popović G., Verbić S. and Pfendt L., Study of acid hydrolysis of bromazepam. *Can. J. Chem.,* 2004; **82**(8): 1260-1265.

[19] Benhamou-Batut F., Demotes-Mainard F., Labat L. and Vincon G., Determination of flunitrazepam in plasma by liquid chromatography. *J. Pharm. Biomed. Anal.,* 1994; **12**: 931-936.

[20] Cornelissen P.J.G. and van Henegouwen G.M.J.B., Photochemical activity of 7-nitro-1,4-benzodiazepines. Pharm. *Pharm. Weekbl,* 1981; **3**: 800-809.

[21] Alfazil A.A. and Anderson R.A., Stability of benzodiazepines and cocaine in blood spots stored on filter paper. *J. Anal. Toxicol.,* 2008; **32**: 511-515.

[22] Robertson M.D. and Drummer O.H., Post-mortem drug metabolism by bacteria. *J. Forensic Sci.*, 1995; **40**: 382-386.

[23] Smith F.M. and Nuessle N.O., Stability of diazepam injection repackaged in glass unit-dose syringes. *Am. J. Hosp. Pharm.*, 1982; **39**: 1687-1690.

[24] Kratzsch C., Tenberken O., Peters F.T., Weber A.A., Kraemer T. and Maurer H.H., Screening, library-assisted identification and validated quantification of 23 benzodiazepines, flumazenil, zaleplone, zolpidem and zopiclone in plasma by liquid chromatography/mass spectrometry with atmospheric pressure chemical ionization. *J. Mass Spectrom.,* 2004; **39**: 856-872.

[25] Peters F.T., Stability of analytes in biosamples-an important issue in clinical and forensic toxicology? *Anal. Bioanal. Chem.*, 2007; **388**: 1505-1519.

[26] Skopp G., Pre-analytic aspects in post-mortem toxicology. *Forensic Sci. Int.*, 2004; **142**: 75-100.

[27] Drummer O.H. and Gerostamoulos J., Post-mortem **drug** analysis: analytical and toxicological aspects. *Ther. Drug Monit.,* 2002; **24**: 199-209.

[28] Drummer O.H., Post-mortem toxicology of drugs of abuse. *Forensic Sci. Int.* 2004; **142**(10): 101-113.

[29] Holmgren P., Druid H., Holmgren A. and Ahlner J., Stability of Drugs in Stored Post-mortem Femoral Blood and Vitreous Humor. *J. Forensic Sci.*, 2004; **49**(4): 820-823.

[30] Al-Hadidi K.A. and Oliver J.S., Stability of temazepam in blood. *Science & Justice,* 1995; **35**: 105-108.

[31] Konig I., Skopp G., Schmitt G. and Mattern R., Storage stability of flunitrazepam, flurazepam, diazepam and metabolites in blood and plasma. *Arch. Kriminol.,* 1997; **200**: 17-24.

[32] Winek C.L., Esposito F.M. and Cinicola D.P., The stability of several compounds in formalin fixed tissues and formalin-blood solutions. *Forensic Sci. Int.* 1990; **44**: 159-168.

[33] Robertson M.D. and Drummer O.H., Stability of nitro-benzodiazepines in post-mortem blood. *J. Forensic Sci.,* 1998; **43**: 5-8.

[34] Drummer O.H., Benzodiazepines-Effects on human performance and behaviour. *Forensic Sci. Rev.* 2002; **14**(1-2): 1-14.

[35] Musshoff F. and Daldrup T., A rapid solid phase extraction and HPLC/DAD procedure for the simultaneous determination and quantification of different benzodiazepines in serum, blood and post-mortem blood. *Int. J. Legal Med.,* 1992; **105**: 105-109.