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Recovery and Analysis of Diazepam from dried bloodstains

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Abstract

This study aims to determine whether it is possible to detect diazepam in bloodstains dried on a glass surface, to assess stability of diazepam in bloodstains over several days and lastly to determine whether dried bloodstains can give reliable quantitative information. One millilitre of blood fortified with different concentrations of diazepam (1, 5, 10, 50 µg/ml) was spotted on a glass surface and allowed to dry at room temperature. Liquid-liquid extraction (LLE) was completed and 100µL of internal standard (flurazepam) was added to the sample, vortexed and allowed to equilibrate for 15 min followed by the addition of 1ml of carbonate-bicarbonate buffer. Diazepam was extracted with 1ml of toluene: heptane (9:1, v/v) and centrifuged for 10min at 4500 rpm. The organic layer collected was analysed by gas chromatography mass spectrometry (GC/MS) using selective ion monitoring (SIM) mode.

The method was checked for linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, extraction recovery and stability. Good linearity was obtained between 1-100 µg/ml (r^2=0.9983) for diazepam in dried bloodstains. LOD and LOQ in bloodstains were 0.5-1 µg/ml and 1 µg/ml respectively. Interday precision was 2-10% and accuracy ranged from -66.3% to -39.3%. A scatter plot showed results obtained from dried bloodstains and whole blood was comparable. However, stability study conducted on five days showed that diazepam was not stable in dried bloodstains since an apparent decrease in the measured concentration of diazepam was observed on each day. It was shown that detection of diazepam in dried bloodstains was possible. Stability study showed that diazepam was not stable in dried bloodstains. Therefore, toxicological analysis of diazepam in bloodstains can assist the court based on qualitative information but not on quantitative information since measured concentration varied on different days. Toxicological analysis of dried bloodstains can be important for the police and lawyers since it can help to determine the chronology of events in a crime and helped the court to reconstruct that crime. The new ideas raised in this study compared to other studies are: glass used as a substrate, diazepam drug, stability and recovery study were assessed on 5 days.

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Keywords

Diazepam, dried bloodstains, stability, LLE, GC/MS, toxicological analysis, chronology

1. Introduction

Dried Blood Spot (DBS) method was first introduced by Ivar Bang in 1913 to determine blood glucose in rabbits and in 1963, Guthrie and Susi were the first to use this method on human blood to detect phenylketonuria in newborns. Recently, DBS method has become well-known not only in newborn screening but also for the analysis of DNA, proteins and small molecules and even post-mortem samples (Rubin et al., 1989, Sirdah, 2014, Choi et al., 2014, Fischer et al., 2004). DBS is an appropriate technique to determine drugs of abuse in cases of driving under the influence of drugs (DRUID). Thus, DBS is not only used clinically but it is well applied forensically also (Odoardi et al., 2014, Sadones et al., 2014).

Numerous studies have been published on the analysis of dried blood on a specific surface namely specialized filter cards (Odoardi et al., 2014, La Marca et al., 2008, Wong et al., 2011, Kostic et al., 2015, Liang et al., 2009, Sadones et al., 2014, Ingels et al., 2010, Tretzel et al., 2014, Reddy et al., 2011, Schutz et al., 2002, Kyriakou et al., 2016) but little is known about the analysis of dried bloodstains on different surfaces such as glass, concrete, textile or wood (Kruger et al., 2013). Therefore, this study will mainly deal with the analysis of diazepam in bloodstains dried on a specific surface such as glass which is a common surface that can be found in different crime scenes (e.g. traffic accident, robbery, murder). Toxicological analysis of dried bloodstains can be important for the police and lawyers since it can help to determine the chronology of events in a crime (Kruger et al., 2013, Schutz et al., 2002). A postmortem toxicological result may reveal that a deceased had consumed drugs but unfortunately cannot give any information on whether the drug had been consumed before or after an incident such as a fight prior to the death of the victim.

Kruger et al., (2013) illustrated the importance of analyzing dried bloodstains found on a carpet in an example given below.

Case history: In a homicide case, dried bloodstains were found on a carpet surface in the house of a victim. Police found that the victim was involved in a fight prior to his death. Heroin consumption was confirmed by postmortem results. Autopsy declared that the victim died of blood aspiration.

The court wanted to know whether heroin was consumed before or after the fight. Presence of heroin in the traces of blood concluded that heroin was consumed before the fight.

Kruger et al., (2013) reported that morphine and codeine were detected on a carpet surface six months after the blood had spilled on the surface. The study showed the good stability of morphine and codeine drugs in dried bloodstains and also showed that dried bloodstains can give a lot of information. Furthermore, Schutz et al., (2002) reported two more cases showing the importance of analyzing blood traces for drugs. Below are the two cases reported:

Case 1: In a waste bin, a handkerchief was found with bloodstains with high amount of opiates detected. Postmortem results confirmed a very low concentration of opiates in the blood of the victim. The dead body was found near a window curtain on which blood traces were found. The court wanted to know whether the blood traces came from the injection of heroin before the incident or from an affray between the victim and his offender on the next day. Analytical results confirmed that bloodstain found on the curtain contained traces of opiates whereas bloodstains on the handkerchief showed full scan spectra of opiates (main components of opiates). From forensic-toxicological analysis, it was deduced that on the previous evening, the victim consumed heroin and wiped away blood with a tissue paper which had accidentally spilled. However, the traces of blood on the curtain were attributed to an affray between the victim and his offender which occurred on the next day. Chronological information helped the court to reconstruct the crime.

Case 2: In a fatal car accident, the injured offender ran away and was caught a few hours later. Cocaine was confirmed present in his blood. Blood traces were collected from the car of the offender and were tested positive with cocaine. The aim was to prove analytically that cocaine was already present in the blood of the offender at the time of the accident.

Based on the examples given, it can be said that drugs can be detected in bloodstains and analysis of drugs in blood traces give important information on the sequences of events happened in a criminal act and can aid scene investigation.

Instability of drugs has always been a real challenge for most toxicologists especially to interpret results because of degradation of the drugs (El Mahjoub et al., 2000, Nilsson et al., 2010, Holmgren

et al., 2004, Morriya, 1996). Tretzel et al., (2014) reported that stability of drugs is better in dried blood spot (DBS) because of the absence of water and hence preventing the activation of degradation of enzymes. The aims behind this study are to determine whether it is possible to detect diazepam from bloodstains left to dry on a glass surface at room temperature and to assess stability of the drug in bloodstains dried for three, four and five days. This may help investigators to detect traces of drug in bloodstains even after a few days have elapsed which will be used to determine chronology of events. Also, the study aimed at determining whether dried bloodstains can give reliable quantitative information which can later be used in court.

2. Experimental

2.1 Materials

Defibrinated horse blood was provided by TCS Biosciences Ltd (Buckingham, UK). Reference drug, diazepam, and internal standard, flurazepam, dichloromethane (DCM) AR grade (99.5%), toluene AR grade (99.5%) and heptane HPLC grade (99%) were obtained from Sigma-Aldrich (Switzerland). Sodium carbonate anhydrous was obtained from *AnalaR*® Standards by BDH Chemicals (Poole, UK) and sodium hydrogen carbonate was purchased from *GPR*® by BDH Chemicals (Poole, UK).

2.2 Methods

2.2.1 Validation Procedure

The validation was conducted in terms of linearity, limit of detection (LOD), limit of quantitation (LOQ), precision, accuracy, extraction recovery and stability. All validation tests were completed using a blood matrix except for the peak identification test. Each prepared solution was spiked in blood samples, allowed to dry, extracted and then injected into gas chromatography mass spectrometry (GC/MS) for analysis. A paired t-test was performed to determine whether the recovered analyte concentrations from dried bloodstains and the corresponding analyte concentrations in whole blood were statistically significant or not (Jantos et al., 2011). A significance level of 0.05 was utilised. A scatter plot was constructed to determine the correlation coefficient between the two types of blood samples (dried and liquid).

2.2.1.1 Linearity study

A series of six calibration standard solutions with diazepam concentration levels of 1, 2, 5, 10, 50 and 100 µg/ml were prepared, spiked in blood samples and allowed to dry. The analyte of interest (diazepam) was then extracted and analysed by GC/MS.

2.2.3.3 Sensitivity study

The same series of solutions used for linearity test was used for the sensitivity test. In addition, two more solutions at concentration level 0.1 and 0.5 µg/ml were prepared.

2.2.3.4 Precision and accuracy study

Quality control (QC) samples were prepared at three nominal concentrations (low: 1, medium: 50 and high: 100 µg/ml) from a stock solution containing 100 µg/ml of diazepam. The samples were analysed on three separate days to determine interday precision and accuracy. Interday precision is expressed as percent relative standard deviation (%RSD) and accuracy as percent relative error (%RE) (Odoardi et al., 2014, Patel et al., 2010, Kostic et al., 2015, Tretzel et al., 2014). %RSD and %RE are calculated using equation 1 (Odoardi et al., 2014) and 2 (Williams et al., 2012) respectively.

$$\%\text{RSD} = \left[\frac{Standard\ Deviation}{Mean}\right] x\ 100 \ldots 1$$

$$\%\text{RE} = \left[\frac{Measured\ concentration}{Nominal\ concentration} - 1\right] x\ 100 \ldots 2$$

2.2.3.5 Extraction recovery study

Working solutions at 1, 5, 10 and 50 µg/ml concentrations were used. The solutions were analysed directly by GC/MS to determine peak area of unextracted diazepam in solvent. 100 µl of each solution was pipetted and spiked in four clean beakers each containing 1 ml of blood. 1ml of blood fortified separately with four concentrations of diazepam (1, 5, 10 and 50 µg/ml) in four beakers. The fortified blood were pipetted and spotted on a glass surface, allowed to dry for three days and finally extracted. The same analyte concentrations were spiked in whole blood and undergone extraction process. Results obtained from dried bloodstains, whole blood and pure solvent were then compared to assess extraction recovery. The percent extraction recovery is calculated using

equation 3 (Reddy et al., 2010, Kostic et al., 2015, Liang et al., 2009, Tretzel et al., 2014, Patel et al., 2010, Lawson et al., 2012).

$$\% \text{ recovery} = \frac{Peak\ area\ of\ extracted\ analyte\ from\ dried\ or\ whole\ blood}{Peak\ area\ of\ unextracted\ standard\ in\ solvent\ solution}\ x\ 100\ \ldots\ 3$$

2.2.3.6 Stability study

The series of four working solutions used for extraction recovery study were used for the stability study. Four blood spots in all were deposited for each concentration to check for stability at day zero, day three, day four and day five. Stability was evaluated by comparing measured concentration of diazepam extracted from bloodstains left to dry at room temperature for three, four and five days (analysed after 72h, 96h, 120h respectively) with measured concentration obtained from freshly made blood spots (day 0).

2.2.4 Extraction procedure

2.2.4.1 Dried bloodstains:

100 µl of each working solutions to be used was transferred into 5ml beakers each containing 1ml of blood. From each beaker the fortified blood, including control blood (no analyte), was pipetted and spotted on a glass surface. The blood spots were allowed to dry for at least 2h at room temperature. The blood spot was scraped using a sterile scalpel and transferred into a pre-weighed 15ml centrifuge tube. The mass of each dried bloodstain was measured and recorded (*Table 1*).

Table 1: Masses of bloodstains recorded on day 0, 3, 4 and 5

	Mass of dried bloodstains (µg/ml)			
Concentration of diazepam spiked (µg/ml)	**Day 0**	**Day 3**	**Day 4**	**Day 5**
0 (control)	0.3377	0.2132	0.2410	0.1994
1	0.2352	0.1880	0.1847	0.1234
5	0.2211	0.2246	0.2051	0.1657
50	0.2410	0.2495	0.2126	0.1876
100	0.3091	0.2525	0.2339	0.1504

100µl of internal standard stock solution containing 100µg/ml of flurazepam was transferred into the centrifuge tube. The mixture was vortexed for 10s and was allowed to equilibrate for 15 min. 1 ml of carbonate-bicarbonate buffer (pH 9.6) was added to the mixture and was vortexed for 1 min. 1 ml of a solvent containing toluene:heptane with a volume ratio of 9:1 was added to the sample. The centrifuge tube was stoppered and vortexed for 30s. The different phases were separated by centrifugation for 10 min at 4500 rpm. The organic layer was formed at the top. The layer was removed and transferred directly into an autosampler GC vial with fixed insert which was injected into a GC/MS for analysis. If the layers had not been clearly separated, the tube was centrifuged again for another 10 minutes.

2.2.4.2 Whole blood:

Extraction of diazepam from whole blood was performed using the same method as reported in section 2.2.4.1 with the exception of the volume of blood used. 5 ml of whole blood was transferred into a centrifuge tube followed by the addition of 100 µl of working solutions of diazepam and 100 µl of internal standard solution containing 100µg/ml of flurazepam. The remaining steps stayed the same.

All glasswares were washed with distilled water, oven-dried and rinsed with acetone before use. Working surfaces were cleaned with Virkon solution after working with blood samples.

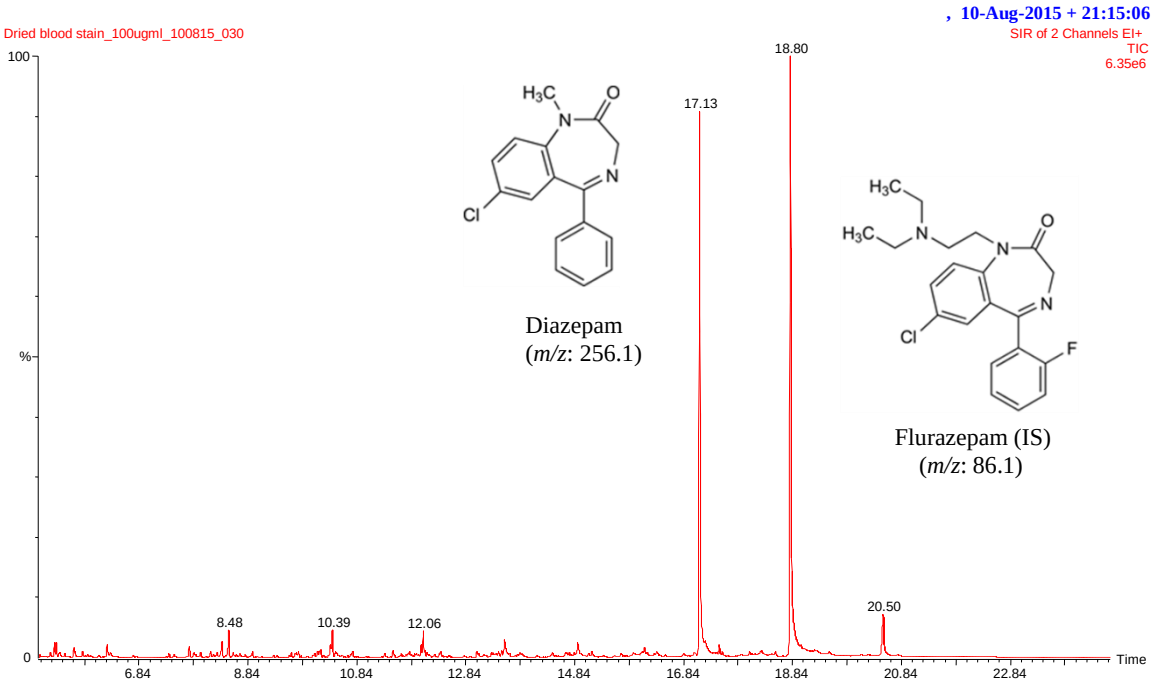
2.5 Chromatographic conditions

The whole and dried blood extracts were analysed using a Perkin Elmer Clarus 500 Gas Chromatograph equipped with a mass spectrometer detector and a silica-coated DBS5 capillary of 30.0m x 320 µm. The sample was injected with a split ratio of 20:1. Helium was used as the carrier gas. Initially the oven temperature was 50^{o}C held for 3 min, then programmed to 300^{o}C at 15^{o}C/min and held isothermally for 6 min. Electron ionization mass spectra were recorded in the range of 50 to 500 Da using the selective ion monitoring (SIM) acquisition mode and a solvent delay of 5 min was selected. The ion monitored for IS was *m/z* 86.1 and for diazepam was *m/z* 257. Source temperature and transfer temperature were set at 200^{o}C and 300^{o}C respectively.

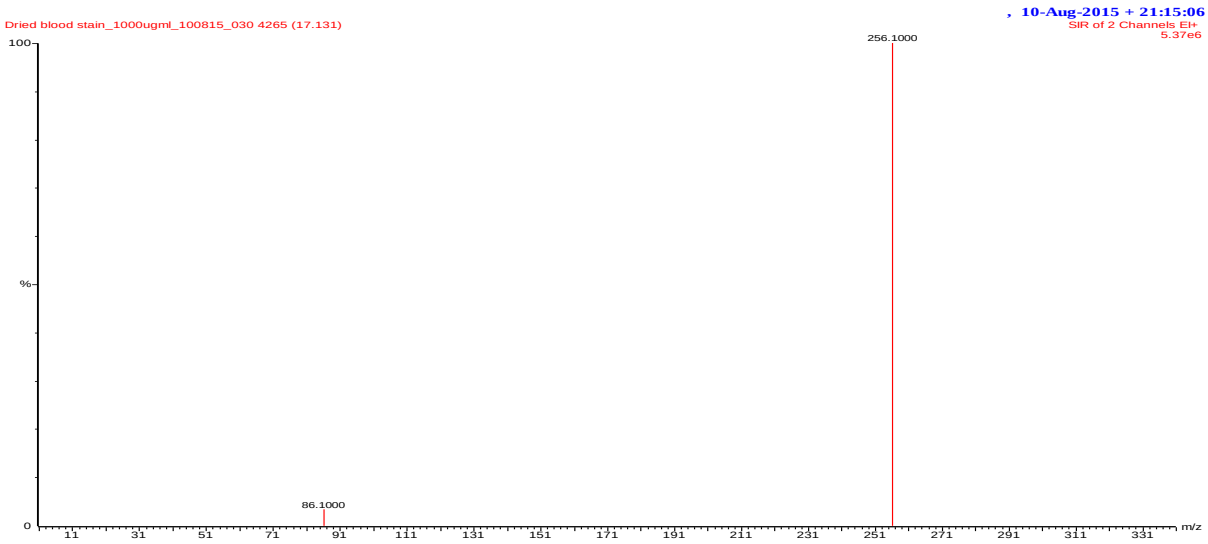
3. Results and Discussion

3.1 Peak identification of diazepam and internal standard (IS)

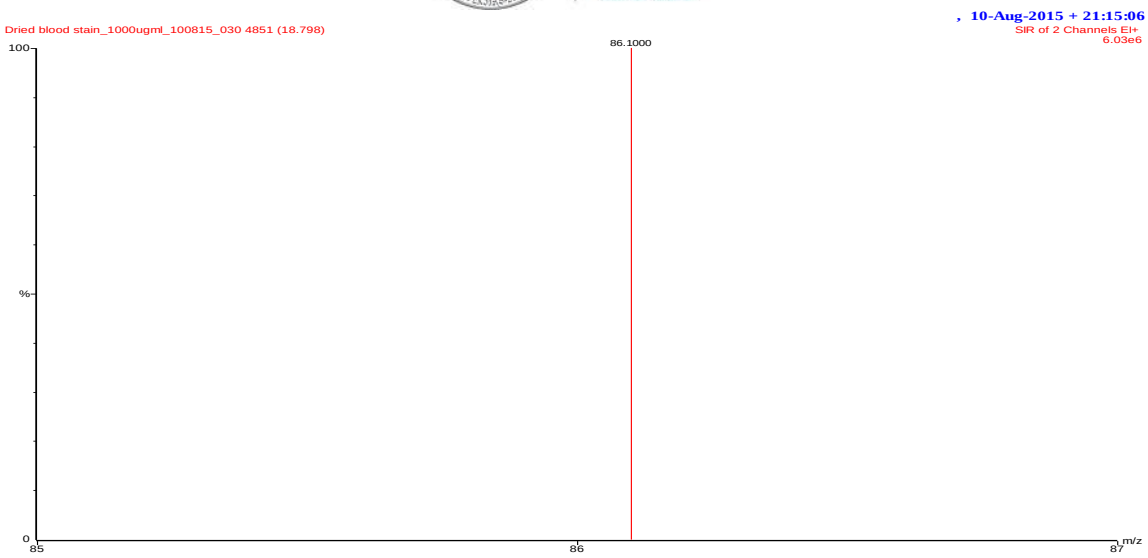
Based on the gas chromatographic conditions used in this study, the retention time of diazepam was 17.13 min and that of flurazepam (IS) was 18.80 min and the major fragment ions monitored for the drugs are *m/z* 257 and *m/z* 86.1 respectively (*figure 1*).



(A)



(B)



(C)

Figure 1: (A) Chromatogram and (B)(C) mass spectra of diazepam and IS

Considering the retention times of diazepam (17.13 min) and flurazepam (18.80 min) in this study, it can be said that flurazepam is a good IS for the quantitative analysis of diazepam since the retention times are close to each other.

3.2 Linearity

Linearity was assessed by plotting a calibration curve of peak area ratio of diazepam (analyte) to internal standard (IS) (P_A/P_{IS}) versus known concentrations of diazepam spiked in blood samples prior drying. A series of six working solutions with analyte concentration levels of 1, 2, 5, 10, 50 and 100 µg/ml was used.

In this study, a calibration curve was built by linear least squares regression of the peak area ratio (analyte/IS) versus concentration of diazepam (analyte) spiked in blood (*Figure 2*).

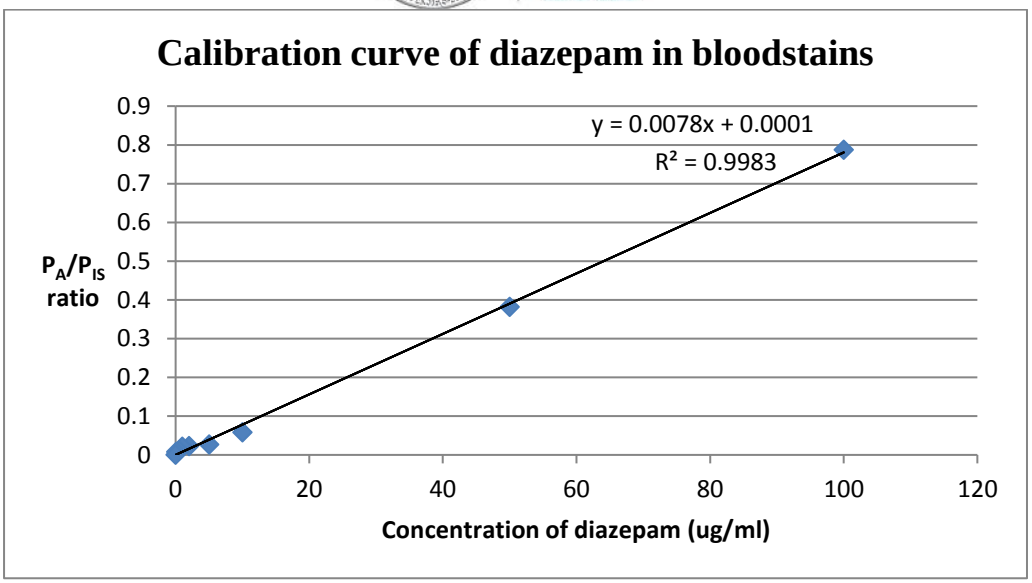


Figure 2: Calibration curve of peak area ratio of diazepam and IS versus concentration of diazepam in bloodstains

The equation of the line of the calibration curve is y = 0.0078 *x* + 0.0001 and the linear regression factor (r^2) is 0.9983. The method developed in this study is said to have a good linearity since the regression factor (r^2) is 0.9983 which is within the linearity acceptance criteria which is reported to be ≥ 0.99 (Patel et al., 2010, Odoardi et al., 2014, Kostic et al., 2015, Tretzel et al., 2014) and ≥ 0.985 (Gunnar et al., 2004). The calibration curve consisted of a blank (no analyte) and eight samples covering a range of 0.1 to 100 µg/ml including limit of quantitation (LOQ).

3.3 Sensitivity

Sensitivity was assessed on limit of detection (LOD) and limit of quantitation (LOQ). LOD and LOQ were determined by calculating signal-to-noise (S/N) ratio as mentioned by Shrivastava et al., (2011). In this study, the LOD in dried bloodstains is 0.5-1.0 µg/ml, the LOQ is 1 µg/ml and the LOD in whole blood is 1 µg/ml and the LOQ is 1-2 µg/ml as tabulated in *Table 2*.

Table 2: LOD and LOQ in dried bloodstains and whole blood

	Dried bloodstains	**Whole blood**
LOD (µg/ml)	0.5-1	1
LOQ (µg/ml)	1	1-2

It is observed that the LOD and LOQ in bloodstains are at a lower concentration level compared to whole blood. The precision of quantification for LOQ in dried bloodstains (1 µg/ml) is 3% (*Table 5*) which is within the limit of precision (< 20%) (Odoardi et al., 2014, Saussereau et al., 2012, Lawson et al., 2012, La Marca et al., 2008, Shrivastava et al., 2011, Gunnar et al., 2004, Armbruster et al., 1994). Thus, it can be said that concentrations above LOQ (1 µg/ml) can be quantified with acceptable precision.

3.4 Precision and Accuracy

Interday precision and accuracy were assessed by analyzing three quality control (QC) samples with low (1µg/ml), medium (50 µg/ml) and high (100 µg/ml) concentration level on three different days. *Table 3* shows the peak area ratios (P_A/P_{IS}) of each nominal concentration on three different days, standard deviation (SD), mean, %RSD (Relative Standard Deviation) (rounded to 1 significant figure, S.F) and %RE (Relative Error) (rounded to 1 S.F).

Table 3: SD, mean and %RSD at each nominal concentration: 1, 50 and 100 µg/ml

Nominal concentration of diazepam (µg/ml)	P_A/P_{IS}			SD	Mean	%RSD	% RE
	Day 1	Day 2	Day 3				
1	0.021	0.021	0.022	0.000577	0.021333	3	-66.3
50	0.382	0.369	0.365	0.008888	0.372	2	-48.8
100	0.787	0.701	0.648	0.07015	0.712	10	-39.3

For both precision and accuracy, %RSD and %RE values should be ≤ 15% to be within the acceptance criteria (Gunnar et al., 2004, Van der Heijden et al., 2009, Patel et al., 2010, Kostic et al., 2015, Tretzel et al., 2014). The %RSD of the three nominal QC concentrations ranged from 2 to 10 % which are within the acceptable limits of precision (< 15%). However, %RE is not within the acceptance accuracy criteria. Therefore, the method presented in this research satisfied interday precision but is not considered optimum.

3.5 Extraction Recovery

Extraction recovery was determined at four concentrations namely: 1 (therapeutic level), 5 (abuse level), 10 and 50 µg/ml. The calculated % recovery (to 1 decimal place) obtained from dried bloodstains (day 3) and whole blood are tabulated in *Table 4.*

Table 4: % recovery from dried bloodstains and whole blood

	% recovery	
Concentration of diazepam (µg/ml)	**Dried bloodstains**	**Whole blood**
1	3.3	1.4
5	2.6	0.8
10	3.4	1.2
50	3.0	1.8

The highest percentage of diazepam extracted was from dried bloodstains at 10µg/ml with 3.4% and the smallest percentage recovery was from whole blood at 5µg/ml (abuse level) with 0.8%. From *Figure 3,* it can be observed that at 1, 10 and 50 µg/ml, the percentage of diazepam recovered from dried bloodstains is approximately 3 times higher than from whole blood. Although the whole blood samples were freshly prepared and the bloodstains were three days old, more diazepam could be extracted from bloodstains compared to whole blood samples. This observation is in accordance with the literature as it is reported that dried bloodspots show a greater stability of drugs than whole blood (Tretzel et al., 2014, Alfazil et al., 2008 and El Mahjoub et al., 2000, Jantos et al., 2011, Sadones et al., 2014). Further tests were made on stability of diazepam in dried bloodstains which are older than three days and the respective results were then compared with freshly deposited bloodspots (Wong et al., 2011).

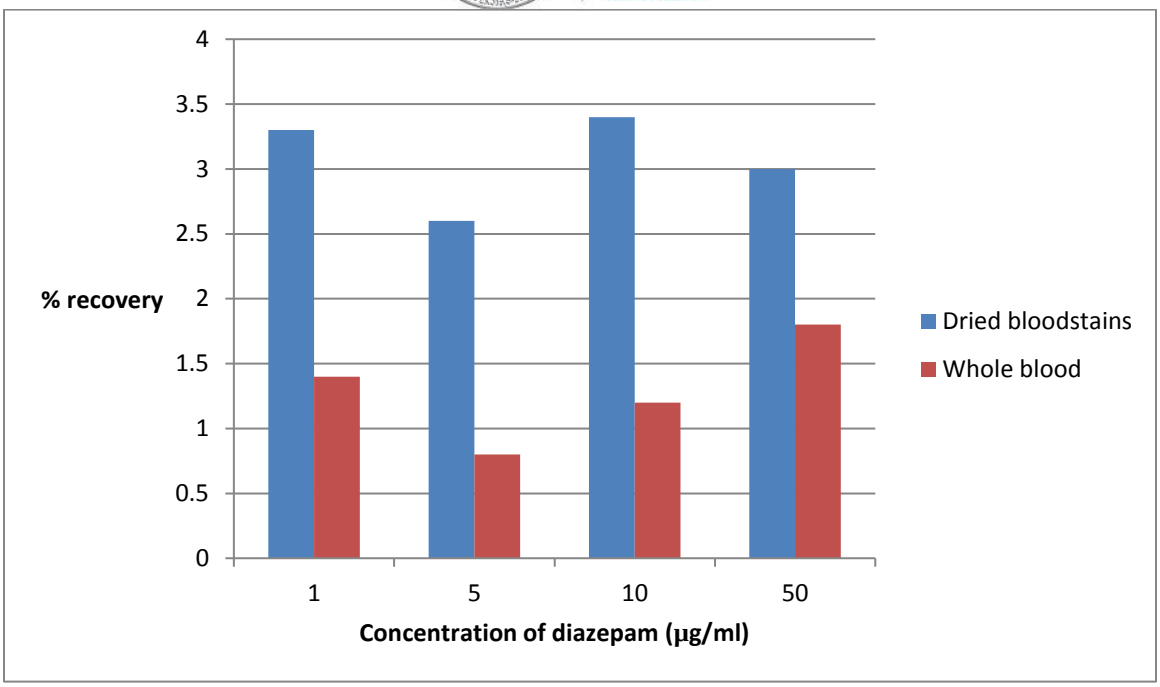


Figure 3: % recovery of diazepam at four concentration levels (1, 5, 10 & 50 µg/ml)

Bloodstains were in a dehydrated state while the whole blood samples contained water molecules which are known to cause the degradation of drug molecules via hydrolysis, oxidation or reduction (Alfazil et al., 2008, Sadones et al., 2014).. Thus, in the absence of water molecules, hydrolysis and oxidation of drug molecules are prevented and thus reducing the risk of drug degradation. So, diazepam is found to be more stable in dried bloodstains compared to whole blood samples, as a higher recovery of diazepam was obtained from dried bloodstains samples.

However, although the %recovery of diazepam from bloodstains was higher compared to whole blood samples it is important to highlight that the overall recovery in both dried bloodstains and whole blood is very low (ranging from 0.8 to 3.4 %). Since bloodstains and whole blood samples were treated with the same extraction steps, it can be said that the poor recovery is related to the extraction procedure. The following reasons are suggested for poor recoveries in dried bloodstains and whole blood:

1. The method of extraction used in this study is liquid-liquid extraction (LLE) which involves the extraction of a solute from a biological matrix (e.g. blood) using two immiscible solvents (Prabu & Suriyaprakash, 2012). Basically, separation of a solute depends on its solubility in a solvent, i.e., if a solute is soluble in a particular solvent, it is

highly probable that the solute is extracted in that solvent (Arneson & Brickell, 2007, Prabu & Suriyaprakash, 2012).

In this study, the organic layer (toluene: heptane) is collected to analyse extracted diazepam. However, considering the % recovery obtained from dried bloodstains and whole blood, it can be deduced that the majority of the diazepam drug was not extracted in that organic solvent (toluene: heptane). Therefore, it can be said that diazepam drug is not soluble enough to be transferred into the organic solvent. Hall et al., (2012) confirmed that it is of paramount importance to choose the correct organic solvent for the extraction of an analyte and to do so it is important to know solubility of the analyte in the selected organic solvent to maximize recovery. Hence, toluene and heptane solvents used were not the appropriate organic solvents for the extraction of diazepam as it resulted in poor recoveries at all concentration levels (1, 5, 10 and 50 µg/ml) for both dried bloodstains and whole blood.

2. Poor recoveries can also be attributed to the adhesion of analytes to tube walls or biological matrices (Hall et al., 2012, Lee, 2012, Brandenberger & Maes, 1997).

   As mentioned earlier in this study, most drugs are bound to plasma proteins (Zech & Freit, 1989). So, to extract the drug analytes from blood samples it is necessary to separate the analytes from the proteins (Collins et al., 1992). Therefore, deproteination is important as it involves breakage of bond between protein and drug molecules and in this way recovery can be increased (Zech & Freit, 1989). Since poor recoveries were obtained, it can be deduced that the deproteination step in the extraction procedure did not work properly as the drug analytes could not be separated from the plasma proteins and hence resulted in low recoveries of drug analytes.

   Since deproteination of blood samples is done with the help of buffer solutions therefore, the poor recoveries obtained can be also linked to a wrong selection of buffer solution (carbonate-bicarbonate buffer). Instead of using carbonate-bicarbonate buffer solution as suggested by Crifasi et al., (2006), phosphate buffer can be used as mentioned by Spector et al., 2007, Inoue et al., 2000, Zech & Freit, 1989, U.S Department of Health & Human Sciences, 1980.

Additionally, it may be possible that the analytes were adsorbed on the plastic surface of the centrifuge tube (Bowen et al., 2010, Smith et al., 1996, Grouzmann et al., 2008) due to hydrophobic interactions between analyte structures and plastic matrix (Wild, 2005, Sood, 2006, Williams et al., 2012) preventing a good extraction of diazepam drug.

3. Diazepam is prone to hydrolysis in both acidic and alkaline medium (Hudecova et al., 2004). Since extraction was done in a basic medium (pH=9.6), diazepam may have degraded resulting in a loss of analyte and consequently allowing only a small amount of diazepam to be recovered (Lee, 2012).

4. The organic layer collected from blood extracts were not cleaned-up (were not purified to obtain cleaner extract) after LLE due to the fact that a small volume of organic layer (< 1 ml) could be collected so it was not possible to do back-extraction to clean-up the sample. Therefore possible presence of endogenous compounds may cause interference and causing a decrease in the peak area of the ion of interest, especially drug analytes at low concentration levels (1 and 5 µg/ml) (Zech & Freit, 1989, Yinon, 1994, Karch, 2007).

3.6 Stability

The equation of the calibration curve (*figure 2*), $y = 0.0078x + 0.0001$, was used to calculate the concentration of diazepam extracted where y represents the peak area ratio (diazepam/IS) and *x* represents the concentration of diazepam in µg/ml. Multiple publications (El Mahjoub et al., 2000, Wong et al., 2011, Tretzel et al., 2014, Nilsson et al., 2010, Rhoden et al., 2014) have worked on stability of drugs on specialized filter cards but not on a particular surface such as glass as used in this study. Control samples were also spotted to ensure glass surfaces were drug-free and thus no contamination.

Histograms (*figure 4*) are built for each nominal concentration to compare measured concentration obtained on day 0 (represented by blue histograms) and with measured concentration obtained on day 3, 4 and 5 (represented by red histograms). *Table 5* shows the measured concentration (to 3 S.F) obtained from bloodstains freshly deposited and bloodstains for three, four and five days.

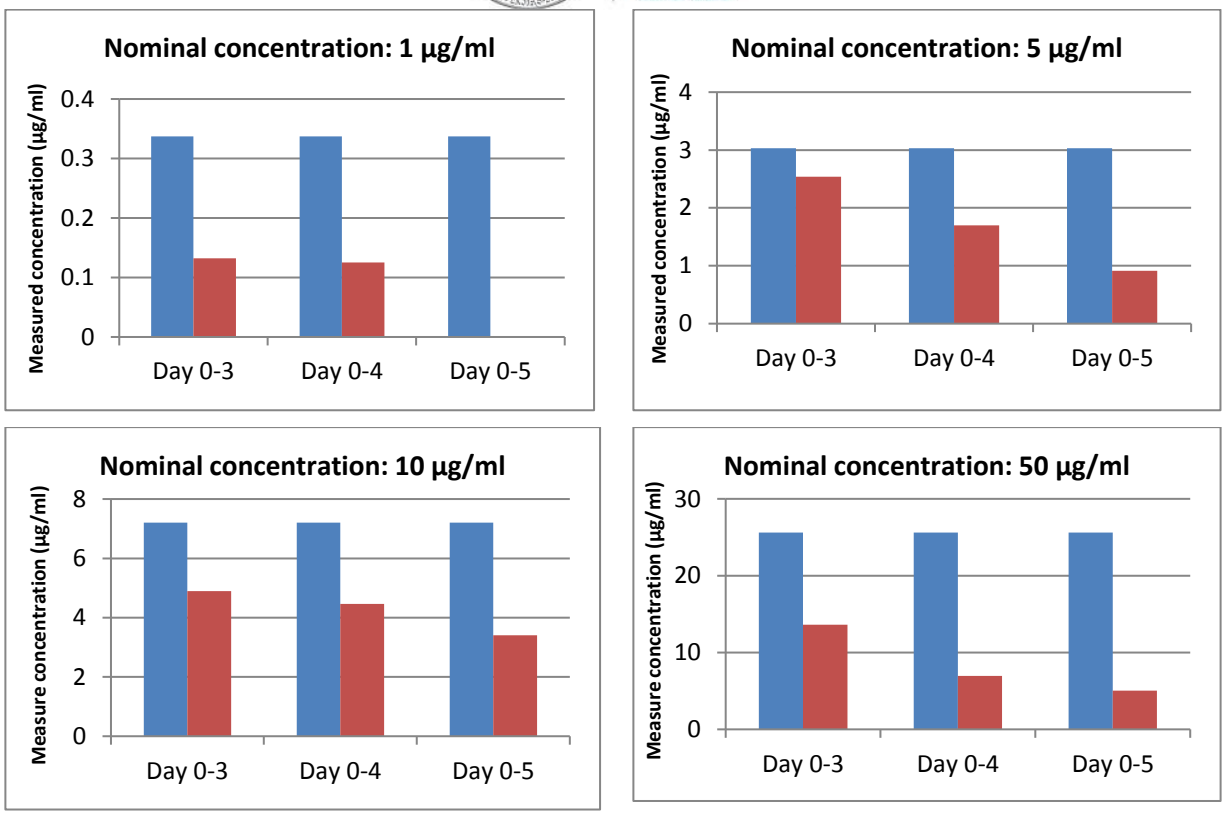


Figure 4: (A)(B)(C)(D) Measured concentration compared to its corresponding nominal concentration in bloodstains dried on different days

Table 5: Measured concentrations obtained from bloodstains on day 0, 3, 4 & 5

Nominal concentration (µg/ml)	Measured concentration (µg/ml)			
	Day 0	Day 3	Day 4	Day 5
1	0.337	0.132	0.125	-
5	3.03	2.54	1.70	0.913
10	7.20	4.89	4.46	3.40
50	25.6	13.6	6.95	5.04

Diazepam could not be detected at therapeutic level (1µg/ml) in dried bloodstains observed by day 5. From *figure 4*, it can be observed that for each nominal concentration a lower concentration is recorded on day 3, 4 and 5 (red histograms) compared to day 0 (blue histograms). Thus, it can be deduced that diazepam continued to degrade even five days after the blood spots were made.

Diazepam in bloodstains dried for 3, 4 and 5 days was not as stable as *figure 5* showed an apparent decrease in the measured concentrations.

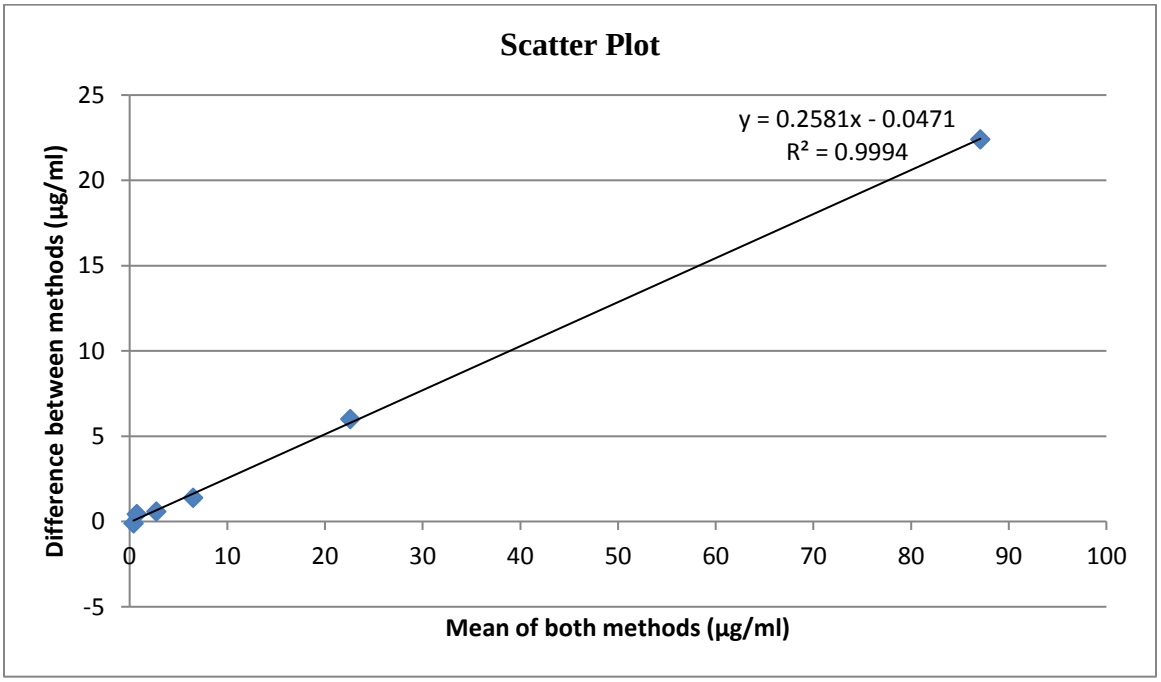


Figure 5: Scatter plot from 6 paired samples obtained from DBS and whole blood

It is noteworthy to point out that the mass of the dried bloodstains, *table 1*, was recorded before analysis and it was noticed that the mass of the dried blood spots continued to decrease and no constant mass was recorded within the four days. Thus, the apparent decrease in the measured concentration of diazepam may be attributed to the possible presence of water molecules in the bloodstains which was causing degradation of the drug molecules.

3.7 Comparison between dried bloodstains and whole blood

Concentration of diazepam obtained from whole blood samples and dried bloodstains (DBS) deposited on day 0 were compared. *Figure 5* illustrates the scatter plot in which the difference in concentration between the two methods is plotted against the mean concentration of the two methods.

A correlation coefficient (r), $-1 \leq r \leq 1$ is considered to be acceptable (La Marca et al., 2008). A perfect positive correlation gives r = 1 where all data points lies exactly on a straight line and a perfect negative correlation gives r= -1. Linear regression was completed and the two methods

appear to be well correlated with a correlation coefficient of 0.9997 which indicates there is a strong relationship between DBS (blood spotted on day 0) and whole blood and also the results obtained from DBS and freshly spiked whole blood can gave comparable results. Additionally the paired t-test gave a p value was 0.2215 (>0.05 significance level) indicating there is no significant difference between the amount of diazepam obtained from DBS and whole blood which means that the measured concentration from a dried bloodspot and whole blood are approximately the same.

4. Conclusion

The aims behind this study were to determine whether it is possible to detect diazepam from dried bloodstains and to assess stability of the drug in bloodstains dried for three, four and five days. Also, the study aimed at determining whether dried bloodstains can give reliable quantitative information which can later be used in court. Lastly, a comparative study was made between dried bloodstains and whole blood based on the recovery and analysis of diazepam. Results showed positive detection of diazepam in bloodstains dried on different days. However, the stability study showed that diazepam was not stable in dried bloodstains since an apparent decrease was observed (*figure 4)* in the measured concentration recorded on different days (day 0, 3, 4 & 5). The degradation is attributed to the possible presence of water molecules. As a result, it can be said that the toxicological analysis of diazepam in bloodstains can assist the court based on qualitative information only, i.e., whether diazepam is present or not. The results can help the court to determine the chronology of events in a crime. However, based on the age of bloodstains quantitative information can vary since degradation of drugs is still possible due to the possible presence of water molecules. This indicates that the concentration of diazepam in bloodstains does not necessarily reflect the concentration of diazepam at the time the blood had been spilled on a glass surface and therefore, quantitative results diazepam in dried bloodstains should be used caustiously. It may be that if the bloodstains are completely dried, which will then be showed by a constant mass of bloodstains recorded, the measured concentration will remain constant. Although, the measured concentration will be constant, it will still not deduce the concentration of drugs at the time blood was spilled. The method presented in this research satisfied interday precision but is not considered optimum since the measured concentrations from bloodstains were not close to the nominal concentration spiked in blood. The %recovery of diazepam from both

dried bloodstains and whole blood was low and could be related to the extraction procedure, degradation of diazepam and adhesion of analytes to tube walls or biological matrices. The comparison made between bloodstains and whole blood showed that a better recovery of diazepam was obtained with dried bloodstains and a correlation coefficient of 0.9997 from a scatter plot concluded that bloodstains and whole blood gave comparable results.

Improvements and future work:

It could be beneficial to use solid phase extraction (SPE) instead of liquid-liquid extraction (LLE) to obtain cleaner extracts reducing interference from the blood matrix and in this way more drugs can be extracted and accuracy can also be increased. Use of deuterated internal standard to increase accuracy of quantification. Derivatisation step can be included in the extraction procedure to enhance thermal stability of diazepam in GC/MS (Carlin & Dean, 2013). Change in carbonate-bicarbonate buffer to phosphate buffer. Use of other organic solvents such as hexane: ethyl acetate (7:3, v/v) (U.S. Department of Health and Human services, 1980). Use of glass centrifuge tubes as glass surfaces are known to have less interaction with analytes (David, Maynard & Wakefield, 2015). Stability can be checked beyond five days to provide more details on stability. Spotting smaller volume of blood (<1ml). Spotting blood on different surfaces namely tiles, wood, cloth, plastic or concrete and finally a comparative study can be made between quantitative results.

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