Quantitative analysis of benzodiazepines in vitreous humor by high-performance liquid chromatography

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Abstract

Objective: Benzodiazepines are frequently screened drugs in emergency toxicology, drugs of abuse testing, and in forensic cases. As the variations of benzodiazepines concentrations in biological samples during bleeding, postmortem changes, and redistribution could be biasing forensic medicine examinations, hence selecting a suitable sample and a validated accurate method is essential for the quantitative analysis of these main drug categories. The aim of this study was to develop a valid method for the determination of four benzodiazepines (flurazepam, lorazepam, alprazolam, and diazepam) in vitreous humor using liquid–liquid extraction and high-performance liquid chromatography.

Methods: Sample preparation was carried out using liquid–liquid extraction with n-hexane: ethyl acetate and subsequent detection by high-performance liquid chromatography method coupled to diode array detector. This method was applied to quantify benzodiazepines in 21 authentic vitreous humor samples. Linear curve for each drug was obtained within the range of 30–3000 ng/mL with coefficient of correlation higher than 0.99.

Results: The limit of detection and quantitation were 30 and 100 ng/mL respectively for four drugs. The method showed an appropriate intra- and inter-day precision (coefficient of variation < 10%). Benzodiazepines recoveries were estimated to be over 80%. The method showed high selectivity; no additional peak due to interfering substances in samples was observed.

Conclusion: The present method was selective, sensitive, accurate, and precise for the quantitative analysis of benzodiazepines in vitreous humor samples in forensic toxicology laboratory.

Keywords

Benzodiazepines, vitreous humor, liquid–liquid extraction, high-performance liquid chromatography, method validation, forensic toxicology

Introduction

Benzodiazepines (BZs) are one of the most prescribed drugs used as anticonvulsant, anxiolytic, muscle relaxant, and sedative-hypnotic agents.[1] They are commonly responsible for accidental overdose, misuse, and suicide attempt especially when consumed simultaneously with other drugs such as opiates, antidepressants, and neuroleptics.[2] Therefore, it is essential to analyze BZs in forensic cases in order to establish cause of death and the magnitude of abuse problem.[3] As the concentration of BZs can be changed after death due to decomposition (e.g. bacterial enzyme activity), and redistribution phenomenon, selecting the best biological sample and efficient sample preparation methods prior to instrumental analysis is important.[4] Vitreous humor (VH) is a transparent, colorless, gelatinous mass that fills the space in the eye between the lens and the retina. It contains a network of collagen fibers with hydrophilic glycosaminoglycan hyaluronan, solids such as sugar, urea, creatinine, electrolytes, and

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98%–99% water.[5,6] VH has served as an alternative matrix in forensic toxicology due to its large volume, easy accessibility, and stability over time after death. It is protected from putrefaction and trauma, is resistant to microbiologic contamination, and less prone to postmortem redistribution. Therefore, it is a suitable sample for postmortem biochemical investigation (e.g. estimation of postmortem interval (PMI) and ethanol quantitation).[7–9]

As many BZs show time-dependent losses and their concentration are changed by postmortem enzymatic and bacterial phenomena, VH analysis for the detection and quantitation of BZs is suggested in many studies.[9–11]

High-performance liquid chromatography (HPLC) with diode array detector (DAD) is a simple, sensitive, and specific method for the analysis of BZs in both clinical and forensic toxicology laboratories, and it is used as a perfect and appropriate method for the analysis of BZs.[1,10–12]

Fernández et al.[13] described HPLC/DAD method for the determination of morphine, methadone, cocaine, and other drugs in VH using solid-phase extraction (SPE). Another autopsy study showed non-significant difference for nordazepam, bromazepam, and oxazepam between VH and blood concentrations.[14] HPLC/DAD method for the determination of six BZs using SPE was validated by Cabarcos et al. In their study, the validation parameters of extraction and detection by HPLC are satisfactory and according to real BZs concentrations in VH specimens. Although many authors considered analysis of BZs technically easier in VH than in other specimens especially in putrefaction and redistribution conditions, setting a sensitive, selective, and rapid method that is cost-efficient for their determination is still required.

Therefore, we used liquid–liquid extraction (LLE) as a commonly employed technique to remove excessive impurities, isolate drugs from VH (as a useful specimen), and HPLC-coupled DAD as a simple, specific, and sensitive method for determination of BZs.

The aim of this study was to optimize a valid method for the determination of BZs in VH using HPLC/DAD and LLE as a sample preparation method.

Methods

Chemicals and reagents

Drug standards (flurazepam, lorazepam, alprazolam, and diazepam) were purchased from Cambrex (Italy). Acetonitrile, methanol, water, sulfuric acid, n-hexane and ethyl acetate were HPLC grade and supplied by Merck (Darmstadt, Germany). NaH_2PO_4 (99.9% purity) was obtained from Sigma-Aldrich (Germany).

VH samples

VH samples were collected by aspiration with a 5-mL syringe and 20-gauge needle to avoid damage to any tissue fragments surrounding the vitreous chamber. Specimens were stored in 4°C and analyzed just during 24 h after autopsy.

Preparation of standard solution

Stock solutions of four BZs were prepared at the concentration of 1 mg/mL. Working solutions of 10 and 100 μg/mL were made. Standard calibration curves were obtained using blank VH spiked with standard solutions to obtain the concentration range of 30–3000 ng/mL (30, 50, 100, 500, 1000, 1800, 3000 ng/mL) of each drug and stored at −18°C.

Sample preparation by LLE

About 500 μL of VH sample was acidified with 500 μL (0.05 M) H_2SO_4 and analytes were extracted by adding 5 mL n-hexane: ethyl acetate (70: 30).

Samples were shaken for 5 min followed by centrifugation at 4500 r/min for 3 min. The organic layer was collected and evaporated to dryness under gentle stream of nitrogen. The extract was reconstituted with 100 μL methanol, and 30 μL was injected to chromatographic system.

Instrumentation

HPLC analysis was performed using a Knauer HPLC system (Berlin, Germany) equipped with a DAD (S 2800-4 channels). Separation of analytes was achieved by using Eurospher-100-5 C18 column (250 mm × 4.6 mm, particle size 5 μm, pore size 100°A) with a smart-1000 pump. Eurospher C18 is made of silicagel and used for reversed phase applications. Elution was performed with a mobile phase consisting of a mixture of acetonitrile and phosphate buffer (pH = 2.32) (37:63) in isocratic mode at a flow rate of 1 mL/min and 400 bar maximum pressure.

BZs were detected at 254 nm, and the purity of peaks was analyzed at 200–400 nm.

Method validation

In validation process, linearity, limit of detection (LOD), limit of quantitation (LOQ), selectivity, precision, and recovery were obtained. The standard addition method (often referred to as "spiking" the sample) was used in this study. In standard addition method, signals are measured as a function of concentration added.

Standard calibration curves for each BZ were illustrated by using blank VH spiked with standard solutions to obtain seven concentration range of 30–3000 ng/mL by triplicate measurements of drugs, and the linearity of method was evaluated.

Sensitivity of the method was determined by evaluation of LOD and LOQ. These parameters were determined by performing analyses at concentrations of interest.

Table 1. Validation parameters and ANOVA regression model for quantitative analysis of four BZs in vitreous humor.

Analyte	Calibration curve equation	r^2	ANOVA regression model	
			F ratio	p-value
Flurazepam	y = 276.83x + 2870	0.9982	2980.26	0.0
Lorazepam	y = 190.66x + 9021	0.9937	2876.38	0.0
Alprazolam	y = 136.22x − 6844	0.9989	31,508.94	0.0
Diazepam	y = 408.16x − 11,786	0.9989	3105.02	0.0

BZ: benzodiazepine; r^2: correlation of coefficient.
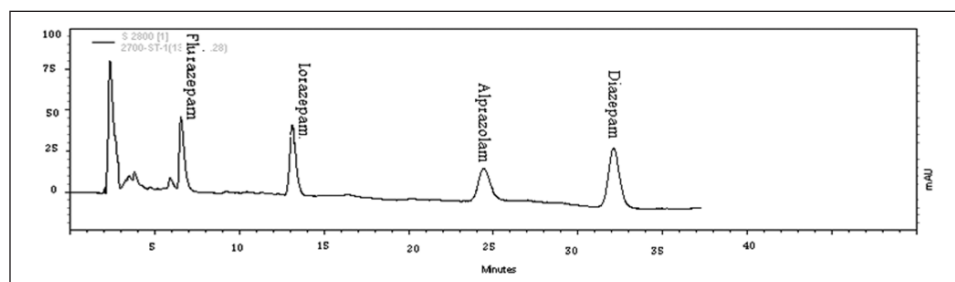
Figure 1. Chromatogram of spiked vitreous humor with 2.7 μg/mL of four benzodiazepines.

The analyte peak must be identifiable and reproducible with precision of maximum 15% (except for the LOQ, where it should not exceed 20% of the coefficient of variation (CV)) and accuracy should be 80%–120%.[15,16]

The precision of an analytical method is determined by five replicates of VH sample with three different concentrations: (low = 400, medium = 1600, high = 2700 ng/mL) on the same day (intra-day precision or repeatability) or on different days (inter-day precision or reproducibility). It has been calculated from the CV and was <10%.[16]

To determine the recovery of the extraction method, spiked VH samples with different concentrations of each drug (400, 1600, 2700 ng/mL) were analyzed using triplicate measurements. Peak areas of spiked VH samples were compared with peak areas obtained from unextracted standards at the similar concentration.

Analysis of BZs in real VH

The method was applied for the quantitative analysis of drugs in 21 authentic VH samples obtained from victims who were referred to Legal Medicine Organization of Tehran, Iran (2015–2016). Studied cases had a history of BZs abused in their death certificate and positive results for BZs using thin layer chromatography (TLC) as screening method.

Statistical analysis

SPSS statistics procedure (version 16) was used to perform one-way analysis of variance (ANOVA) to validate the regression data. Linear regression models are statistically significant when p-values are lower than 0.05.

Ethical review

According to Legal Medicine Research Center of Iran ethics Committee, the information about human cadavers was fully confidential. The study protocol conformed to the ethical guidelines of 1975 Declaration of Helsinki, as revised in 1983.

Results

This study achieved validation of a satisfactory LLE for the detection and quantitation of flurazepam, lorazepam, alprazolam, and diazepam using HPLC-DAD in VH.

Linearity

The calibration curves were linear over these levels, and correlation coefficients (r^2) were higher than 0.99 and CV < 10% for four drugs. The linear regression equation and correlation coefficient (r^2) and ANOVA regression parameter are shown in Table 1.

Detection and quantification limit

Table 1 shows validation parameters and ANOVA regression model for quantitative analysis of four BZs in VH. The LOD for flurazepam, lorazepam, alprazolam, and diazepam were 30 ng/mL. The LOQ was 100 ng/mL. The method has proved to be sensitive.

Selectivity

Figure 1 shows a chromatogram of spiked VH sample with 2.7 μg/mL of four BZs. The retention time of drugs were

Table 2. Inter- and intra-day precision and accuracy for four benzodiazepines detection in vitreous humor.

Drug	Concentration added (ng/mL)	Concentration mean ± SD	CV (%)	Accuracy (%)
Flurazepam	Inter-day (n = 5)			
	400	361 ± 41	9.35	90
	1600	1486 ± 105	7.06	92
	2700	2597 ± 182	7.06	96
	Intra-day (n = 5)			
	400	385 ± 24	6.23	96
	1600	1548 ± 119	7.68	96
	2700	2681 ± 222	8.23	99
Lorazepam	Inter-day (n = 5)			
	400	382 ± 31	8.1	95
	1600	1570 ± 101	6.4	98
	2700	2791 ± 169	9.3	100
	Intra-day (n = 5)			
	400	390 ± 35	8.9	97
	1600	1621 ± 93	5.7	101
	2700	2769 ± 158	5.7	102
Alprazolam	Inter-day (n = 5)			
	400	431 ± 43	9.8	107
	1600	1569 ± 97	6.1	98
	2700	2601 ± 183	5.1	96
	Intra-day (n = 5)			
	400	376 ± 38	8.9	97
	1600	1671 ± 99	5.7	104
	2700	2745 ± 159	5.7	101
Diazepam	Inter-day (n = 5)			
	400	362 ± 31	8.1	95
	1600	1515 ± 81	6.4	98
	2700	2809 ± 119	9.3	100
	Intra-day (n = 5)			
	400	358 ± 54	15	90
	1600	1502 ± 101	6.7	94
	2700	2561 ± 181	7.0	95

SD: standard deviation; CV: coefficient of variation.

6.5 min for flurazepam, 13.3 min for lorazepam, 24.2 min for alprazolam, and 32.4 min for diazepam. No interfering peak was observed at the BZs retention times. This validated method showed high selectivity.

Inter- and intra-day precision and accuracy

Summary of inter- and intra-day precision (CV %) and accuracy are shown in Table 2. Coefficients of variation were less than 11.5% especially when determining high concentrations. The accuracies in intra-day analyses were higher than inter-day in all drugs. Results showed that validated method was adequately precise and accurate (<15%).

Recover (extraction efficiency)

Table 3 shows satisfactory recovery results for each BZs (CV<10%). The results show that recovery ranged

Table 3. Recovery data for four benzodiazepines in vitreous humor samples.

Drugs	Extraction recoveries (%)	CV (%)
Flurazepam	82.31	9.81
Lorazepam	91.48	6.59
Alprazolam	89.93	5.43
Diazepam	83.77	4.34

CV: coefficient of variation.

from 82.31% to 91.43% and was independent of drug concentration.

Analysis of BZs in real VH

The developed method was applied to analysis of 21 VH samples that were taken from BZs consumers to demonstrate the presence of this class of drug. Analysis of BZs in real VH was examined on the calibration curve of spiked VH by standard solutions. The BZ concentrations in authentic samples are shown in Table 4. Results showed that flurazepam was the most frequently detected, followed by diazepam, alprazolam, and lorazepam.

Discussion

The aim of this study was to validate a sensitive, accurate, and selective method to determine four BZs in VH using LLE and HPLC/DAD.

BZs are frequently used or misused, either as a single substance exposure or in combination with other substances.[1,3,17] Also, BZs are categorized as controlled substances and drugs in Iran. Therefore, detection and analysis of these controlled drugs is an important task in clinical and forensic toxicology cases. Several analytical techniques have already been published for the isolation and quantitation of BZs in biosamples.[1,3,17–19] Many BZs are detected in biological matrices obtained at autopsy examination in Iran. In this study, only flurazepam, lorazepam, alprazolam, and diazepam were analyzed because they are the most commonly found BZs in biological matrices in Forensic Toxicology Laboratory, Tehran, Iran. Loss or degradation of BZs in biological samples during bleeding, burning, postmortem changes, and redistribution could be biasing forensic analytical toxicology results.

VH is a relatively simple matrix, easily accessible, and clean in comparison to other autopsy matrices. It is one of the samples that require simple preparation process before toxicology analysis.

A biochemical analysis of VH assists in estimating PMI and determining cause of death (e.g. testing glucose levels of diabetic-related as well as alcohol- and drug-related fatalities).[5,9,20–22]

There are many reports about correlation of BZ concentration in VH and other biological samples for forensic interpretations. Robertson and Drummer[23] had used simple and sensitive

Table 4. The concentrations of benzodiazepines in real vitreous humor samples.

Number	Analyte	Concentration (ng/mL)
1	Flurazepam	515.75
2	Diazepam	275.21
3	Alprazolam	1868.28
4	Flurazepam	122.4
5	Flurazepam	2473.65
6	Flurazepam	2730.13
7	Diazepam	118.92
8	Flurazepam	1787.32
9	Alprazolam	809.56
10	Diazepam	2557.3
11	Flurazepam	1884.05
12	Flurazepam	276
13	Lorazepam	1076.97
14	Alprazolam	1697.07
15	Alprazolam	168.18
16	Diazepam	1839.12
17	Alprazolam	291.52
18	Diazepam	115.71
19	Flurazepam	811.99
20	Diazepam	467.51
21	Diazepam	128.69

Conc: Concentration.

HPLC method using LLE for the determination of many BZs and reported r=0.626 for nitro-BZs (nitrazepam, flunitrazepam, and clonazepam) and r=0.746 for their metabolites in blood and VH. Scott and Oliver[24] evaluated confidence of determination (r^2) of 0.788 for temazepam and 0.723 for diazepam and 0.068 for nordazepam in the similar samples. In another study, Teixeira et al.[25] found a 10 times lower concentration of diazepam in VH compared to plasma between 1 and 2 h after death because VH is a matrix protected against postmortem redistribution from abdominal cavity. In animal study of Bevalot et al.,[26] no significant correlation between VH and blood concentrations was observed for diazepam(r=0.512).

The influence of several parameters such as intake-to-death interval, time to autopsy, temperature, and light exposure for determination of BZs levels still remain controversial in several studies. Rowshan[3] reported that BZs are relatively resistant to postmortem redistribution effect and their concentrations don't change after death. Melo P et al. believed that concentration of BZs can be influenced by factors like time between death and autopsy and time of storage. They demonstrated that negative temperature causes suitable stability of BZs, and VH is better sample for quantitation of these drugs.[4] In another study, diazepam level in VH at 24 h after death was higher than at the time of death.[25] Bevalot et al.[14] recorded that the interval between intake and death may vary greatly according to the circumstances of death that are rarely known.

Due to some physicochemical properties such as neutral or weak acidic chemical structure and high protein binding,

BZs have low diffusion rate into VH. Therefore, it seems that quantitative interpretation of these drugs in VH represents a wide scatter in the result and cannot consist in straightforward extrapolation of blood or urine concentration.

In this study, we developed HPLC/DAD method as a reference and acceptable method for the determination of BZs. Validation parameters results in this research were in accordance with the results of other studies.[1,2,10,12,14,15]

This study is a simple, rapid, inexpensive procedure that can be used in forensic toxicology laboratories to determine cause of death. These are advantages of our proposed method over the other methods.

BZs have tertiary amines in their structure and are chemically unstable under strong alkaline conditions and high temperature; therefore, HPLC combined with DAD has been proposed for their analysis due to mild analytical conditions used in this instrumentation (low temperature and pH).[1,11,27]

In our study, significant differences in retention time of four BZs allow their detection potentially.

Several methods have been developed for the extraction of BZs from biological samples.[2,10–13]

Magalhães et al.[28] used an innovative extraction–clean up procedure, namely, LLE with low-temperature partitioning (LLE-LTP) and analysis by liquid chromatography combined with high-resolution mass spectrometry (LC-HRMS).

In our research, BZs' extraction from VH was carried out with a mixture of n-hexane:ethyl acetate as extraction solvent to obtain higher recovery and less interfering peaks. Extraction by adding of sulfuric acid to samples can avoid the loss of drugs. No internal standard and high temperature is needed in this method. Plasma and urine samples were successfully extracted using this extraction method in other studies.[29,30]

The recoveries of four BZs ranged from 84% for flurazepam to 91.41% for lorazepam (CV<10%). The efficiency of the extraction procedure for BZs was comparable to other studies.[17,31,32] Fernández et al.[13] reported that average recoveries for BZs were higher than 78% in their study except for EDDP (methadone metabolite) with a value of 66.4%. Data obtained from linearity test showed coefficient of correlation higher than 0.99 for four drugs, and ANOVA was performed to validate the regression data (p value<0.05).

The LOD in this study was 30 ng/mL for four BZs that is comparable to those published by Cabarcos et al.[10] Several authors evaluated different LOD for BZs but their results cannot be compared with our findings due to analysis in different matrices using other methods.[12,33,34]

The intra- and inter-day validation parameters showed satisfactory results with coefficient of variation less than 10%. These values are similar to those reported by Fernández et al.[31,35]

Analysis of 21 real VH samples showed that flurazepam was the most frequently detected BZs. In the study of Scott and Oliver,[24] the concentrations of drugs found for BZs

varied from 0.10 to 6.84 µg/mL that were within the toxic levels and can be the cause of death as described by other studies.[13]

Comparison between BZ levels detected by other authors with this study is difficult due the difference between the ranges of extracted drugs and the method used. Therefore, an experimental approach in a significant autopsy population, with the aim of comparison blood, tissue, and bile levels and known uncertainly based on VH concentrations, would be recommended.

We faced some limitations in performing this study. There is no VH in all cadavers that referred to Legal Medicine center, for example, in death to head injury and burning. Therefore, we were unable to take VH samples from all cases and this caused long time to gather enough samples.

Conclusion

The results of our study demonstrated the suitability of VH as an alternative biological matrix for the detection and quantitation of BZs using the extraction procedure (LLE) and HPLC-DAD method.

Validated method showed satisfactory results, and good absorption spectra and retention time. The application of the method to real samples shows that we can use VH in forensic toxicology laboratories for routine case work to determine cause of death in victims.

References

1. Toyo'oka T, Kumaki Y, Kanbori M, et al. Determination of hypnotic benzodiazepines (alprazolam, estazolam, and midazolam) and their metabolites in rat hair and plasma by reversed-phase liquid-chromatography with electrospray ionization mass spectrometry. *J Pharmaceut Biomed* 2003; 30(6): 1773–1787.
2. Anderson RA, Ariffin MM, Cormack PA, et al. Comparison of molecularly imprinted solid-phase extraction (MISPE) with classical solid-phase extraction (SPE) for the detection of benzodiazepines in post-mortem hair samples. *Forensic Sci Int* 2008; 174(1): 40–46.
3. Rowshan H. Postmortem detection of benzodiazepines. *Open Sci J Pharm Pharmacol* 2014; 2(1): 1–8.
4. Melo P, Bastos ML and Teixeira HM. Benzodiazepine stability in postmortem samples stored at different temperature. *J Anal Toxicol* 2012; 36(1): 52–60.
5. Bévalot F, Cartiser N, Bottinelli C, et al. Vitreous humor analysis for the detection of xenobiotics in forensic toxicology: a review. *Forensic Toxicol* 2016; 34: 12–40.
6. Mulla A, Lorne MK and Kalra J. Vitreous humor biochemical constituents. *Am J Forensic Med Pathol* 2005; 26(2): 146–149.
7. Baniak N, Campos-Baniak G, Mulla A, et al. Vitreous humor: a short review on post-mortem application. *J Clin Exp Pathol* 2015; 4(6): 1–7.
8. Gagajewski A, Murakami MM, Kloss J, et al. Measurement of chemical analytes in vitreous humor: stability and precision studies. *J Forensic Sci* 2004; 49(2): 371–374.
9. Bazmi E, Behnoush B, Akhgari M, et al. Interpretation of ethanol analysis in postmortem specimens. *Iran J Toxicol* 2008; 2(1): 2–7.
10. Cabarcos P, Tabernero MJ, Álvarez I, et al. Analysis of six benzodiazepines in vitreous humor by high-performance liquid chromatography-photodiode-array detection. *J Anal Toxicol* 2010; 34(9): 539–542.
11. Fernández P, Lago M, Alvarez I, et al. Chromatographic determination of benzodiazepines in vitreous humor after microwave-assisted extraction. *Anal Method* 2013; 5: 4999–5004.
12. Fernández P, Vazquez C, Lorenzo RA, et al. Development of a liquid chromatographic method for the simultaneous determination of six benzodiazepines in human plasma after solid-phase extraction. *Anal Lett* 2010; 43(6): 1075–1084.
13. Fernández P, Seoane S, Vázquez C, et al. Chromatographic determination of drugs of abuse in vitreous humor using solid-phase extraction. *J Appl Toxicol* 2013; 33(8): 740–745.
14. Bevalot F, Fanton L, Maujean G, et al. Correlation study between blood concentrations and vitreous concentrations; case of meprobamate and some benzodiazepines (bromazepam, nordazepam, oxazepam). In: *Proceedings of the 60th Anniversary scientific meeting of American academy of forensic sciences*, Washington, DC, 18–23 February 2008, p. 418. American Academy of Forensic Sciences (AAFS).
15. Bressolle F, Bromet-Petit M and Audran M. Validation of liquid chromatographic and gas chromatographic methods. Applications to pharmacokinetics. *J Chromatogr B Biomed Appl* 1996; 686(1): 3–10.
16. Pushpa Latha E and Sailaja B. Bioanalytical method development and validation by HPLC: a review. *J Med Pharm Innov* 2014; 1(6S): 1–9.

17. Laloup M, Ramirez Fernandez Mdel M, De Boeck G, et al. Validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of 26 benzodiazepines and metabolites, zolpidem and zopiclone, in blood, urine, and hair. *J Anal Toxicol* 2005; 29(7): 616–626.

18. Szaltowska P, Koba M, Kosliuski P, et al. Analytical methods analysis for determination of benzodiazepines. A short review. *Cent Eur J Chem* 2014; 12(10): 994–1007.

19. Uddin MN, Samanidou VF and Papadoyannis LN. Stability study of six 1,4 benzodiazepine in bio-fluids stored at −20°C. *Chiang Mai J Sci* 2010; 37(3): 451–463.

20. Chandrakanth HV, Kanchan T, Balaraj BM, et al. Postmortem vitreous chemistry—an evaluation of sodium, potassium and chloride levels in estimation of time since death (during the first 36 h after death). *J Forensic Leg Med* 2013; 20(4): 211–216.

21. Jashnani KD, Kale SA and Rupani AB. Vitreous humor: biochemical constituents in estimation of postmortem interval. *J Forensic Sci* 2010; 55(6): 1523–1527.

22. Palmiere C and Mangin P. Postmortem chemistry update part I. *Int J Legal Med* 2012; 126(2): 187–198.

23. Robertson MD and Drummer OH. Postmortem distribution and redistribution of nitrobenzodiazepines in man. *J Forensic Sci* 1998; 43(1): 9–13.

24. Scott KS and Oliver JS. The use of vitreous humor as an alternative to whole blood for the analysis of benzodiazepines. *J Forensic Sci* 2001; 46(3): 694–697.

25. Teixeria HM, Reis F, Proenca P, et al. Vitreous humour as a complementary sample to blood for the detection/confirmation of diazepam: ante-mortem and post-mortem studies in an animal models. *Hum Exp Toxicol* 2004; 23: 571–574.

26. Bévalot F, Cartiser N, Bottinelli C, et al. Correlation of bile and vitreous humor concentrations with blood drug concentrations for forensic interpretation: a comparative study between animal experimental and human postmortem data. *Forensic Toxicol* 2015; 33(1): 131–140.

27. Griffith S and Jody L. *An investigation of the use of alternative matrices in clinical and forensic toxicology*. M.Phil. Thesis, University of Birmingham, Birmingham, 2013.

28. Magalhães EJ, Nascentes CC, Augusti R, et al. Fast determination of benzodiazepines in human urine via liquid-liquid extraction with low temperature partitioning and LC-HRMS. *Am J Anal Chem* 2012; 3(2): 118–124.

29. He W, Parissis N and Kiratzidis T. Determination of benzodiazepines in forensic sample by HPLC/DAD. *J Forensic Sci* 1998; 43(5): 1061–1066.

30. Elliott SP and Hale KA. Applications of an HPLC-DAD drug-screening system based on retention indices and UV spectra. *J Anal Toxicol* 1998; 22: 279–289.

31. Fernández P, Aldonza M, Bouzas A, et al. GC-FID determination of cocaine and its metabolites in human bile and vitreous humor. *J Appl Toxicol* 2006; 26: 253–257.

32. Drummer OH. Requirements for bioanalytical procedures in postmortem toxicology. *Anal Bioanal Chem* 2007; 388(7): 1495–1503.

33. Pelander A, Ristimaa J and Ojanpera I. Vitreous humor as an alternative matrix for comprehensive drug screening in postmortem toxicology by liquid chromatography-time-of-flight mass spectrometry. *J Anal Toxicol* 2010; 34: 312–318.

34. Kintz P, Villain M, Cheze M, et al. Identification of alprazolam in hair in two cases of drug-facilitated incidents. *Forensic Sci Int* 2005; 153(2–3): 222–226.

35. Fernández P, Rodriguez P, Bermejo AM, et al. Simultaneous determination of cocaine and benzoylecgonine in vitreous humor by HPLC. *J Liq Chromatogr* 1994; 17: 883–890.