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Simultaneous analysis of eight benzodiazepines in blood and urine matrix by gas chromatography–mass spectrometry: Implications for air crash investigation

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ABSTRACT

Introduction: Benzodiazepines are the most commonly prescribed class of drugs in India and are capable of impairing the performance of an aviator in therapeutic to subtherapeutic levels. Detection of benzodiazepines, particularly in blood, is not easy, since the concentrations present, especially following prescribed medical use, can be very low. Several publications have addressed estimation of benzodiazepines in plasma or serum; however, few have attempted their detection in whole blood. Urine, although a better specimen, benzodiazepines due to their extensive metabolism, its metabolites are excreted in urine instead of the parent compounds.

Materials and Methods: In our laboratory, a method was developed for simultaneous detection and quantification of eight benzodiazepines in whole blood and urine matrix by gas chromatography–mass spectrometry selective ion monitoring (SIM) method. Chromatographic separation was optimized and achieved for separation of all 8 compounds using Agilent DB-5MS column. Retention time, selectivity and sensitivity were achieved by measuring each analyte in SIM mode. The developed method was tested and validated on actual biological samples for lorazepam, temazepam, diazepam, clonazepam, and nitrazepam.

Conclusion: A single method was developed for the detection and quantification of eight benzodiazepines in whole blood and urine matrix by GC–MS SIM method. The method was also tested on limited number of actual biological samples for the lorazepam, temazepam, diazepam, clonazepam, and nitrazepam.

Keywords: Benzodiazepines, Gas chromatography–mass spectrometry, Air crash investigation

INTRODUCTION

Benzodiazepines are the most commonly prescribed class of drugs having a history of abuse primarily due to their extensive potential for abuse. Benzodiazepines mainly come under the category of central nervous system (CNS) depressants.[1,2] Therapeutically benzodiazepines are used to produce sedation, induce sleep, relieve anxiety/panic disorders and muscle spasms, and to prevent seizures by slowing the CNS.[3-5] The side effects of benzodiazepines include drowsiness, dizziness, decreased alertness, and/or memory loss, all of which can lead to diminished ability to properly control an aircraft.[6,7] Further benzodiazepines when used along with other drugs or alcohol, the performance impairing capacity of these medication increases. Determination of these medications in the autopsy specimens of aircraft accident victims is an important part of toxicological screening can help in determining the cause of the accident and potentially result in serious legal consequences.

Detection of benzodiazepines, particularly in blood, is not without difficulty, since the concentrations present, especially following prescribed medical use, can be low. Several publications have addressed the issue of their analysis in plasma or serum; however, few have attempted the detection in whole blood. Gunnar *et al*.[8] determined several benzodiazepines in whole blood using extraction, derivatization, and gas chromatography–mass spectrometry (GC–MS) analysis. Urine was found to be a better specimen for analysis of benzodiazepines than blood; however, benzodiazepines are known to undergo extensive metabolism and many a times, instead of the parent compound, its metabolites are excreted in urine.[9]

An aircraft accident investigation is the unique and important role of the Department of Aviation Pathology and Toxicology at Institute of Aerospace Medicine as this department is a nodal center in India for conducting air crash investigation of all fatal air crashes from both civil and service. As blood samples collected post-crash are usually whole blood and urine, the present study was taken up with the broad objective to develop a GC–MS based detection tool for estimation of commonly used benzodiazepines in whole blood and urine so that it can be adapted in toxicological screening of autopsy specimens of fatal air crashes.

MATERIALS AND METHODS

Benzodiazepine multicomponent mixture-8 solution (250 µg/mL each component in acetonitrile, ampule of 1 mL) from Cerilliant sigma (#B-033) which is a certified reference material grade for routinely tested benzodiazepines (alprazolam, clonazepam, diazepam, flunitrazepam, lorazepam, nitrazepam, oxazepam, and temazepam) was used as a standard in the present this study. This multicomponent mix is used for development of instrument method, sample preparation method, and preparation of matrix standards in blood and urine for linearity check and quantitative application. Agilent DB-5MS column and Agilent 7890 A GC and 5975 C mass selective detector (MSD) were used in the present study to develop a GC–MS selective ion monitoring (SIM) method for eight benzodiazepines. All solvents were of high-performance liquid chromatography (HPLC) grade or better; all reagents were of the American Chemical Society grade and purchased from Sigma Aldrich.

The standard operating procedures were used to develop a GC–MS method for benzodiazepines. Briefly, determination of retention time (Rt) and identification of candidate SIM ions, for all the compounds was the first and utmost important step in method development. 100 ng of multicomponent drug standard was injected and scanned from 100 to 600 amu on DB-5MS column for checking the spectrum of the compounds planned to analyze by SIM. All the peaks were characterized, Rt for each compound/peak noted. Two ions (Target and Q1) for each compound were determined by analyzing a standard in full scan mode following the procedure outlined by the instrument manufacturer. Ions (Target and Q1) for each compound were selected by examining the spectrum for candidate SIM ions, the ions that are unique to the compound, higher in mass and abundant are selected and tabulated. The details of the scan mode method were used to set up a SIM method for benzodiazepines.

GC and MS conditions standardized for the GC–MS SIM method are as below:

GC: Agilent 7890 A with autoinjector and tray
Inlet: EPC split/splitless
Mode: Constant pressure
Injection type: Splitless
Injection volume: 2.0 µl
Inlet temperature: 280°C
Septum purge flow: 3 ml/min
Purge flow to split vent: 50 ml/min for 0.4 min
Gas type: Helium
Oven
Equilibration time: 0.5 min
Initial oven hold: 100°C for 0.25 min
Ramp rate: 40°C/min
Final temp: 325°C
Run time: 9 min
Column: DB-5MS
MSD type: Agilent 5975
Mode: SIM
Solvent delay: 4 min
Quad temperature: 180°C
Source temperature: 280°C
Transfer line temperature: 300°C

After categorizing the method for separating all 8 compounds on Agilent DB-5MS column, we went ahead with developing a method to extract these drugs from whole blood and urine. A series of calibration solutions were prepared in blood/urine of healthy persons with no previous history of consumption of medication by spiking benzodiazepine multicomponent mix stock solution. Briefly, 500 µL of human blood/urine was spiked with different volume of multicomponent mix stock solution (250 µg/mL each component in acetonitrile, ampule of 1 mL) to prepare 250 ppb, 500 ppb, and 750 ppb final concentration of the drugs in blood/urine matrix calibration standards in a microfuge tube. Blanking was done with 500 µL of blood/urine having no previous history of medication. Bond Elut Captiva ND Lipid from Agilent (P/N: A5300635) along with Vac Elut 12 Manifold from Agilent (P/N: 5982–9110) was used for solid-phase extraction. All the matrix match standards and blank were processed by a simple protein precipitation method adopted in the laboratory. In brief, 1500 µl of acetonitrile was added to 500 µl of blood or urine which spiked with known concentration of

standards drug mix and vertexed for 30 s. The supernatant was transferred to Bond Elut Captiva ND Lipid cartridges after centrifuge at 6000 rpm for 5 min at 8°C. The vacuum was applied and the elute collected was vacuum concentrated by nitrogen concentrator. The vacuum concentrated samples were reconstituted with 150 µl of toluene and injected into system. The developed method was also tested on limited number (15) of actual blood and urine samples of persons visited Psychiatry Department, CHAF, Bengaluru, Karnataka, India. The details of the medication including dose were not provided.

RESULTS

Chromatographic separation was optimized and achieved for all the eight target compounds using Agilent DB-5MS column. Rt, selectivity, and sensitivity were achieved by measuring each analyte in SIM mode [Table 1]. A simple protein precipitation method was used to extract target compounds from blood/urine samples. This method was quick and amenable to clinical research. The optimal separation of the selected compounds was achieved within a run time of <7 min [Figure 1]. All calibration curves were generated with linear curve fitting and were weighed (1/x). A linear dynamic range of 250–750 ng/mL was achieved for all the analytes with an R^2 value >0.9 [Figure 2].

Table 1: Details of benzodiazepines, retention time, detection window, target ion, and Q1 ion standardized for gas chromatography–mass selective detector selective ion monitoring method.

Compound	Rt	Detection window	Targetion	Q1
Oxazepam	4.552	4.052–5.052	77	269
Lorazepam	4.742	4.242–5.242	239	274
Diazepam	4.802	4.302–5.302	256	283
Temazepam	5.149	4.649–5.649	271	300
Flunitrazepam	5.193	4.693–5.693	312	285
Nitrazepam	5.514	5.014–6.014	253	280
Clonazepam	5.682	5.182–6.182	280	286
Alprazolam	5.881	5.381–6.381	279	204

The standard curve thus obtained revealed a working range of 250–750 ppb. The cyclic voltammetry was >0.9 with sensitivity of 250 ppb for all compounds. Recovery study revealed a recovery of 80–90% for all drugs. Repeatability study also revealed similar Rt, peak shape, and response after six injections. The specificity of the developed method was attributed to the Rt, product ions, and ion ratio.

Attempt to test the developed method on actual patient samples on benzodiazepines from the psychiatry ward of CHAF Bengaluru resulted in detection and estimation of only few benzodiazepines such as lorazepam, temazepam, diazepam, clonazepam, and nitrazepam among the samples tested.

DISCUSSION AND CONCLUSION

Benzodiazepines remain one of the most widely prescribed classes of drugs used to manage anxiety, insomnia, seizures, muscle relaxation, and for management of other conditions. Benzodiazepines are also frequently encountered in forensic toxicology.

Benzodiazepines have many adverse effects, all of which would be incongruent with the operation of an aircraft. In combination with other drugs that depress the CNS, such as alcohol, this effect is even more harmful due to their synergistic effect.[6,7,9,10]

Civil Aerospace Medical Institute (CAMI), Federal Aviation Administration studied benzodiazepine use in pilots of civil aviation accidents from 1990 to 2008. The study revealed that of 6062 fatal aviation accident cases received at CAMI, and 96 (~1.6%) pilots were found positive for a benzodiazepine. In ~74% of pilots found positive for benzodiazepine(s), more than one additional compound was often present.[11]

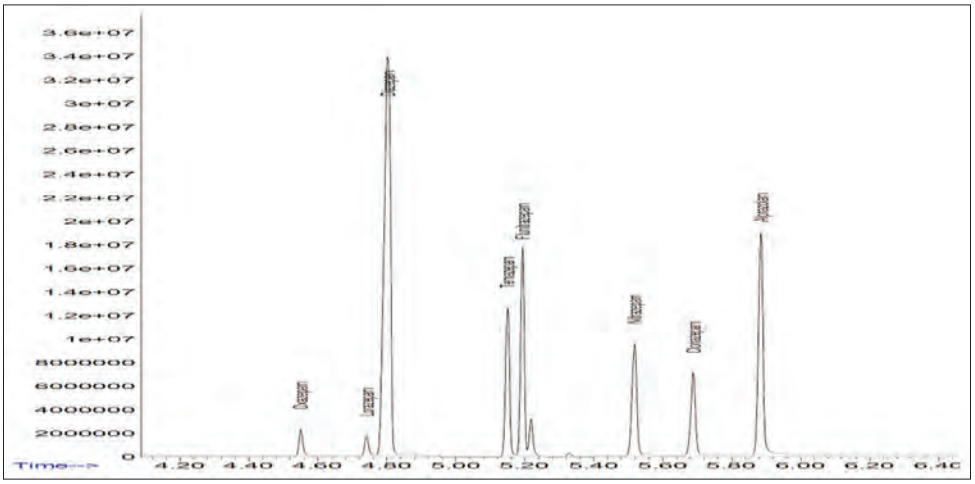

Figure 1: Total ion chromatogram showing separation of all eight benzodiazepines.

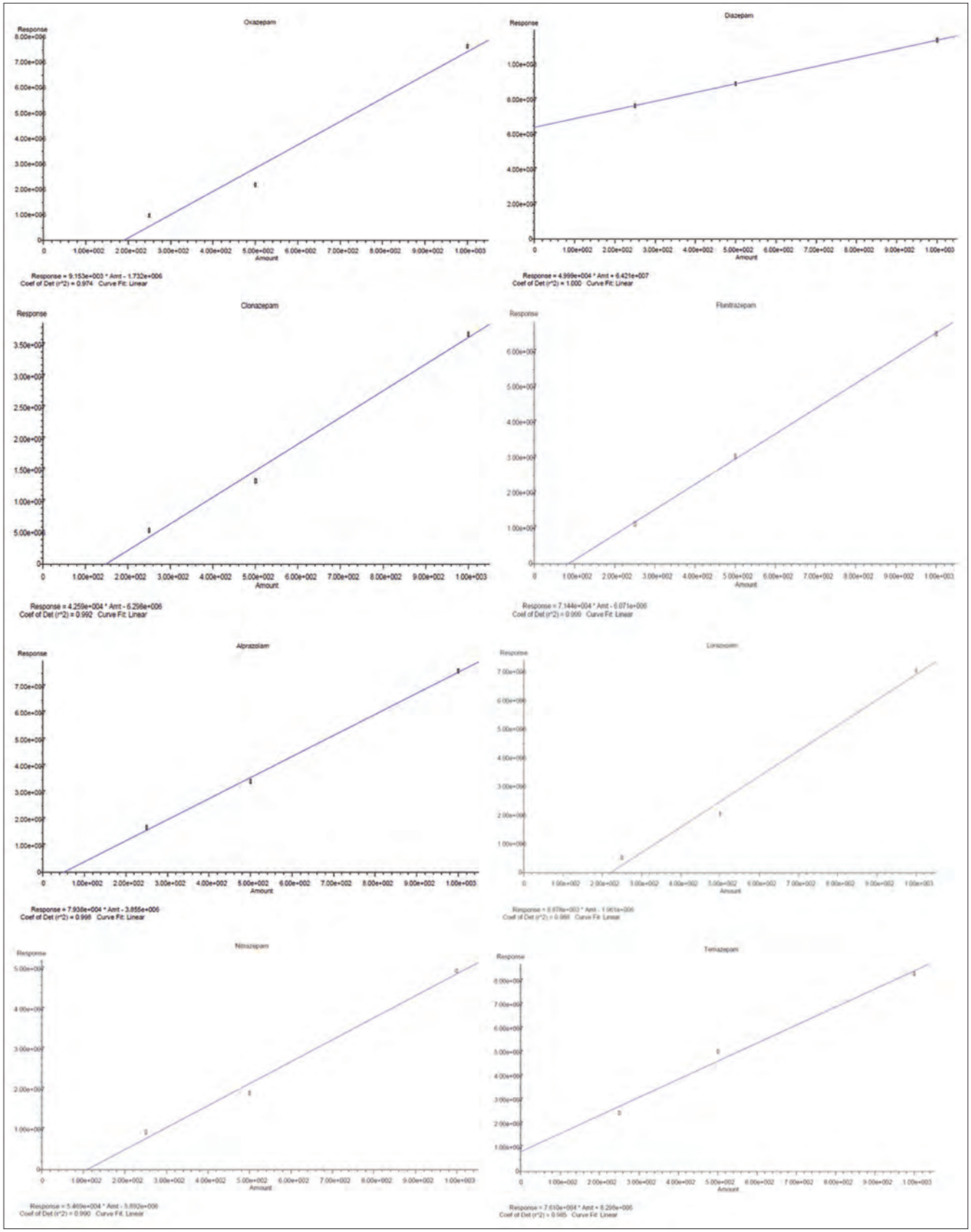

Figure 2: Standard curves generated for all the eight benzodiazepines.

There are various methods for determination of benzodiazepines in blood and urine, some laboratories use immunoassay screen for detecting the presence of benzodiazepines; radioimmunoassay, latex agglutination, and enzyme-linked immunosorbent assay. The screening methods have high incidence of false-positive and false-negative reports, which is a sensitive issue like aircraft accident investigation would give rise to various complications. Therefore, confirmation of screening techniques is required to assist in developing a definitive clinical diagnosis. A number of confirmatory techniques are widely used for benzodiazepines, such as HPLC, followed by ultraviolet detection, GC–MS, or GC-electron capture detection.[9]

Detection of benzodiazepines, particularly in blood, is not easy, since the concentrations present, especially following prescribed medical use, can be very low. Several publications have addressed estimation of benzodiazepines in plasma or serum; however, few have attempted their detection in whole blood.[8] Urine although a better specimen for analysis of benzodiazepines; benzodiazepines are known to undergo extensive metabolism and many a times, instead of the parent compounds, its metabolites are excreted in urine.[9] In our laboratory, a single method was developed for detection and quantification of eight benzodiazepines in whole blood and urine matrix by GC–MS SIM method. Chromatographic separation was optimized and achieved for all the 8 target compounds using Agilent DB-5MS column. Rt, selectivity, and sensitivity were achieved by measuring each analyte in SIM mode.

Several laboratories have developed confirmatory methods for determination of benzodiazepines from biological samples by adopting different advanced analytical tools.[2,9-12] Some of the laboratories developed a method for quantification of benzodiazepines in whole blood by electron impact – GC–MS with sensitivity of 50 ng/ml.[10] Other laboratories have developed methods using HPLC, GC–MS and recently even learning content management systems platforms also used with sensitivity ranging from 50 ng/ml to 200 ng/ml.

The present study developed a method for determination of eight benzodiazepines in blood and urine matrix using GC–MS with sensitivity of 250 ng/ml. The sensitivity of the developed method is slightly less compared to some of the previous developed methods. The present method used simple sample preparation without derivatization of the analytes. The previous published methods adopted a separate derivatization process to enhance sensitivity. However, when the method was validated on actual biological samples of patients visiting Psychiatry Department, CHAF, Bengaluru, it resulted in successful detection of lorazepam, temazepam, diazepam, clonazepam, and nitrazepam from blood and urine samples. All the benzodiazepines could not be studied on actual patient samples due to paucity of specimens with known history of consumption of the above benzodiazepines as well as extensive metabolism of the parent compounds in the body. The details of the medication and the dose were also not there to cross-check the results. The method developed in the present study is truly based on molecular ions specific to drugs and their Rt in the column in which the method was standardized suggesting no scope for false positivity. The method developed in the present study can be further improved by adopting derivatization process to enhance the sensitivity and by analyzing metabolites of benzodiazepines like midazolam in the panel, which are bound to be present due to extensive metabolism of parent compounds.

The unique about the present study is the methodology adopted certain steps which are special like in the present study eight different benzodiazepines are analyzed simultaneously in blood and urine matrix successfully. The second important thing about the study is that we have successfully developed and adopted a simple sample preparation tool for blood matrix, very few studies attempted analysis of benzodiazepine in blood, which is difficult. The third uniqueness of the study is that the method for blood and urine developed without laborious derivatization step and still achieving the required sensitivity, which makes the method simple.

In the present study, a single method was developed for the detection and quantification of eight benzodiazepines in whole blood and urine matrix by GC–MS SIM method. The developed method adopted a simple sample preparation step without sample derivatization. Chromatographic separation was optimized and achieved for all the eight target compounds using Agilent DB-5MS column. The developed method was also successfully tested on limited number of actual biological samples with history of taking drugs successfully for the lorazepam, temazepam, diazepam, clonazepam, and nitrazepam.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Drugs of Abuse, A DEA Resource Guide. 2017 edition. Available from: http://www.Justice.Gov/Dea/Concern/Benzodiazepines. [Last accessed on 2010 Feb].
2. Hegstad S, Øiestad EL, Johansen U, Christophersen AS. Determination of benzodiazepines in human urine using solid-phase extraction and high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. J Anal Toxicol 2006;30:31-7.
3. Fenton JJ. Toxicology: A Case Oriented Approach. Boca Raton,

FL: CRC Press; 2002.
4. Hoffman EJ, Mathew SJ. Anxiety disorders: A comprehensive review of pharmacotherapies. Mt Sinai J Med 2008;75:248-62.
5. Lader M. Effectiveness of benzodiazepines: Do they work or not? Expert Rev Neurother 2008;8:1189-91.
6. Mills KC, Spruill SE, Kanne RW, Parkman KM, Zhang Y. The influence of stimulants, sedatives, and fatigue on tunnel vision: Risk factors for driving and piloting. Hum Factors 2001;43:310-27.
7. Vgontzas AN, Kales A, Bixler EO. Benzodiazepine side effects: Role of pharmacokinetics and pharmacodynamics. Pharmacology 1995;51:205-23.
8. Gunnar T, Ariniemi K, Lillsunde P. Determination of 14 benzodiazepines and hydroxy metabolites, zaleplon and zolpidem as tert-butyldimethylsilyl derivatives compared with other common silylating reagents in whole blood by gas chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2005;818:175-89.
9. Valentine JL, Middleton R, Sparks C. Identification of urinary benzodiazepines and their metabolites: Comparison of automated HPLC and GC-MS after immunoassay screening of clinical specimens. J Anal Toxicol 1996;20:416-24.
10. Tiscione NB, Shan X, Alford I, Yeatman DT. Quantitation of benzodiazepines in whole blood by electron impact-gas chromatography-mass spectrometry. J Anal Toxicol 2008;32:644-52.
11. Sabra R, Botch RD, Jhonson EM, Ricaurte MS. Benzodiazepine Use in Pilots of Civil Aviation Accidents: 1990-2008 Toxicology and Autopsy Findings. CAMI, FAA Report. DOT/FAA/AM-11/12; 2011.
12. Sauve EN, Langødegård M, Ekeberg D, Øiestad AM. Determination of benzodiazepines in ante-mortem and post-mortem whole blood by solid-supported liquid-liquid extraction and UPLC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci 2012;883-884:177-88.

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