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An analytical approach to drug-facilitated traffic accident: Investigation of benzodiazepine and alcohol in biological samples

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Abstract

Benzodiazepines are prescribed for the treatment of sleep disorder, anxiety and muscle pain and also have addiction potential due to their effects on central nervous system. These frequently abused substances may also cause impaired driving skill. The aim of this prospective study was to develop an optimized Gas Chromatography – Mass Spectrometry (GC-MS) method for some benzodiazepines in serum and to perform the method on real samples collected from injured drivers. It was also aimed to simultaneously determine the frequency of ethyl alcohol use during traffic accidents. Samples were collected from 44 injured drivers involved in traffic accidents. Serum samples (n=40) were extracted by Solid Phase Extraction (SPE) method and analyzed by GC-MS for benzodiazepines, and whole blood samples (n=37) were analyzed by Head Space/GC-MS for alcohol analysis.The linear range was 1.25-30 µg/mL for diazepam, 5-120 µg/mL for alprazolam and 10-60 µg/mL for clonazepam with $r^2 \geq 0.997$ correlation coefficients while the recovery value was ≥70.2%. Limits of Detection for diazepam, alprazolam and clonazepam were 0.09 µg/mL, 0.2 µg/mL and 0.77 µg/mL respectively. All these substances did not determine in any of the serum samples. Considering the alcohol analysis, 16.2% of drivers were drunk with 2-348 mg/dL alcohol concentrations. Although the numbers of samples were limited, surprisingly the rate of drunk drivers was substantially high. As a conclusion, an analytical method has been developed for determination of the mostly used benzodiazepines in Turkey, which may impair driving ability; it is needed to conduct further studies with more cases to reveal the effect of prescribed drugs on traffic accidents.

Keywords: Benzodiazepines, alcohol, traffic accident, diazepam, alprazolam, clonazepam

Introduction

Driving under the influence of drugs (DUID) is a term used to describe individuals' driving action that may hinder traffic safety following the illicit or medical drug consumption [1]. Although driving is a daily activity, it is an action in which the organs work in coordination with the central nervous system, and that needs to act quickly by thinking fast [2, 3]. Some prescribed psychotropic drugs also used in everyday life affect the driving performance and increase the risk of accident due to the effects and/or side effects that cause loss of attention and reflexes, decreased vision and decision-making, increased self-confidence and risk-taking behaviors [4, 5].

In the project of Driving Under the Influence of Drugs, Alcohol and Medicines in Europe (DRUID Project) analyses were carried out in the blood and oral fluids of 50,000 drivers from 13 countries. As a result of the project, the alcohol rate was found 3.48% and illegal substance was 1.9% and medications were 1.36% [6]. The International Alcohol, Drugs and Traffic Safety Council (ICADTS) has divided medical drugs into 3 categories according to their effects on driving performance. Benzodiazepines are generally classified in Category 2, which has moderately negative effects on driving, and Category 3 which is considered potentially dangerous as well [7]. Besides, the risk of having an accident by a driver using alcohol increases 4-10 times as the blood alcohol concentration increases. In a study conducted in the USA in 2013, it was reported that approximately half of the drivers who died as a result of traffic accidents used alcohol. In Turkey, according to the final declaration of Symposium on Effectively Fighting with Alcohol and Drugs in Traffic, it was stated that the alcohol detection rate between the drivers varies between 15.4% and 35.2% [8].

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In a 5-year study in Australia, blood samples were analyzed in order to research prevalence of alcohol and drugs in injured drivers, and alcohol rate was 15.8% and diazepam was the most common benzodiazepine [9]. In another study conducted with injured drivers coming to the emergency departments of 6 different hospitals, blood samples were analyzed. Among 900 drivers, the rate of positive alcohol usage, which was above the legal limit, was 26%, diazepam ratio was 1.1%, and alprazolam was 0.2% [10]. In a similar study, between 2010-2011 in Turkey, one psychoactive substance was detected in 91 of 4274 drivers in addition to alcohol. The rate of using alcohol alone was in 44% of those, while cannabis, benzodiazepines, barbiturates, antidepressants, cocaine and amphetamines were detected at the rest. Blood samples were analyzed with Head Space – Gas Chromatography (HS-GC) for alcohol and with Cloned Enzyme Donor Immunoassay (CEDIA) and Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) for drugs/substances [4]. Additionally, in a study conducted in Turkey stated that 82.3% of drug facilitated crime cases was related with benzodiazepines especially for clonazepam, diazepam, alprazolam. Benzodiazepines were one of the most abused prescribed drugs in the files evaluated in the Council of Forensic Medicine in 2008-2009 within the scope of "substances whose production is subject to the permission of the official authorities or prescription which is regulated by the authorized physician, and which have a drug or stimulant effect" [11].

In this study, the prevalence and the risks of alcohol and frequently used benzodiazepines (diazepam, alprazolam and clonazepam) to the traffic safety, which are in the class of controlled prescribed drugs were investigated. For this purpose, whole blood and serum samples were collected from injured drivers (n=44) in an emergency service. For the quantitative and qualitative determination of benzodiazepines, a GC-MS method was developed and samples were analyzed. For alcohol analysis, head space GC-MS method was used as well.

Materials and Methods

Chemicals and Equipment

Stock solutions of diazepam, alprazolam and clonazepam were purchased from Lipomed (Arlesheim, Switzerland) and internal standard (IS), n-docosane were purchased from Sigma (Taufkirchen, Germany). Methanol (MeOH), isopropanol, acetonitrile (ACN), n-butanol, acetic acid, ethanol (EtOH), β-glucuronidase-arylsulfatase enzyme and sodium acetate trihydrate (for buffer solution) were supplied from Merck (99.8–100%, Darmstadt, Germany). Oasis HLB (3mL, 60 mg) Solid Phase Extraction (SPE) cartridges were purchased from Waters (Milford, MA, USA). GC-MS analyses were carried out using Agilent 7820A GC and Agilent 5977E MS for benzodiazepines (Agilent Technologies, Palo Alto, CA USA) and alcohol analysis were carried out HP 6890 GC and Hewlett Packard 5973 MS with Agilent 7694E HS. Evaporation under nitrogen stream was conducted with a HyperVap HV-300 from Gyrozen (Daejeon, Rep. of Korea). A Direct-Q UV 3 ultrapure water system was acquired from Millipore (18.2 MΩ cm) (Molsheim, France). SPE negative pressure vacuum manifolds with 24 ports were used for the extraction of the samples.

Instrumental Analysis of Benzodiazepines

Method validation: Stock solutions of reference standards (diazepam, clonazepam and alprazolam) were prepared as 1 mg/mL concentrations in methanol. The stock solutions were used to obtain mixed working solutions (calibration solutions) containing all targeted substances in order to achieve the calibration curves. Calibration solutions, containing 5 µg/mL n-docosane as IS were prepared in incremental amounts including blank solution (diazepam: 1.25, 2.5, 5, 7.5, 12.5, 15, 20, 25, 30 µg/mL; alprazolam: 5, 10, 20, 30, 50, 60, 80, 100, 120 µg/mL and clonazepam: 10,15, 20, 30, 40, 50, 60 µg/mL). Linearity was achieved by six replicates of each calibration point. Calibration curves were obtained using peak area ratios of each analyte to internal standard.

Limit of Detection (LOD) was calculated using 3 σ/slope of calibration curves of standards and Limit of Quantitation (LOQ) was calculated using 10 σ/slope of calibration curves of standards.

Extraction efficiency and RSD% calculations were based on analyte responses of the blank serum sample. They were estimated by spiking two different concentrations (15 and 40 µg/mL for clonazepam, 7.5 and 20 µg/mL for diazepam, and 30 and 80 µg/mL for alprazolam) into blank serum samples in six replicates.

Sample preparation and SPE procedure: 900 µL of serum sample was mixed well with 900 µL acetonitrile (1:1) and centrifuged at 5000 rpm for 15 min to precipitate proteins. Then the supernatant was collected and 2.5 mL of 0.1 M acetate buffer (pH 5.5) and 50 µL enzyme β –glucuronidase/arylsulfatase were added into the test tube and incubated for 60 min at 55°C. The SPE cartridges (3mL, 60mg) were conditioned with 2 mL methanol and 2 mL ultra pure water. The samples were transferred and slowly passed through the cartridge under the vacuum. Afterwards, cartridges were washed using 2 mL ultra pure water and acetonitrile (H_2O: ACN) (80:20) and dried under a full vacuum for 5 min. Following the elution step, analytes were eluted using 0.8 mL methanol and 0.8 mL methanol-isopropanol (3:1). The eluate was evaporated to dryness under a stream of nitrogen at 37°C and reconstituted to 200 µL methanol which contains 5 µg/mL n-docosane solution. It was injected into the GC/MS system in 3 µL injection volume [12-15].

GC-MS analysis: Separation was performed using a fused silica capillary column (30m*250µm*0.25µm) (HP-5MS 5% Phenyl Methyl Siloxane – Agilent Part No. 190991S-433). Total analysis time was 19.5 min and in the temperature gradient mode: 0 min at 160°C, held for 1 min at 160°C and increases by 30°C per minute and reaches 265°C and analysis continues for 15 min at this temperature. Solvent delay time was 4.8 min and in the split mode (8:1). Helium was used as a carrier gas. The mass spectrometer was performed in scan mode between 50-600 m/z. Detection and identification of compounds of this study was conducted in selected ion monitoring (SIM) and scan mode together.

Instrumental Analysis of Alcohol

Routine alcohol analysis procedure of Istanbul University – Cerrahpaşa, Institute of Forensic Sciences and Legal Medicine, Forensic Toxicology Laboratory was applied for alcohol analysis.

Sample preparation for HS-GC/MS: One mL whole blood sample

was analyzed by adding 500 µL n-butanol (IS) solution (in 1.6 g/L concentration).

Separation was performed using a fused silica capillary column HP-5MS (30m*250µm*0.25µm). Total run time was 3.5 min and data analysis was started at 0 min. After waiting at 37°C for 2 min, temperature increases 2°C per minute and reached to 40°C. Device was analyzed in split mode (50:1) and in selected ion monitoring (SIM) mode.

Sample Collection

This prospective study was approved by University of Health Sciences, Haseki Education Research Hospital Ethical Committee with approval number of 242 on 22nd July of 2015. Biological samples were collected by the staff of the emergency department from the drivers who applied to University of Health Sciences, Haseki Research Education Hospital Emergency Department as a result of traffic injuries and who approved to participate in this study by signing the "Informed Consent Form". The clinicans of the related emergency department also obtained the consent of the patients. Between August 2016 – November 2017, a total of 37 whole blood samples for alcohol analysis and 40 serum samples for benzodiazepine analysis were obtained from 44 drivers in total and were analyzed in Istanbul University-Cerrahpaşa, Institute of Forensic Sciences and Legal Medicine, Forensic Toxicology Laboratory. All injured drivers who signed the form to participate to this study between these dates were included in this study. Since some of participants did not approve to give both serum and whole blood samples, number of analyzed biological samples was not equal. Whole blood samples were collected into EDTA containing tubes (2 mL) and serum samples were also collected into dry vials (2 mL). Alcohol-free sampling procedure was conducted to avoid the contamination for alcohol analysis during whole blood collection. The collected samples were kept at -20°C until analysis. The sociodemographic information obtained from the "Participant Information Form" of 44 drivers was given in Table 1.

Table 1. Distribution of participants due to age, sex and accidents.

Number of drivers	44
Sex	Female: 2 (4.6%) Male: 42 (94.4%)
Mean age ± SD	32.4 ± 11.9
Age range	16-63
Motorcycle accidents (n, %)	15 (34.1%)
In-vehicle traffic accidents (n, %)	29 (65.9%)

Results

Analytical Method

Retention times, m/z parameters, correlation coefficient (r^2), linearity, LOD and LOQ values for benzodiazepine analysis in serum samples and for alcohol analysis in whole blood samples were calculated, precision studies were conducted in 6 replicates. The correlation coefficients were $r^2>0.997$ with reference materials. LOD and LOQ values were calculated according to the ratio of standard deviation of the lowest concentration point to the slope of calibration curve (LOD = 3 x σ/Slope and LOQ = 10 x σ/ Slope) [16]. For recovery studies, 2 different calibration points in 6 replicates, representing low and high levels, were added to the blank serum sample before the extraction procedure, and were analyzed in GC-MS. Recovery%, RSD% and other performance parameters were shown in Table 2. Recovery values were determined between 70.23%-76.67%, the RSD values were determined as <15% for all substances. A couple of chromatograms for the mixed calibration solution were given in Figure 1. The chromatogram (upper) shows the result of the scanning mode and the chromatogram seen at the bottom shows the result of the SIM mode for diazepam, alprazolam, clonazepam. The chromatograms of serum sample spiked with reference standards of diazepam, alprazolam and clonazepam are given in Figure 2.

Table 2. Chromatographic method parameters of diazepam, clonazepam, alprazolam and ethanol

Compound	Rt±SD min	m/z	Lin. Range (µg/mL)	Cor. Coef. (r^2)	Conc. (µg/mL)	Rec. % (SD)	RSD %	LOD (µg/mL)	LOQ (µg/mL)
Diazepam	7.414±0.005	256 283	1.25-30	0.9997	7.50 20	76.67 (0.47) 73.3 (1.15)	8.17 7.85	0.09	0.27
Clonazepam	12.540±0.011	280 314 234	10-60	0.9987	15 40	74.6 (1.05) 70.23 (2.87)	9.38 10.23	0.77	2.34
Alprazolam	14.637±0.054	279 204 308	5-120	0.9990	30 80	74.54 (1.85) 74.08 (5.64)	8.27 9.52	0.2	0.6
Ethanol	1.567± 0.036	31 46	0.01-1*	0.9976	-	-	-	2*	5,9*

*mg/dL

Table 3. Positive alcohol results of drivers

Sample ID	Alcohol concentration (mg/dL)
6	184
9	192
10	12
13	205
25	348
33	2

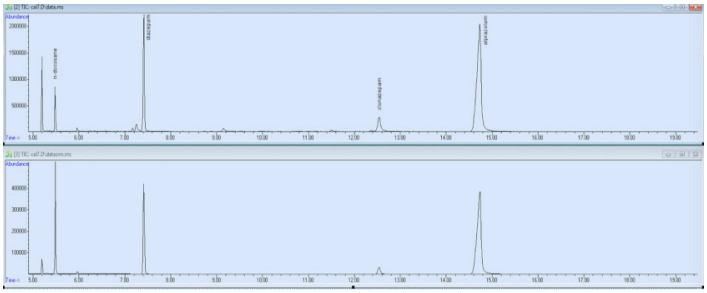

Figure 1. The chromatograms of 5 µg/mL n-docosane, 20 µg/mL diazepam, 40 µg/mL clonazepam and 80 µg/mL alprazolam (with scan and SIM methods)

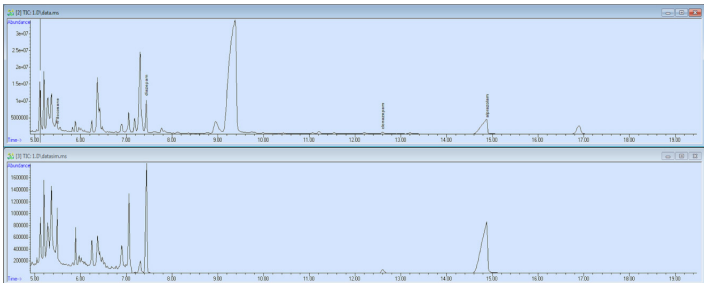

Figure 2. The spiked serum sample's chromatogram 40 µg/mL diazepam, 80 µg/mL clonazepam, 160 µg/mL alprazolam (with scan and SIM method)

Analysis of Drivers' Samples

As a result of experiments, diazepam, clonazepam and alprazolam active substances were not found in 40 serum samples collected from drivers. Considering the alcohol analysis, ethyl alcohol was found in the blood samples of 6 drivers (16.2%) among 37 ones. Alcohol concentration levels were given in Table 3 and a representative chromatogram with positive alcohol result was given in Figure 3.

Table 4. Comparative findings of studies using chromatographic methods performing benzodiazepine analysis in biological material

Reference	Method	Compund	r2	Lineer Range (µg/mL)	LOD	Recovery Concentration	Recovery %	RSD %
Adamowicz et al. 2010 [12]	SPE, GC-EI-MS urine	Diazepam	0.9659	0.4-50	0.3 µg/mL	-	16	-
		Alprazolam	0.9831	0.3-50	0.2 µg/mL	-	67	-
		Clonaze-pam-TMS	0.9984	20-50	12 µg/mL	-	16	-
Borrey et al. 2001 [13]	SPE, GC-MS urine	Alprazolam	0.9996	0.05-1	16 ng/mL	5 ng/mL	92.67	-
						100 ng/mL	99.27	-
						1000 ng/mL	94.8	-
Borrey et al. 2001 [14]	SPDE, LC/TOF-MS serum, urine	Serum Diazepam	>0.998	0.5-2	1-10 ng/mL	50 ng/mL	93.7	3.0
						500 ng/mL	98.7	4.2
		Alprazolam	>0.998	0.5-2	1-10 ng/mL	50 ng/mL	104.8	3.6
						500 ng/mL	104.7	3.1
		Urine Diazepam	>0.998	0.5-2	1-10 ng/mL	50 ng/mL	99.4	3.4
						500 ng/mL	91.5	4.1
		Alprazolam	>0.998	0.5-2	1-10 ng/mL	50 ng/mL	99.1	3.5
						500 ng/mL	100.1	4.2
This study	SPE, GC-MS serum	Diazepam	0.9997	1.25-30	0.09 µg/mL	7.5 µg/mL	76.67	8.17
						20 µg/mL	73.3	7.85
		Alprazolam	0.9990	5-120	0.2 µg/mL	30 µg/mL	74.54	8.27
						80 µg/mL	74.08	9.52
		Clonazepam	0.9987	10-60	0.77 µg/mL	15 µg/mL	74.6	9.38
						40 µg/mL	70.23	10. 23

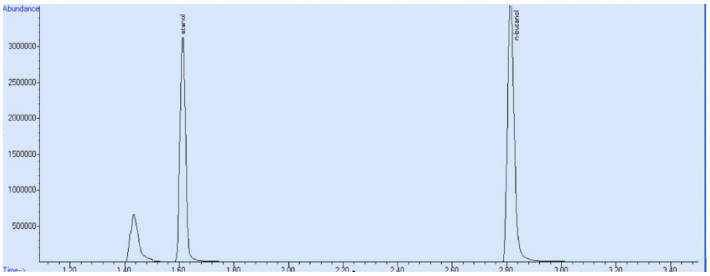

Figure 3. Total ion chromatogram of a whole blood sample with 205 mg/dL alcohol

Discussion

Benzodiazepines have become one of the psychotropic drug groups that are frequently prescribed in many countries and are widely abused in addition to medical use with their introduction into medical practice in the 1960s [17, 18]. They are generally considered safe because of their side effects being more easily tolerated, less serious side effects in overdose, low drug interaction and rapid onset of effects. However, they increase the risk of home and traffic accidents, especially when they used with other substances such as alcohol, illegal substances, tranquilizers and antidepressants. In addition, they can be used in drug-facilitated crimes, especially in sexual assaults. Because of all these reasons, green prescription application was validated in Turkey for their usage [8, 17]. When consider the frequency of usage and misuse of these substances, in a study on depression complaints, approximately one-third of patients were prescribed benzodiazepines [19]. In another study conducted in the USA between 2004-2011, the applications to the emergency services increased due to the misuse of benzodiazepines [20]. Similarly in Turkey, in 2011, the benzodiazepine prescribing frequency was 2.06% and often-preferred benzodiazepines were alprazolam (76.4%), clonazepam (11.4%) and diazepam (6.8%) [21]. And in a study conducted with high school students in Istanbul, it was concluded that benzodiazepines were used at least once after hookah, alcohol, cigarettes and volatile substances [22]. Due to their widespread use, various reliable and selective analytical methods have been applied for the determination of benzodiazepines [17]. For example, a gas chromatography-electron impact-mass spectrometry (GC-EI-MS) method has been developed using SPE [12]. In a sample preparation method, it was stated that the use of water and acetonitrile both in the washing step reduces the matrix effect in the SPE stage [13]. In the method developed by Saito et al. for the analysis of some benzodiazepines including alprazolam and diazepam using solid-phase dispersive extraction (SPDE) and liquid chromatography/time-of-flight mass spectrometry (LC/TOF-MS), serum and urine samples were studied. Protein precipitation and enzyme hydrolysis steps were used for the serum samples [14]. For the analysis of benzodiazepine and tricyclic antidepressants, it was suggested that acetonitrile was also used in the washing step of the solid phase extraction so as to minimize the matrix effect [15]. Validation findings obtained from the above-mentioned studies were detailed in the Table 4 for comparison with the results of this study. In this study for the analysis of diazepam, alprazolam and clonazepam, an analysis method was developed based on the methods of Adamowicx and Kala, utilizing the methods of Saito et al. and Mazhar and Binder [12, 14, 15]. In sample preparation, protein precipitation was performed before the SPE method, thus the SPE method became more applicable. Moreover, various trials were carried out to reduce the matrix effect in the SPE step. For this, only the ultra-pure water specified in the method of Adamowicz and Kala, as well as the washing step of 5% MeOH and ultra-pure water found in the procedure of the manufacturer company of the Oasis HLB SPE cartridge were compared separately. However, in the attempts using the literature [13, 15], it was observed that using acetonitrile with ultrapure water instead of MeOH gave the best result for the benzodiazepine analysis to reduce the matrix effect. Compared to the similar studies seen in Table 4, quantitation results of this study had higher correlation coefficient value especially for diazepam and alprazolam and had similar results for clonazepam. As for the linear range, there were different results due to the chromatographic techniques and detectors were used.

In our study, the recovery values were between 70.23-76.67% and RSD% values were <%15 for all substances. Considering Table 4, it was seen that there were similar recovery results for diazepam, alprazolam and clonazepam [12]. Eventhough the extraction efficiency of our method was <80%, low RSD% found throughout the experimental steps were within acceptable limits [23]. According to studies listed in Table 4, wide ranges of results were found at ng/mL and µg/mL concentrations for LOD, LOQ values and linear calibration interval. Especially in method using LC/TOF-MS, lower LOD and LOQ concentrations were found [14]. It is thought that a lower LOD concentration was reached due to the differences in sample preparation and analysis conditions such as temperature, sample and injection volume as well as different analytical equipment. In our study, the results were similar to studies using GC-MS method.

According to the World Health Organization's 2015 road safety report, although deaths pertaining to traffic accidents were in the 9th place among all causes of death, it is foreseen that this will increase to the 7th place in 2030. Additionally, it is also included in the report that deaths caused by injuries resulted from traffic accidents among the first causes of all deaths between the ages of 15-29 [24]. According to the data of Turkish Statistical Institute belonging to 2018, as a result of 186,532 traffic accidents with deaths and injuries of 307,071 people wounded. And, when faults are considered resulting in accident, faults emanating from the driver is at rate of 89.5% [25]. Due to the side effects of benzodiazepines such as sedation and impaired cognitive performance, the behaviors of drivers such as steering, road tracking and ability to make decisions in emergencies may be affected [3, 26]. The risk is higher particularly, when they are used at high doses and are in the first weeks of use. Besides, benzodiazepines with long half-life have more negative effects on driving performance [3, 17]. For this reason, studies on the effects of benzodiazepines on driving safety become important.

Alcohol also causes behavioral changes in individuals. Due to the impairment of cognitive functions and psychomotor performance, deterioration in decision-making behaviors and perception, weakening visual and auditory functions, problems in compliance capabilities and in memory, and negative effects in driving performance are observed [27]. In the analysis of blood samples of 275 Korean drivers, who gave positive results for alcohol in roadside tests, the rate of substance use was examined using SPE and GC-MS. Totally, in 4 of 14 positive results for diazepam and its metabolites were found [28].

In this study, whole blood and serum samples were collected from the injured drivers who applied to the hospital as a result of traffic accident and were analyzed with this developed method. For alcohol analysis, routine blood alcohol analysis procedure of Istanbul University-Cerrahpaşa, Institute of Forensic Sciences and Legal Medicine Forensic Toxicology Laboratory was used. None of investigated benzodiazepines were in any of the 40 serum samples collected from drivers. Ethyl alcohol was in 6 (16.2%) of 37 whole blood samples collected for alcohol analysis. However, according to the legal limit of 50 mg/dL, which is applied only to private car drivers, a positive result was found for 4 drivers (10.8%). Compared to the researches on the driving under the influence of substances, drugs and alcohol, there are different results for benzodiazepines and alcohol according to the place, time and purpose of the studies. For example, in the study of Acar et al., 91 of 4273 cases were suspected of substance and alcohol. Among these suspects, benzodiazepine substances were detected only in the blood of 4 drivers [4].

Conclusion

Benzodiazepines and alcohol can compromise driving skills and endanger the traffic safety. In this study, a selective and reliable method has been developed in serum samples for the simultaneous determination of alprazolam, diazepam and clonazepam which are the most prescribed benzodiazepines in our country.

In this study, a general evaluation was tried to make among the samples from traffic accident injuries coming to the emergency department of only one hospital. However, when evaluating the results of our study, it should be taken into consideration that the number of collected samples is lower than expected due to the reasons such as the working conditions and workload of the emergency departments in the hospital. Additionally, the samples could not be collected due to the state of health of the patient. Another limitation is that, clonazepam as an aromatic 7-nitro compounds can partially degrade at high temperatures and depending on the duration time of the analyte remains in the columns in some cases. A derivatization step is recommended to improve the thermal stability and to get better peaks when working with GC-MS.

The lack of sufficient studies in our country about the effects and prevalence of this drug groups in traffic, especially with its medical use, as well as its abuse, makes such studies important. Considering the mortality and injury rates caused by traffic accidents, more studies are recommended to reveal the effect of benzodiazepines on drivers, their use with alcohol and detection in traffic safety. For this reason, it is expected that this developed method will contribute to benzodiazepine analysis and driving safety studies with different biological samples to be taken from different regional hospitals in the long term. Further studies are planned to continue our research in the future with cooperation of more emergency departments to expand the monitored drug spectrum

Conflict of interests

The authors declare that they have no competing interests.

Financial Disclosure

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Ethical approval

This study was approved by University of Health Sciences, Haseki Education Research Hospital Ethical Committee with approval number of 242 on 22nd July of 2015.

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