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Research Paper

Quantitative determination of bromazepam in bulk and crime scene applications using GC-FID without derivatization.

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ABSTRACT
Sensitive, simple, rapid, selective, reliable and expeditious GC–FID method without derivatization for the quantitative analysis of bromazepam (BZ) drug has been developed for different analytical and forensic toxicology laboratories in pure form, pharmaceutical preparations, urine, blood, biscuits and beverages. The chromatographic system was carried out using a GC- 17AGas Chromatograph SHIMDZU in conjunction with a column (DP – 5, length: 30 meter and ID: 0.25 ml) was validated. The validation guidelines in terms of linearity, accuracy, precision, matrix effects, stability, selectivity, and recovery have been validated as per US-FDA bioanalytical guidelines. The method was linear over the concentration range of 0.6–800 µg ml^{-1} with limits of detection ad quantification of 0.0253 and 0.108 µg ml^{-1}, respectively. The intraday and interday precisions and accuracy expressed by the relative standard deviation (RSD, %) and the relative standard error (RE, %) were both not more than 4.52 % and 2.5 %, 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 BZ in the presence of all endogenous applications components was studied and the new GC–FID method was successfully employed.

KEYWORDS: *Bromazepam; Clonazepam; GC–FID; Extraction; Stability; crime scene applications; Urine; Blood; Biscuits and Beverages.*

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I. INTRODUCTION

Benzodiazepines are one of the most frequently detected substances in drug facilitated crimes (DFC), due to sedative effect and ability to induce amnesia. A typical pro-active DFC involves covert drugging by drink/food spiking in order to make the unaware victim an easy prey for the offender. The analysis of the evidence at the crime scene is very important since it may help to clarify sexual assault or robbery cases, especially when the episode is reported with delay so that any drug would have been eliminated from blood and urine (1–2). Benzodiazepines identification and confirmation their presences in human body are important in emergency rooms and toxicology laboratory; various reliable and specific techniques are required (3). Bromazepam (1,4-benzodiazepines) is one of the most frequently observed compound in cases of drug-facilitated crime (DFC) and drug-facilitated sexual assaults (DFSA) (4–7). Bromazepam (BZ) has become one of the most commonly used drugs for their sedative, hypnotic, anti-depressive, tranquilizer, anti-convulsant properties and also used as pre-medication for induction or general anaesthesia as widely prescribed throughout the world (8–11). Bromazepam (7-bromo-l,3-dihydro-5-(2-pyridinyl)-2H-1,4-benzodiazepin-2-one) was synthesized in 1963 and following a single administration of 12 mg to 10 subjects, an average peak plasma concentration of bromazepam of 131 ng/ml (107–173 ng/ml) was achieved between 1 and 4 h, declining with an average half-life of 11.9 h (7.9– 19.3 h) ad 3-hydroxy-bromazepam is reported to be its main metabolite in urine (12–14). Clonazepam (CZ), 7-nitro-5-(2-chlorophenyl)-3H- 1,4- benzodiazepine-2(1H)-one as internal standard (I.S.) and bromazepam are considered the most important 1,4-benzodiazepines (Fig. 1) 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 management of alcohol withdrawal syndrome (15–18). Several analytical methods have been reported for bromazepam determination in pharmaceutical and biological fluids including spectrophotometry (19–26), spectrofluorimetry (19–27), spectroscopy (28, 29), HPLC (30–46), GC–MS (47–50), TLC (51), capillary electrophoresis (52, 53), ion-selective electrode (54, 55), potentiometric (56) and voltammetric (21, 57) methods.

This study is important to develop and validate a simple, rapid, sensitive and accurate method for BZ determination using GC–FID without derivatization. This method is successfully applied on bulk, tablets, biological, urine, blood, biscuits and beverages. All methods of extractions were validated to be applicable in different toxicological and analytical laboratories. All past chromatographic methods were based on derivatization process that 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 (58).

II. MATERIALS AND METHODS

2.1. Instrumentation and chromatographic separation

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

2.2. Chemicals

The purity of all standards was at least 99.9%, as certified by the manufacturer, then further evaluation 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. Bromazepam and clonazepam were kindly gifted from (EIPICO, 10th of Ramadan, Egypt). Methanol, ethanol, n-hexan, ethyl acetate, 2-propanol, acetonitrile and PTFE filters (0.2µm×25mm) were supplied from Merck (Darmstadt, Germany). Ammonium hydroxide and Potassium hydroxide were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Deionised water (18.1 MΩ) was obtained from a Millipore-Q water system (Bedford, MA, USA).

2.3. Preparation of Stock Solutions.

Stock solution of BZ was prepared in methanol at 1000 µg ml^{-1} and stored frozen no more than 3 months at -20 ºC. Working solutions were made in methanol and aliquots of the working solutions were evaporated under nitrogen and then reconstituted in biscuits, beverages, blood and urine. The calibration range of BZ was 0.6–800 µg ml^{-1} and the concentration of internal standard is 1µg ml^{-1}.

2.4. Preparation of Calibration Standards and QC Samples.

Working solutions were prepared by further dilution with methanol of BZ stock solution with internal standard (IS). The prepared working solution was used to prepare calibration standards, QC and IS by dissolving in methanol and spiking working standard solutions into blank biscuits, beverages, blood and urine. Seven concentration levels of the calibration standards were prepared to obtain final concentration levels of 0.6, 50, 100, 200, 400, 600, and 800 µg ml^{-1} for BZ. In the same way, four QCs samples at 1, 250, 500, and 750 µg ml^{-1} for BZ 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 BZ (Lexatonil® 6 mg, Roche Co) 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 BZ 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 (59).

Soft drink and urine samples Preparation. Four definite concentrations of BZ were taken from working solutions were evaporated under nitrogen then reconstituted. 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 pH 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 BZ 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 pH 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, BZ definite concentrations of tablets and extractant of all other applications was calculated using the corresponding regression equation of the appropriate calibration graph.

2.6. 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 (61). 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, 250, 500, and 750 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 LLE. 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 (61). 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 BZ 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 BZ quantification of and CZ as shown in **Table 1**. BZ was linear over the range 0.6–800 µg ml^{-1} with a correlation coefficient (r^2 value) of 0.999, 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 BZ and CZ are shown in **Figures 3 and 4**. 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 to be 0.0253 µg ml^{-1} 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 0.108 µg ml^{-1} for all analytes.

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, 250, 500 and 750 µg ml^{-1} 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 –3.2 to 2.5 % 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 0.98 to 4.52% 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 BZ (47–50).

BZ 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 BZ 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 (62). All results of BZ stability for all applications in the different conditions were summarized in **Table 3**; the results indicated that BZ had the required stability without considerable degradation to be kept at room temperature for 1 h with RSD % ranging from 1.53 to 3.95 %, and this time is enough for sample pretreatment. Moreover, BZ was stable with RSD % ranging from 1.08 to 4.06 % 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 0.89 to 3.42 %. Additionally, the drug of interest was exhibited good stability after three thaw−freeze cycles with RSD % ranging from 1.23 to 3.47 % 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) BZ 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 BZ 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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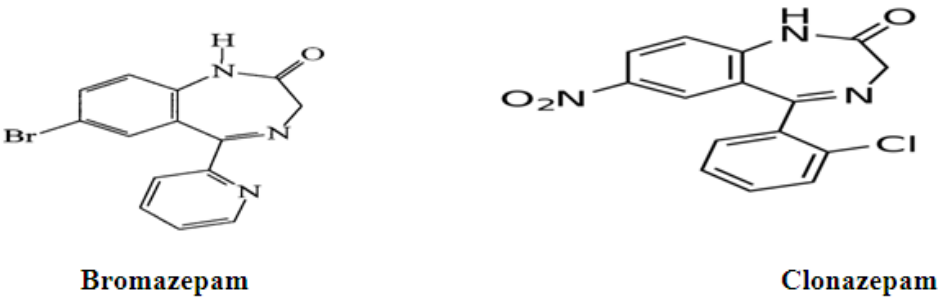

Bromazepam **Clonazepam**

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

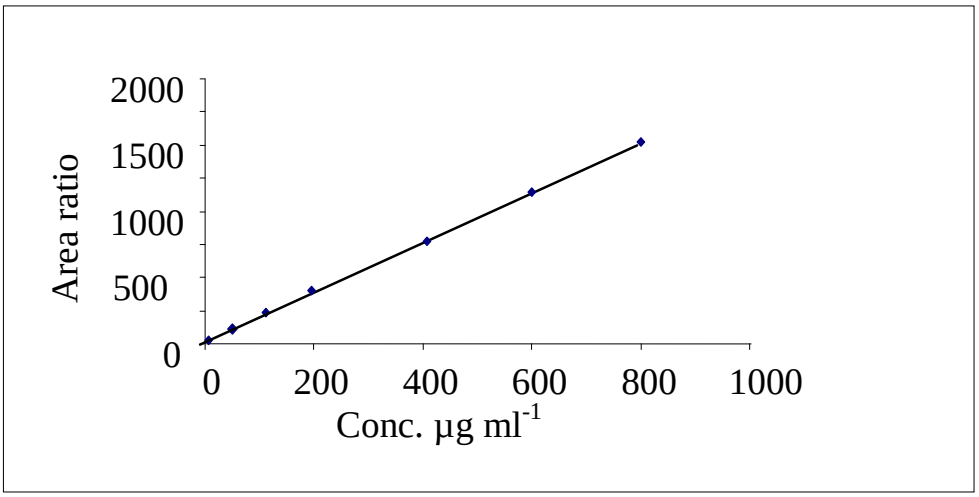

Figure 2. Standard calibration curve of BZ.

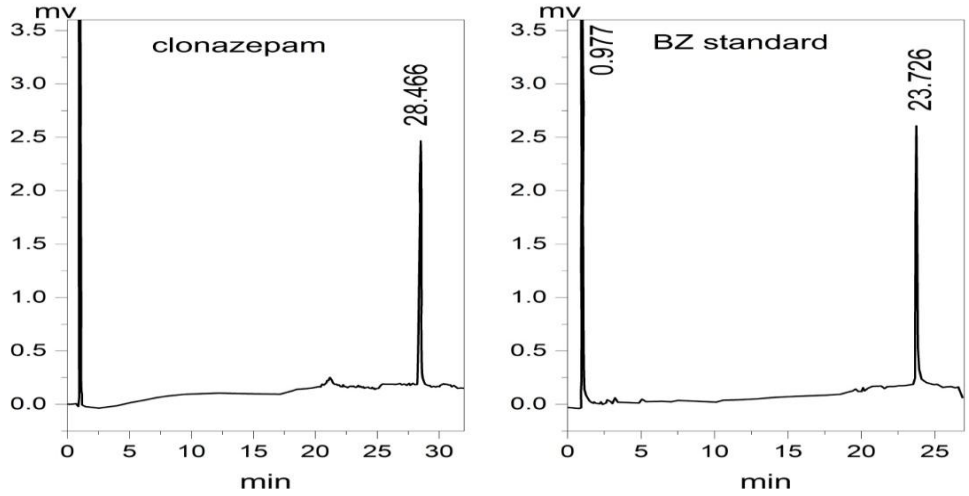

Figure 3. GC–FID chromatogram of clonazepam (CZ) and bromazepam (BZ).

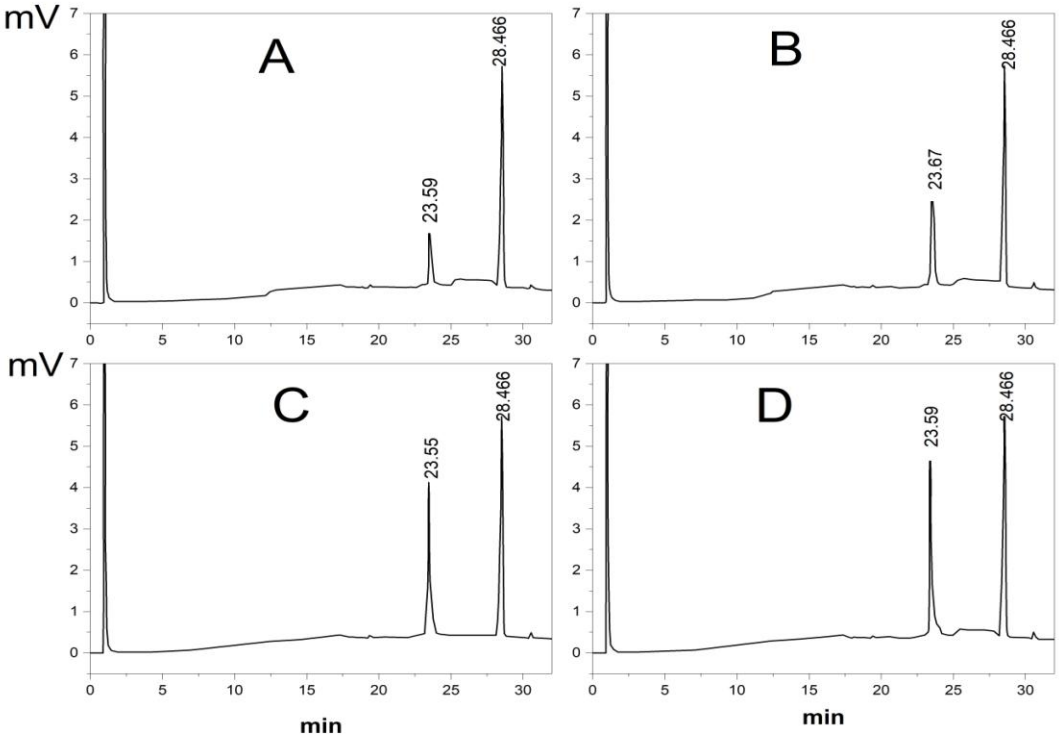

Figure 4. GC–FID chromatogram of BZ in four spiked urine samples: (A) 1 µg ml^{-1}; (B) 2500 µg ml^{-1}; (C) 500 µg ml^{-1}; (D) 750 µg ml^{-1}.

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

Parameters	Results
Ramping rate	A. Oven was initially set1 min to 80 °C for 1 min. B. Ramping rate 12 °C min^{-1} up to 180 °C. C. Ramping rate 15 °C min^{-1} up to 290 °C. D. 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	23.65±0.09 min (BZ) and 28.46±0.06 min (CZ)
Linearity range (µg ml^{-1})	0.6–800
correlation coefficient (r^2)	0.999
Linear regression equation	A= a+bC
Intercept (a)	0.182
Slope (b)	0.04
S.D. of slope (S_b)	3.75×10^{-4}
S.D. of intercept (S_a)	0.001
LOD, µg ml^{-1}	0.0253
LOQ, µg ml^{-1}	0.108

Table 2. Intraday and interday precision and accuracy of BZ 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	0.982	98.2	1.25	–1.8	0.984	98.4	2.23	–1.6
	250	249	99.6	0.98	–0.4	248.25	99.3	2.52	–0.7
	500	495	99	1.55	–1	491	98.2	3.14	–1.8
	750	751.5	100.2	2.37	0.2	738.75	98.5	1.81	–1.5
biscuits	1	0.988	98.8	1.7	–1.2	1.005	100.5	2.9	0.5
	250	248.5	99.4	2.38	–0.6	249.25	99.7	2.28	–0.3
	500	491	98.2	3.46	–1.8	510	102	1.25	2
	750	747	99.6	3.17	–0.4	760.5	101.4	1.38	1.4
beverage	1	1.01	101	3.88	1	0.993	99.3	2.49	–0.7
	250	249	99.6	2.56	–0.4	245.25	98.1	3.02	–1.9
	500	501	100.2	3.24	0.2	492	98.4	3.46	–1.6
	750	754.5	100.6	1.96	0.6	742.5	99	2.72	–1
Urine	1	0.98	98	3.76	–2	0.986	98.6	3.6	–1.4
	250	248	99.2	3.47	–0.8	251.25	100.5	3.59	0.5
	500	487.5	97.5	2.9	–2.5	495.5	99.1	4.25	–0.9
	750	733.5	97.8	1.58	–2.2	757.5	101	4.03	1
blood	1	0.968	96.8	2.61	–3.2	1.025	102.5	2.98	2.5
	250	245.25	98.1	3.49	–1.9	254.25	101.7	3.62	1.7
	500	487.5	97.5	4.15	–2.5	490.5	98.1	4.52	–1.9
	750	742.5	99	4.23	–1	741.75	98.9	3.14	–1.1

Table 3. Stability data of BZ 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	0.99	1.53	1.12	1.55	0.93	0.92	0.84	1.46
	250	252	1.98	258.25	1.43	236.4	1.11	238.25	1.53
	500	492.2	1.7	488.57	1.08	476	1.72	490.4	2.5
	750	760.8	1.13	742.8	2.12	766.8	1.66	762.5	1.98
biscuits	1	1.002	2.06	1.06	1.96	0.86	0.89	0.81	3.02
	250	248	2.72	262.75	2.87	266	1.23	236.5	2.72
	500	448.5	3.18	510	2.52	472.6	1.81	428.12	2.59
	750	738.7	2.78	738.75	2.99	725.25	1.99	720.4	2.76
beverage	1	0.96	1.99	0.82	3.44	0.81	1.65	0.8	3.11
	250	240.8	2.42	238.5	2. 6	228	1.72	254.25	3.28

	500	485.6	3.18	480	3.15	462.2	2.86	459.4	1.86
	750	742.5	2.83	735.8	2.16	720	3.02	722.46	2.4
Urine	1	0.92	3.72	0.83	1.91	0.8	2.53	0.83	3.47
	250	240.2	2.45	236.5	2.77	236	2.71	224.5	1.23
	500	515.6	3.15	482	3.08	468.4	3.42	472.4	2.11
	750	772.25	3.86	734.6	4.06	730.88	2.48	715.42	3.56
blood	1	0.83	2.12	0.79	3.1	0.85	1.89	0.78	2.9
	250	233.8	3.89	222.8	3.84	232.2	2.63	215	1.87
	500	480.8	3.95	467	3.55	479.25	2.9	460.12	3.11
	750	738.4	3.47	786.75	3.97	729	3.42	710.5	3.29