IOSR Journal of Applied Chemistry (IOSR-JAC)
e-ISSN: 2278-5736.Volume 14, Issue 3 Ser. I (March. 2021), PP 31-38
www.iosrjournals.org

GC-FID without derivatization method for the quantitative determination of Drug-Facilitated Crimes (Diazepam) in Bulk, Pharmaceuticals, urine, blood, biscuits and beverages.

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Abstract
Diazepam (DZ) drug-facilitated crime is frequently encountered in clinical and forensic toxicology cases due to misuse or abuse. This paper reports simple, sensitive, selective, reliable, rapid and expeditious GC–FID method for the quantitative analysis of diazepam (DZ) drug (1,4-benzodiazepines) in pure form, pharmaceutical preparations, urine, blood, biscuits and beverages, 1µg/ml clonazepam (CZ) as internal standard (I.S.) has been developed for different forensic toxicology laboratories. The chromatographic system of GC–FID was carried out without derivatization using a GC- 17AGas Chromatograph SHIMDZU in conjunction with a column (DP – 5, length: 30 meter and ID: 0.25 ml) was validated. The proposed method has been validated as per US-FDA bioanalytical guidelines in terms of linearity, accuracy, precision, matrix effects, stability, selectivity, and recovery. The method was linear over the concentration range of 0.8–1000 µg ml^{-1} with limits of detection ad quantification of 0.0308 and 0.128 µg ml^{-1}, respectively. The intraday and interday precisions and accuracy expressed by the relative standard deviation and the relative standard error were both less than 6.12 % and 1.8 %, respectively. The proposed method was successfully applied in pure form, pharmaceutical preparations, urine, blood, biscuits and beverages, 100 µg ml^{-1} clonazepam (CZ) as internal standard (I.S.) has been developed for different forensic toxicology laboratories. The stability of DZ in the presence of all endogenous applications components was studied and the new GC–FID method was successfully employed.

Keywords: *Diazepam; Clonazepam; GC–FID; Extraction; Stability; Urine, Blood, Biscuits and Beverages.*

Date of Submission: 04-03-2021 Date of Acceptance: 18-03-2021

I. Introduction

Benzodiazepines identification and confirmation their presences in human body are important in emergency rooms and toxicology laboratory; various reliable and specific techniques are required (1). Benzodiazepines remain the primary targets in clinical and forensic urine drug testing as it's related to suicide attempts, road traffic offenses, drug facilitated sexual assault, sudden deaths, car accidents, rapes, burglaries and robbery (2–5). Diazepam (1,4-benzodiazepines) is one of the most frequently observed compound in cases of drug-facilitated crime (DFC) and drug-facilitated sexual assaults (DFSA) (6–10). This is due to Diazepam (D Z) has become the most commonly used drugs for their anti-convulsant, anaesthetic, anti-depressive, hypnotic, tranquilizer and sedative properties. They are also used as pre-medication and for induction or general anaesthesia and are widely prescribed throughout the world (11–14). Apart from their therapeutic applications, benzodiazepines are often abused by drug addicts; as a consequence, these drugs are frequently involved in both clinical and forensic cases (15). Diazepam (DZ) and clonazepam (CZ) as internal standard (I.S.) are considered the most important 1,4-benzodiazepines (Figure 1) where diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H- 1,4-benzodiazepin-2-one) drug enhances the activity of *gamma*-aminobutyric acid, the most common inhibitory neurotransmitter in the central nervous system which an abused drug in which sudden withdrawal, particularly from high dosage, carries the risk of epileptic seizures that used in the treatment of severe anxiety disorders, as a hypnotic in the short-term management of insomnia, as a sedative and premedicant, as an anticonvulsant, in the management of alcohol withdrawal syndrome (16–19). Hepatic *N*-demethylation results in the formation of an active metabolite, desmethyldiazepam (nordiazepam). This metabolite is hydroxylated to form oxazepam, which is also active and is metabolised to form oxazepam glucuronide. Aminor active metabolite is temazepam, which can be in turn demethylated to oxazepam or glucuronidated (20). Various analytical methods have been described for the determination of diazepam (DZ) in pharmaceutical preparations,

biological fluids, different organs and hair. The methods cited in the literature for the determination of (DZ) include spectrophotometric (21–26), fluorimetric (27–30), electrochemical (31–35), and different chromatographic methods (36–43).

The monitoring of diazepam is important for quality assurance in preparations and for obtaining optimum therapeutic concentrations in body fluids to minimize the risk of toxicity. This study is important to develop and validate a rapid, accurate, simple and sensitive method for DZ determination without derivatization using GC–FID. Most notably, derivatization is required prior to GC instrumental analysis such as conversions of the benzodiazepines (e.g., to the corresponding benzophenone) that can occur during pretreatment and GC-MS measurement spoil the identity of the analytes (44).

This method is applied on bulk, tablets, biological fluids (urine and blood), biscuits and beverages. All methods of extractions were validated to be applicable in different analytical ad toxicological laboratories. The proposed method has been successfully applied for the determination of DZ in pharmaceutical formulations, biological fluids, biscuits and beverages.

II. Materials and methods

2.1. Instrumentation and chromatographic separation

The analysis was performed using a GC- 17AGas Chromatograph SHIMDZU in conjunction with a column (DP – 5, length: 30 meter and ID: 0.25 ml) were used for all the measurements. The oven was initially set to 80 ^{o}C hold for one min, then a ramp rate of 12 ^{o}C min^{-1} until a temperature of 180 ^{o}C was reached. At this point, the ramping rate was increased to 15 ^{o}C min^{-1} until a final temperature of 290 ^{o}C was achieved. This final temperature was held for 20 min. Temperature of both injector and detector (FID) were 280 ^{o}C and 300 ^{o}C, respectively.

2.2. Chemicals

All standards had a purity of at least 99.9%, as certified by the manufacturer. The purity of the standards was further evaluated by checking the standards for cross-talk interference by injecting each analyte individually, under the established chromatographic conditions. All solvents were high performance liquid chromatography grade. Diazepam and clonazepam were kindly gifted from (EIPICO, 10th of Ramadan, Egypt). Ethanol, methanol, ethyl acetate, n- hexan, 2-propanol, acetonitrile and PTFE filters (0.2µm×25mm) were supplied from Merck (Darmstadt, Germany). Deionised water (18.1 MΩ) was obtained from a Millipore-Q water system (Bedford, MA, USA). Potassium hydroxide and ammonium hydroxide were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France).

2.3. Preparation of Stock Solutions.

Stock solution of DZ was prepared in methanol at 1 mg ml^{-1}. They were stored frozen at -20 ºC for no more than 3 months. Working solutions were made in methanol and aliquots of the working solutions were evaporated under nitrogen and then reconstituted in urine, blood, biscuits and beverages. The calibration range of DZ was 0.8–1000 µg ml^{-1} and the concentration of internal standard is 1µg/ml.

2.4. Preparation of Calibration Standards and QC Samples.

DZ stock solution with internal standard (IS) was further diluted to prepare working solutions with methanol, which were then used to prepare calibration standards, QC, and IS. By dissolving in methanol and spiking working standard solutions into blank urine, blood, biscuits ad beverages, seven concentration levels of the calibration standards were prepared to obtain final concentration levels of 0.8, 50, 100, 250, 500, 750, and 1000 µg ml^{-1} for DZ. In the same way, four QCs samples at 1, 200, 400, and 800 µg ml^{-1} for DZ were also prepared either by dissolving in methanol or by spiking at four levels; LLQC, LQC, MQC, and HQC. For optimization and validation, both standards and QC samples were kept at −80 °C before use. Without deterioration, the samples prepared in methanol can be held at 4 °C for 1 week.

This investigation conforms to the Egyptian Community guidelines for the use of humans in experiments. Blood and urine samples were obtained from healthy (normal liver, kidney functions and electrocardiogram) male, informed, adult volunteers were instructed to abstain from all medications**.**

2.5. Extraction methods

Pharmaceutical preparations, At least ten tablets of DZ (Valinil® 5 mg, Nile Co, Egypt) were weighed to obtain the mean tablet weight and then ground to a homogenized powder. A quantity of the powdered tablets equivalent to 50 mg was transferred into a 50 ml calibrated flask and dissolved in methanol then filtered. The first portion of the filtrate was rejected, and a measured volume of the filtrate was diluted quantitatively with methanol to yield suitable concentrations that were in the linear range.

Biscuit samples preparation, Biscuit samples were grounded and homogenized then each 1 gm was added to four definite concentrations of DZ that taken from working solutions and 100 µl of IS (1000 µg ml^{-1}). Methanol is added to allow centrifugation and the filtrate evaporated under nitrogen before injection the target analytes into the GC–FID system (45).

Soft drink and urine samples Preparation. All specimens were centrifuged after mixing each 1000 µl of spiked samples with 100 µl of IS (1000 µg ml^{-1}).Vortex 10-15 seconds then P^H adjusted at 8.5. Extraction for 5 min at 3000 rpm by 3 ml n- hexan: ethyl acetate: 2-propanol (85: 14: 1, v/v/v). Separate organic layer then evaporate before injection the target analytes into the GC–FID system.

Blood samples Preparation, Four definite concentrations of DZ were taken from working solutions were evaporated under nitrogen then reconstituted with 1000 µl of blood and the sample was spun for 20 s at room temperature with 100 µl of IS (1000 µg ml^{-1}). The second step is adding 500 µL acetonitrile followed by centrifugation for 5 min at 3000 rpm then adjust P^H at 8.5 for the supernatant before extraction for 10 min at 3000 rpm by 3 ml n- hexan: ethyl acetate: 2-propanol (85: 14: 1, v/v/v). Separate organic layer then evaporate before injection the target analytes into the GC–FID system.

For the proposed method, DZ definite concentrations of tablets and extractant of blood, urine, beverage and biscuits was calculated using the corresponding regression equation of the appropriate calibration graph.

2.7. Validation

All blood and urine samples used in the preparation of quality controls was obtained from staff volunteers within the hospital facility, and was screened prior to preparation of quality controls to ensure that it was drug free, using the assay procedure and quality control samples (LLQC, LQC, MQC, and HQC) analytes were used during clinical samples analysis. All QCs, working standards, and stock solutions were stored frozen at -20 ºC prior to use. CZ as an internal standard was used to prepare µg ml^{-1} during all extraction. The regression line was calculated using linear regression model. Calibration curves using the same concentrations were performed for 5 days. Accuracy, intra- and inter-day precisions for all analytes were evaluated according to the requirements of FDA guideline on bioanalytical method validation (46). The intra- and inter-day accuracy and precision values were analyzed on one day and over 7 days were analyzed on one day and over 7 days of four QCs samples at 1, 200, 400, and 800 µg ml^{-1}. Accuracy was determined by the percentage deviation of the mean calculated concentration compared to the spiked concentration. Precision was determined by calculating the coefficient of variation (%RE) at each concentration level based on the mean concentration and the standard deviation.

The extraction efficiency was determined by injecting five replicates of four QCs samples. Blank blood, urine, beverage and biscuits were fortified with analyte solution and internal standard before and after SPE. Matrix effect was calculated by dividing peak areas of each analyte and the internal standard in samples from set 2 (five extracts of each different drug-free spiked with analytes after extraction) by those in samples from set 1(five neat standards) matrix effects were evaluated according to (47). Peak area ratios (analyte/I.S.) were used for determination of concentration from extracted matrix.

Carryover was evaluated for each application by injecting blank sample containing (I.S.) immediately after a sample spiked with 100 ng ml^{-1} of all target analytes. The measured concentration of the blank sample was used to calculate the carryover rate. Carryover was considered negligible if the measured concentration was below the LOQ.

The relative recoveries at all QCs concentrations and limit of quantifications were measured by comparing the response obtained for samples that were subjected to the extraction procedure with those obtained from blank extracts that were spiked post extraction to the same nominal concentrations. Recoveries were calculated using peak ratio (peak area of analyte divided by peak area of I.S.).

III. Results and discussion

It is important our knowledge, there is no analytical method for quantitative determination DZ using GC–FID without derivatization has been created. This paper introduces large varieties of applications in different fields such as quality control laboratories of pharmaceutical industries ad forensic chemistry laboratories. Herein, therefore, we established a GC–FID analytical method in an attempt to obtain a better therapeutic drug application and different forensic chemistry applications study. Developing a validated method needs the optimization of the following parameters.

The method was validated for DZ quantification of and CZ as shown in **Table 1**. DZ was linear over the range 0.8–1000 µg/ml with a correlation coefficient (r^2 value) of 0.9999, no interferences occurred with any of the standards under the method. Accuracy and precision was calculated for all samples and RSD, % was found to be less than 5%. The validation of thermal program was carried out and the best program is announced in (table 1). A good peaks resolution without interferences and retention times of both DZ and CZ are shown in

(**Fig. 3**). The limit of detection (LOD, signal-to-noise higher than 3:1) was calculated for each analyte based on each blank noise and this gave a limit of detection less than 0.0308 µg/ml for all analytes. The limit of quantification (LOQ, signal-to-noise higher than10:1) was to be measured with a relative standard deviation percent (RSD, %) less than 10% for accuracy and precision to be 0.128 µg/ml for all analytes.

Method accuracy and precision, In order to satisfy this identification criterion, intraday and interday days were assessed at four QC concentrations (low, middle, and high concentrations) 1, 200, 400 and 800 µg/ml as shown in **Table 2**. The method accuracy was assessed by calculating the relative standard error RE % ((found concentration – add concentration/add concentration) × 100) of the proposed method either intraday or interday and ranged from –3.8 to 1.8% for all applications (Pharmaceutical, biscuits, beverage, urine and blood). The proposed method showed good reproducibility for all applications, calculated by the relative standard deviation (RSD %), ranging from 0.89 to 6.12% across four QC samples as presented in **Table 2**. The proposed method is characterized and introduced multi applications in terms of simplicity, sensitivity, sample volume, and sample processing than the previous methods reported for the determination of DZ (42–45).

DZ stability in five applications, the new GC–FID method was successfully employed to investigate the target drug stability in the presence of Pharmaceutical, biscuits, beverage, urine and blood components. Under different storage conditions DZ stability was tested, such as short-term RT stability for 1 h and also refrigeration stability for 24 h at 4 °C, stability for 3 thawing cycles, and long-term storage stability at –80 °C for 1 month (48). All results of DZ stability for all applications in the different conditions were summarized in **Table 3**; the results indicated that DZ had the required stability without considerable degradation to be kept at room temperature for 1 h with RSD % ranging from 0.98 to 3.68 %, and this time is enough for sample pretreatment. Moreover, DZ was stable with RSD % ranging from 0.83 to 3.2 % when storing for 1 day in the refrigerator at 4 °C, the time required for sample injection. The target drug also exhibited reasonable stability when kept in the freezer at –80 °C for one month with RSD % ranging from 0.69 to 2.99 %. Additionally, the drug of interest was exhibited good stability after three thaw−freeze cycles with RSD % ranging from 0.87 to 3.45 % as indicated in **Table 3**.

IV. Conclusion

Herein, a novel analytical method has been developed for the quantitative determination of the widely administered anti-convulsant, anaesthetic, anti-depressive, hypnotic, tranquilizer, sedative and drug-facilitated crime (1,4-benzodiazepines) DZ drug, for different applications in pharmaceuticals and forensic cases after simple ad rapid extraction process and gas chromatography (GC) with flame ionization detector (FID) without derivatization and to avoid using the toxic derivatization reagents used by the former GC methods.. The current method has been validated and successfully applied to the most important applications (Pharmaceutical, biscuits, beverage, urine and blood) study. To the best of our knowledge, this the first validated method for quantification studies of DZ without derivatization and very good resolution using GC–FID with high resolution and no interferences. Moreover, this study may collect the most important applications and it might provide useful tools to be used in the analytical, clinical and toxicological laboratories. Despite that the stability data of the current method have been obtained; this study may contain a means for the quality control units in drug manufacturing and results interpretation in forensic ad toxicology labs.

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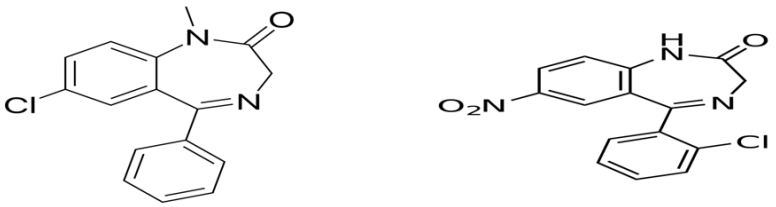

Diazepam **Clonazepam**

Figure 1. Chemical structures of DZ and CZ as (IS)**.**

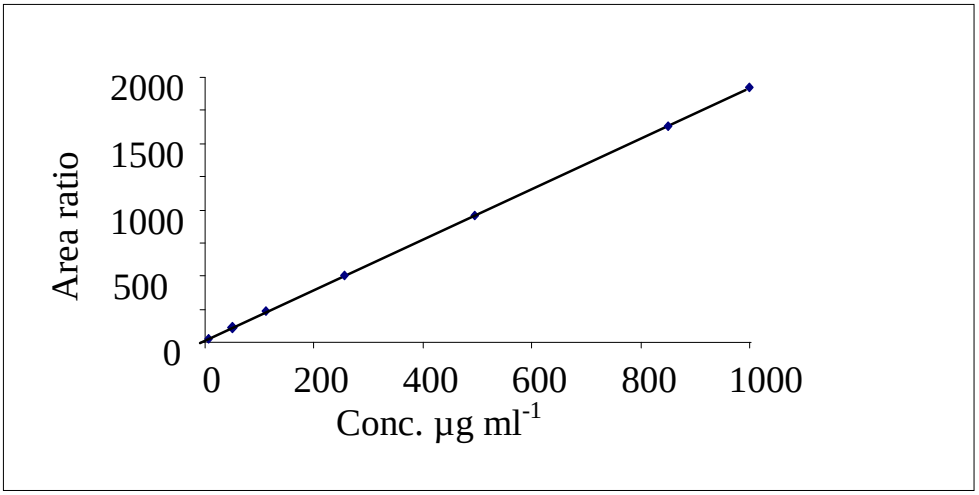

Figure 2. Standard calibration curve of DZ.

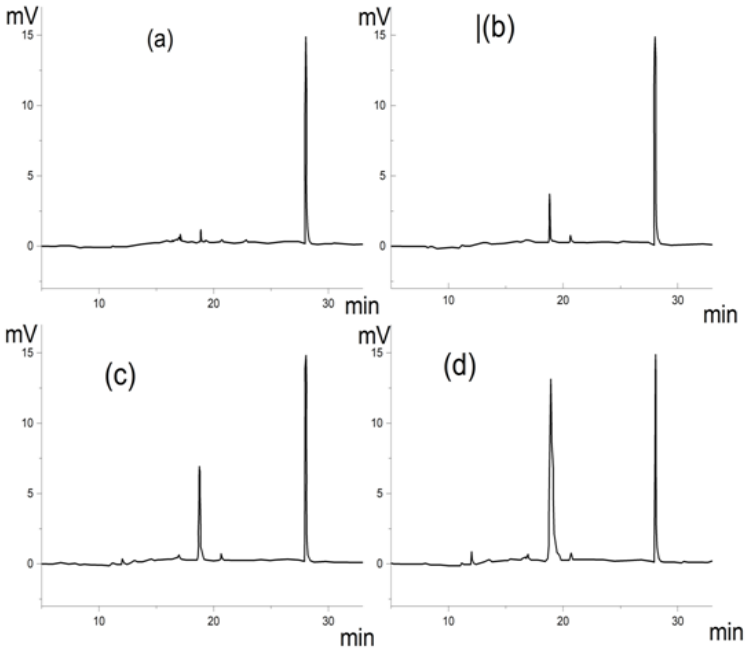

Figure 3. GC–FID chromatogram of DZ in four spiked biscuit samples (RT = 18.44 min) and CZ as internal standard (RT = 28.46 min): (a) 1 µg ml^{-1}; (b) 200 µg ml^{-1}; (c) 400 µg ml^{-1}; (d) 800 µg ml^{-1}.

Table1. Validation parameters and statistical data of the regression equations of the proposed method.

Parameters	Results
Thermal program steps	1– The oven was initially set to 80 °C (1 min). 2– Ramping rate 12 °C min^{-1} up to 180 °C. 3– Ramping rate 15 ◦Cmin^{-1} up to 290 °C. 4– This final temperature was held for 20 min.
Injector temperature	280 °C
Detector temperature	300 °C
Internal standard (IS) conc.	Clonazepam (CZ) 100 µg ml^{-1}
Retention times	18.445±0.078 min (DZ) and 28.466±0.06 min (CZ)
Linearity range (µg ml^{-1})	0.8–1000
correlation coefficient (r^2)	0.9999
Linear regression equation	A= a+bC
Intercept (a)	0.194
Slope (b)	0.044
S.D. of slope (S_b)	3.9×10^{-4}
S.D. of intercept (S_a)	0.001
LOD, µg ml^{-1}	0.0308
LOQ, µg ml^{-1}	0.128

Table 2. Intraday and Interday Precision and Accuracy of DZ in applications

Applications	Conc.	Intraday accuracy and precision				Interday accuracy and precision			
		Measured conc. µg ml^{-1}	Recovery, %	RSD, %	RE, %	Measured Conc. µg ml^{-1}	Recovery, %	RSD, %	RE, %
Pharmaceutical	1	0.99	99	1.66	–1	0.988	98.8	2.44	–1.2
	200	198.8	99.4	1.89	–0.6	198.4	99.2	1.65	–0.8
	400	401.2	100.3	2.56	0.3	395.2	98.8	2.11	–0.2
	800	808	101	0.89	1	792	99	1.34	–1
biscuits	1	0.98	98	2.25	–2	0.984	98.4	3.00	–1.6
	200	197.8	98.9	1.63	–1.1	196.4	98.2	2.46	–1.8
	400	398	99.5	3.51	–0.5	404.8	101.2	1.68	1.2
	800	804.8	100.6	2.72	0.6	802.4	100.3	1.26	0.3
beverage	1	0.974	97.4	3.11	–2.6	0.982	98.2	3.31	–1.8
	200	198.4	99.2	4.23	–0.8	197.8	98.9	4.86	–1.1
	400	394.4	98.6	3.65	–1.4	397.6	99.4	4.28	–0.6
	800	795.2	99.4	2.51	–0.6	801.6	100.2	3.19	0.2
Urine	1	0.978	97.8	4.85	–2.2	0.972	97.2	5.04	–2.8
	200	196	98	3.80	–2	197.2	98.6	4.53	–1.4
	400	407.2	101.8	3.35	1.8	397.6	99.4	3.17	–0.6
	800	809.6	101.2	4.09	1.2	792	99	2.85	–1
blood	1	0.964	96.4	6.12	–3.6	0.972	97.2	4.98	–2.8
	200	194.4	97.2	4.98	–2.8	192.4	96.2	5.36	–3.8
	400	393.6	98.4	5.25	–1.6	396	99	4.67	–1
	800	780.8	97.6	3.89	–2.4	788.8	98.6	3.20	–1.4

Table 3. Stability Data of DZ in applications

Applications	Conc.	bench-top stability		refrigerator (4 °C) for 24 h		−80 °C temperature for 1 month		three freeze−thaw cycles (each at −80 °C)	
		Measured conc. µg ml^{-1}	RSD, %	Measured conc. µg ml^{-1}	RSD, %	Measured conc. µg ml^{-1}	RSD, %	Measured conc. µg ml^{-1}	RSD, %
Pharmaceutical	1	0.98	1.01	0.96	0.83	0.92	1.98	0.86	1.13
	200	204	1.68	192	1.57	196	1.25	190	0.87
	400	391	1.77	392	1.16	386	1.34	378	2.15
	800	783	0.98	788	2.03	808	2.22	776	2.8
biscuits	1	0.94	1.87	1.08	1.98	0.88	0.69	0.86	2.08
	200	208	2.63	190	2.15	192	0.85	176	2.52
	400	415	2.26	382	2.86	382	1.13	371	2.39
	800	780	1.79	770	2.68	768	1.18	753	1.76
beverage	1	1.11	1.85	0.88	2.14	0.92	2.58	0.86	2.12
	200	218	2.06	222	1.96	184	1.36	178	2.59
	400	368	2.39	416	1.48	373	1.67	362	2.81
	800	760	2.96	836	2.86	829	2.50	748	2.16
Urine	1	0.86	2.63	0.8	1.48	0.9	1.63	1.18	1.42

	200	182	2.89	173	1.86	213	1.95	172	0.83
	400	416	3.28	420	1.98	371	2.48	376	2.78
	800	838	3.54	759	2.46	752	2.36	815	2.96
blood	1	0.80	2.59	0.82	2.16	0.84	1.63	0.76	2.69
	200	176	3.11	180	3.28	182	2.14	169	2.47
	400	358	3.68	369	3.04	370	2.85	353	3.15
	800	742	3.08	755	3.2	766	2.99	723	3.45

Moftah A. Moustafa. "GC-FID without derivatization method for the quantitative determination of Drug Facilitated Crimes (Diazepam) in Bulk, Pharmaceuticals, urine, blood, biscuits and beverages. ." *IOSR Journal of Applied Chemistry (IOSR-JAC)*, 14(3), (2021): pp 31-38.