EFFECT OF PUTREFACTION ON POSTMORTEM DETECTION OF ETHANOL AND DIAZEPAM : AN EXPERIMENTAL STUDY

BY

Safaa A. Amin and Amira M. El Seidy

Department of Forensic Medicine and Clinical Toxicology

Faculty of Medicine- Menoufiya University

ABSTRACT

Drugs and alcohol concentrations are regularly used as evidence in criminal and civil litigations. The interpretation may be critical to the thorough investigations of different types of forensic cases whether antemortem or postmortem. So, the aim of the present work is to study the effect of putrefaction on the postmortem detection of ethanol and diazepam. The study was conducted on 96 adult male albino rats divided randomly to four groups 24 rats for each and every group is further subdivided into 4 subgroups (a, b, c, and d) 6 animals for each subgroup. Group I: control group received 1ml distilled water. Group II: rats received lethal dose of ethanol. Group III: rats received lethal dose of diazepam. Group IV: rats received lethal dose of both ethanol and diazepam. After death of treated animals, control animals were sacrificed at the same time and immediate dissection of subgroup a was done. Rats of subgroups b ,c and d were dissected after death at 18 hours, 24 hours and 48 hours respectively. The livers were extracted for detection of ethanol and diazepam. The control group showed increased liver ethanol in subgroups I-c and I-d. Liver ethanol of group II and IV showed gradual elevation by increasing post mortem interval while there was no considerable change in liver diazepam of group III. As regards liver tissue diazepam of group IV, a non-significant elevation was detected in the subgroups IV-b, IV-c and IV-d as compared to the subgroup IV-a.

Key words: *postmortem, putrefaction, ethanol, diazepam, detection.*

INTRODUCTION

Biochemical processes, which are strictly controlled in the living organism, can be changed drastically in the course of disease and can influence the concentration of many substances (Kala and Chudzi-Kiewicz, 2003).

Death is defined as cardiac arrest, respiratory arrest and stopping cerebral functions, after this fact, all processes in the dead body veer towards thanato-chemical changes (Senkowski et al., 1990).

In the early phase of postmortem

decomposition, the dead body is exposed to autolysis, i.e. dissolution of organs under the impact of endogenous enzymes and parallel to that putrefaction is begun mainly by reductive processes due to the action of endogenous bacteria and their enzymes (Melvin et al., 1984).

Postmortem degradation does not only concern volatile compounds but also many other substances such as benzodiazepines, tricyclic anti-depressant and phenothiazines which could be metabolized by microorganisms during the putrefactive process (Robertson and Drummer, 1995).

So, the present study aimed to study the effect of putrefaction on the detection of ethanol and diazepam in liver tissue of adult male albino rats.

MATERIALS AND METHODS

1. Chemicals :

Absolute ethanol HPLC grade (Sigma Aldrich).

Diazepam "standard powder" (Nile pharmaceutical Co.).

Chloroform (Fisher Scientific).

Formalin (El-Nasr pharmaceutical chemical Co.)

Potassium dichromate (El-Nasr pharmaceutical chemical Co.)

Sulfuric acid (Sigma Aldrich).

Picric acid (universal fine chemical PVT. TD. India).

2. Equipment :

Steam distillation apparatus (Scientific equipment factory).

Hot plate.

Test tubes and pipettes.

A syringe with large curved spinal needle.

Glass homogenizer.

Single beam spectrophotometer (T60U PG instruments limited).

3. Animals :

This study was done on 96 male albino rats. Their weights ranged between 150-200 gm. They were divided into 4 groups as follows:

Group I (control group) comprised 24 animals divided into 4 equal subgroups: I-a, I-b, I-c and I-d. Group II (ethanol group) comprised 24 animals divided into 4 equal subgroups: II-a, II-b, II-c and II-d. Group III (diazepam group) comprised 24 animals divided into 4 equal subgroups: III-a, III-b, III-c and III-d. Group IV (ethanol and diazepam group) comprised 24 animals divided into 4 equal subgroups: IV-a, IV-b, IV-c and IV-d.

Methods :

Animal experimentation was carried out in an ethical manner according to guidelines set by ethical committee of Menoufiya University.

Rats are housed at room temperature and were fed standard laboratory diet ad labium.

Normal and healthy environment was maintained for all rats through taking strict hygienic measures. They were left to accommodate for four days, then they were randomly divided into 4 equal groups (24 rats for each group) as follows:

Group I (control group): 24 animals received distilled water (1ml) orally and were divided into 4 subgroups:

Subgroup I-a: 6 animals were sacrificed by cervical dislocation at the same time of death of other treated subgroups, then immediately dissected.

Subgroup I-b: 6 animals sacrificed, and left for 18 hours at room temperature, then dissected.

Subgroup I-c: 6 animals sacrificed, and left for 24 hours at room temperature, then dissected.

Subgroup I-d: 6 animals sacrificed, and left for 48 hours at room temperature, then dissected.

Livers were taken from all animals for detection of ethanol and diazepam.

Group II (ethanol treated): 24 animals received the oral lethal dose of absolute ethanol 8000mg/kg (Gable, 2004) then divided into 4 subgroups (6 animals for each subgroup):

Subgroup II-a: animals were dissected immediately after death.

Subgroup II-b: animals were left for 18 hours after death at room temperature, and then dissected.

Subgroup II-c: animals were left for 24 hours postmortem at room temperature, and then dissected.

Subgroup II-d: after death, animals were left for 48 hours at room temperature, and then dissected.

Livers were taken from all animals for ethanol detection.

Group III (Diazepam treated): 24 rats received the oral lethal dose of diazepam 1240 mg/kg (Thomson healthcare Micromedex, 2000) then divided into 4 subgroups (6 animals for each subgroup):

Subgroup III-a: animals were dissected immediately after death.

Subgroup III-b: after death, animals were left for 18 hours at room temperature, and then dissected.

Subgroup III-c: animals were left for 24 hours after death at room temperature, and then dissected.

Subgroup III-d: animals were left for 48 hours postmortem at room temperature, then dissected

Livers were taken from all animals to detect diazepam.

Group IV (Diazepam and ethanol treated): 24 rats received the oral lethal dose of both diazepam and ethanol then

divided into 4 subgroups (6 animals for each subgroup):

Subgroup IV-a: animals were dissected immediately after death.

Subgroup IV-b: animals were left for 18 hours postmortem at room temperature, and then dissected.

Subgroup IV-c: after death, animals were left for 24 hours at room temperature, and then dissected.

Subgroup IV-d: animals were left for 48 hours after death at room temperature, and then dissected.

Animals of group II and III died within 1 hour, while animals of group IV died earlier i.e. after one hour.

After dissection, 300mg of liver tissue was taken for extraction and detection of ethanol and diazepam according to Moffat et al., (1986).

Standard curve for ethanol and diazepam:

Different concentrations of diazepam and ethanol were prepared (25, 50,100, 150,200mg/L) and zero standard curve point was measured using distilled water.

Potassium dichromate was added and ethanol concentration was measured by single beam spectrophotometer at wave length 585nm.

Diazepam was dissolved in chloroform, then diluted formalin was added (formalin: water = 6:4) and its concentration was measured by single beam spectrophotometer at wave length 240nm.

Standard curves for diazepam and ethanol for absorbencies and concentrations were obtained. Any unknown concentration could be measured using this curve. (Figures 1 and 2).

Extraction of ethanol and diazepam from liver tissue: preparation of tissue followed by steam distillation for ethanol and solvent extraction by chloroform for diazepam were done according to Meloe et al., (2012) and Moffat et al. (1989).

Statistical processing of the data was performed using SPSS version 11.0 software for windows. For all tests, P values less than 0.05 were considered to be statistically significant.

RESULTS

All animals are observed after administration of ethanol and/or diazepam till death. Animals of both ethanol and diazepam groups show decreased activity as compared to the control group. The animals remain in one corner of the cage but they move on shaking the cage, where rats of the control group are moving freely in the cage. After 30 minutes, 40% of the

treated animals of both groups become comatose but respond to painful stimuli (catching and squeezing the tail by forceps), while the rest of animals respond to shaking of the cage.

After 60 minutes, 50% of animals die, and the other 50% go into deep coma (not respond to painful stimuli). After 90 minutes, all treated animals die.

As regards animals receiving both ethanol and diazepam, the previous observations start earlier. Death of all animals occur within 60 minutes.

In liver tissue of animals of the control group, diazepam is not detected at all after preparing the tissue by the same method of treated groups.

On measuring ethanol in liver tissue of the control group, traces are detected in subgroups I-a and I-b (mean = 0.001), elevation of ethanol level is detected in subgroups I-c and I-d (mean = 0.002). These values cannot be computed as the standard deviations of both groups were 0 (Table 1).

Table (2) shows gradual increase in liver tissue ethanol on prolongation of postmortem interval. The increase is highly significant (p = 0.000).

Table (3) shows that the mean values of diazepam in different subgroups are nearly the same (0.0213±0.00137, 0.0228 ±.00183, 0.0218 ±0.00075 and 0.0218 ±.00098 for subgroups III-a, III-b, III-c and III-d respectively) and p values are not significant. As regards liver tissue ethanol in group IV, the mean values of ethanol show gradual elevation with increase of postmortem interval (0.0117± 0.00082, 0.0333±0.00489, 0.0705± 0.00723 and 0.0918±0.00691 for subgroups IV-a, IV-b, IV-c and IV-d respectively) and p values are highly significant as seen in table (4).

Table (5) shows that the mean values of liver tissue diazepam of ethanol and diazepam treated group (group IV) are non-significantly increased in subgroups IV-b, IV-c and IV-d (0.0372 ± 0.0553, 0.0207±0.01194 and 0.0175± 0.00164 respectively) as compared to subgroup IV-a (0.0150±0.00210). Table (6) illustrates that the comparison between liver ethanol of subgroups II-a, II-b, II-c and II-d is highly significant.

On the other hand, changes in liver tissue diazepam of groups III and IV are non-significant.

DISCUSSION

Drugs and alcohol concentrations are regularly used as evidence in criminal and civil litigations. The forensic toxicologist is frequently asked during the routine work

to give an expert opinion and the interpretation may be critical to the thorough investigations of different types of forensic cases whether antemortem or postmortem (Athanaselis et al., 2005).

In this study, animals treated by ethanol showed progressive depression of their activity till coma and death. Santhakumar et al. (2007) in their work stated that ethanol is a central nervous system depressant, and acute alcohol intoxication induces activity in the gamma-aminobutyric acid (GABA) system which is known to inhibit brain activity. Animals treated by diazepam in this work showed also progressive depression of motor activity due to the inhibitory effect of diazepam on CNS. This was also proved by Ramakrishnan et al. (2011), who recorded that reduction in motor activity of animals treated by diazepam is due to the reduction of CNS excitability level that means CNS depression.

Both ethanol and diazepam are classified as depressants of central nervous system. This similar mechanism of action and the fact that sedative effects are likely to be additive make ethanol and diazepam a potentially dangerous combination in overdose (Holmgren and Jones, 2003).

In this study, animals of the control group showed low level of ethanol in liver tissue. This could be explained by fermentation caused by Candida albicans present in the normal gut microflora causing release of ethanol traces as animals were fed freely till time of scarification and their food contains carbohydrates. This was also reported in the study of Petkovi et al. (2008) who applied the term 'endogenous alcohol' to spontaneous ethanol production in the body via different metabolic pathways. The ethanol values were extremely low, close to the detection threshold of modern analytical equipment.

Animals of this group showed elevated levels of liver tissue ethanol after 24 and 48 hours. This could be explained by postmortem microbial neo-formation. This is consistent with the study of Ziavrou et al. (2005).

In the present study, a highly significant increase in liver tissue ethanol of groups II and IV started after 18 hours, this could be attributed to the effect of both endogenous and exogenous bacteria. These data were consistent with that of Kala and Chudzikiewicz (2003) who found that transmigration of normal microbiota (selected bacterial and mycotic organisms) through the wall of the small intestine occurs within 2 to 3 hrs at 37^{o}C and 5 to 6 hrs at 25^{o}C. They stated that Escherichia coli and Candida albicans are the primary causes of postmortem ethanol synthesis. They also reported in their study that body tissues remain bacteriologically sterile from exogenous infection for at

least 20 hours after death. Other researchers also reported that ethanol could be formed postmortem in variable and non-predictable amounts, as a function of the type and number of microorganisms present either in corpses or specimens collected at autopsy (Ziavrou et al., 2005, Yajama et al., 2006). The progressive increase of ethanol level with prolongation of time between death and autopsy i.e. after 24 and 48 hours was also found in the study of Petkovi et al. (2005) who stated that postmortem ethanol production occurs in different tissues, and it is directly related to time. The increase of ethanol in liver tissue autopsy occurs as time between death and autopsy is prolonged. This could be attributed to the liver's highest glucose storage capacity in the form of glycogen that can be converted into glucose postmortem (O'Neal and Poklis, 1996).

Benzodiazepine drugs have been implicated in sudden and unexplained deaths specially when co-ingested with alcohol (Holmgren and Jones, 2003). In the present study, animals given both ethanol and diazepam died earlier (within 60 minutes) than those given ethanol or diazepam alone (died within 90 minutes).

Regarding animals given the lethal dose of diazepam either alone or in combination with ethanol (groups III and IV respectively), the subgroups "b", "c" and "d" where diazepam was detected 18, 24 and 48 hours respectively after death showed non-significant elevation of liver diazepam as compared to the subgroup "a". These findings coincided with Shiota et al. (2004) who reported that triazolam concentrations of 24 hours after death were increased in liver, kidney, lung and heart blood and similar findings were also observed with diazepam. They stated that these results indicated that diazepam as well as triazolam in the stomach diffuse through the stomach wall into tissues inside the abdominal cavity. The phenomenon of postmortem drug changes by drug diffusion from gastrointestinal tract has been reported for alcohol and several drugs in both humans and experimental animals and few studies reported the postmortem changes of benzodiazepines (Pelissier et al., 2003).

Liver tissue diazepam in subgroups "c" and "d" of groups III and IV showed gradual decrease as compared to subgroup "b" of both groups. This may be due to degradation of diazepam by microorganisms. This is in agreement with Robertson and Drummer (1995) who stated that many non-volatile compounds, drugs among them, could be metabolized by microorganisms during the putrefactive processes. The authors also reported that the nitrobenzodiazepines, clonazepam, nitrazepam and flunitrazepam are metabolized to their 7-amine metabolites in the liver, lungs, myocardium, kidney, and skeletal

muscle by enterobacteria. Also, Melo et al. (2012) concluded that the data obtained from their study suggest that results from samples with benzodiazepines stored long -term should be cautiously interpreted.

The present study concluded that the possibility of post-mortem production of ethanol makes correct interpretation of ethanol detection in forensic autopsy samples difficult. Diazepam level is elevated on increasing postmortem time before autopsy; also it is affected by putrefaction. It is recommended to compare the efficiency and the results of other different extraction and detection techniques.

Table (1): Effect of postmortem interval on detection of ethanol in control group.

Groups	Subgroups	Liver ethanol (mg/dl) X ± SD	t-test	p
Group I (Control group)	I-a I-b	.001±.000 .001±.000	----	------
	I-a I-c	.001±.000 .002±.000	----	------
	I-a I-d	.001±.000 .002±.000	---	-----

Table (2): Changes in ethanol level in liver tissue of ethanol treated group.

	Subgroups	Liver ethanol (mg/dl) X ± SD	t-test	p
Group II (Ethanol treated)	II-a II -b	.0113±.00082 .0343±.00242	-22.041	.000**
	II -a II -c	.0113±.00082 .0703±.00528	-27.055	.000**
	II -a II -d	.0113±.00082 .0917±.00641	-30.460	.000**

Significance <0.05 High significance = **

Table (3): Effect of postmortem interval on detection of liver tissue diazepam.

	Subgroups	**Liver diazepam (mg/dl) X ± SD**	**t-test**	**p**
Group III (Diazepam treated)	III -a III -b	.0213±.00137 .0228±.00183	-1.6	0.14
	III -a III -c	.0213±.00137 .0218±.00075	-0.79	0.45
	III -a III -d	.0213±.00137 .0218±.00098	-0.73	0.48

Significance<0.05.

Table (4): Effect of postmortem interval on liver tissue ethanol in ethanol and diazepam treated group.

	Subgroups	**Liver ethanol(mg/dl) X ± SD**	**t-test**	**P**
Group IV (Diazepam and ethanol treated)	IV-a IV -b	.0117±.00082 .0333±.00489	-10.715	.000**
	IV -a IV -c	.0117±.00082 .0705±.00723	-19.801	.000**
	IV -a IV -d	.0117±.00082 .0918±.00691	-28.216	.000**

High significance = **

Table (5): Effect of postmortem interval on liver tissue diazepam in ethanol and diazepam treated group.

	Subgroups	Liver diazepam (mg/dl) X ± SD	t-test	P
Group IV (Diazepam and ethanol treated)	IV -a IV -b	.0150±.00210 .0372±.0553	-0.98	0.35
	IV -a IV -c	.0150±.00210 .0207±.01194	-1.15	0.28
	IV -a IV -d	.0150±.00210 .0175±.00164	-2.29	0.44

Significance <0.05

Table (6): Comparison between mean ethanol and diazepam concentrations in liver tissue regarding different postmortem intervals.

Groups	Subgroups	Liver ethanol (mg/dl) X ± SD	F	P
Group II	II -a II -b II -c II -d	.0113±.00082 .0343±.00242 .0703±.00528 .0917±.00641	410.822	.000**
Group III		**Liver diazepam (mg/dl) X ± SD**		
	III -a III -b III -c III -d	.0213±.00137 .0228±.00183 .0218±.00075 .0218±.00098	1.404	.271
Group IV (ethanol level)		**Liver ethanol (mg/dl) X ± SD**		
	IV -a IV -b IV -c IV -d	.0117±.00082 .0333±.00489 .0705±.00723 .0918±.00691	250.662	.000**
Group IV (diazepam level)		**Liver diazepam (mg/dl) X ± SD**		
	IV -a IV -b IV -c IV -d	.0150±.00210 .0372±.0553 .0207±.01194 .0175±.00164	1.071	.384

Significance <0.05 ** = High significance

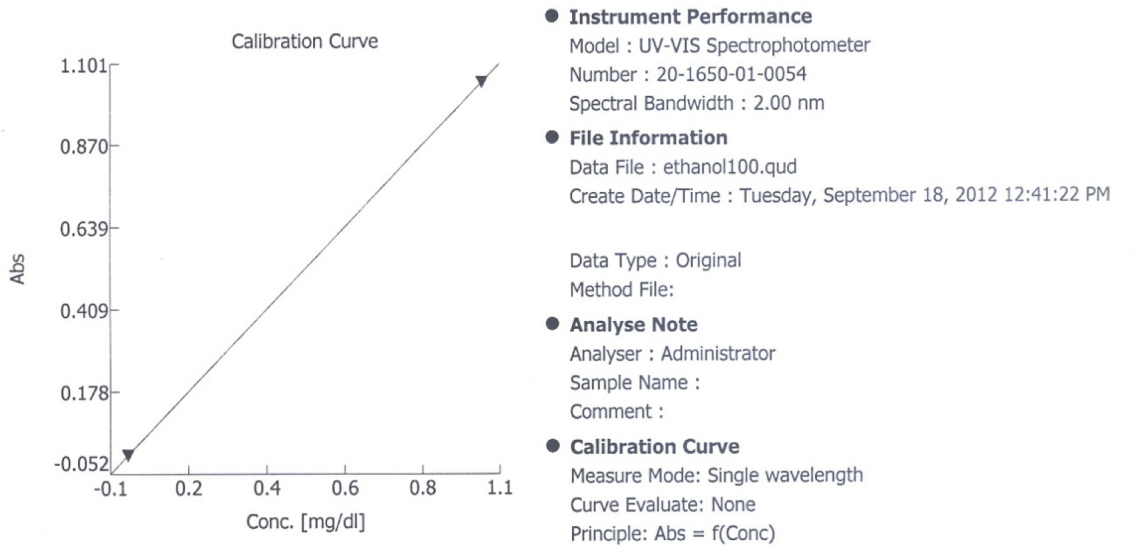

Figure (1) : Calibration curve for different prepared standard concentrations of ethanol.

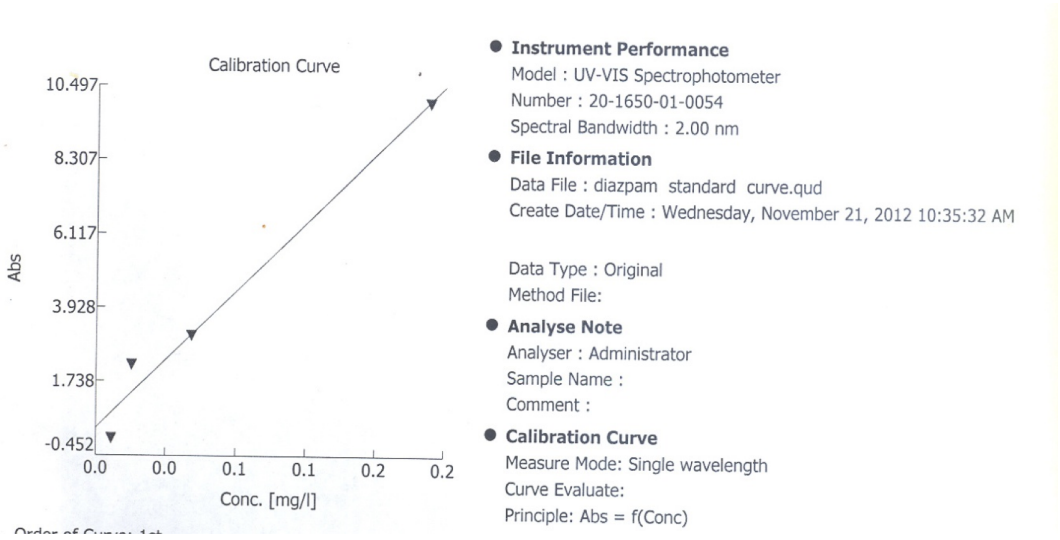

Figure (2) : Calibration curve for different prepared standard concentrations of diazepam.

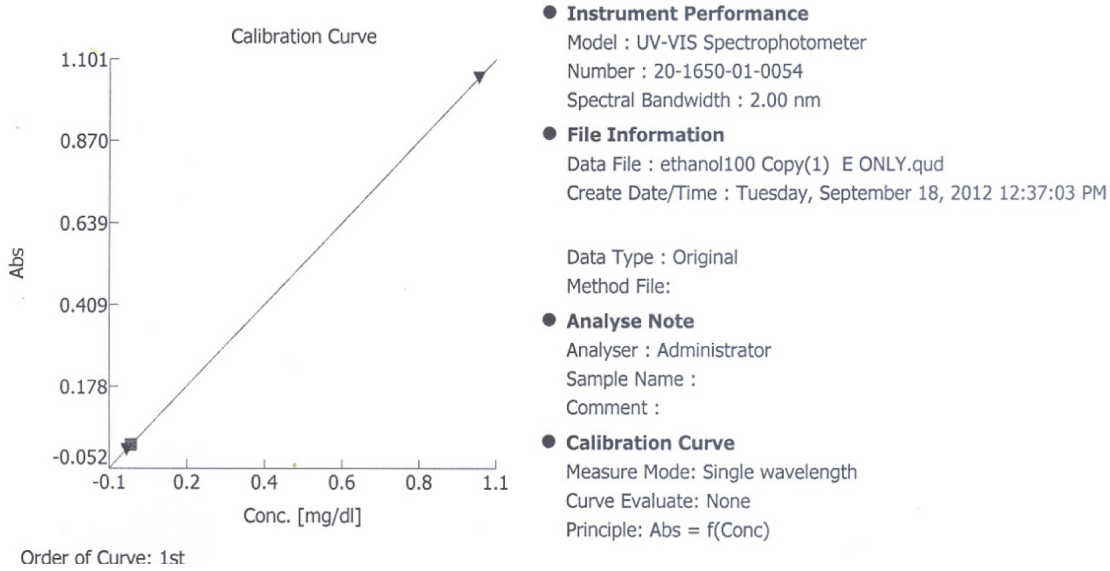

Figure (3) : Ethanol liver tissue concentrations in rats received lethal dose of ethanol only mediately after death on ethanol calibration curve.

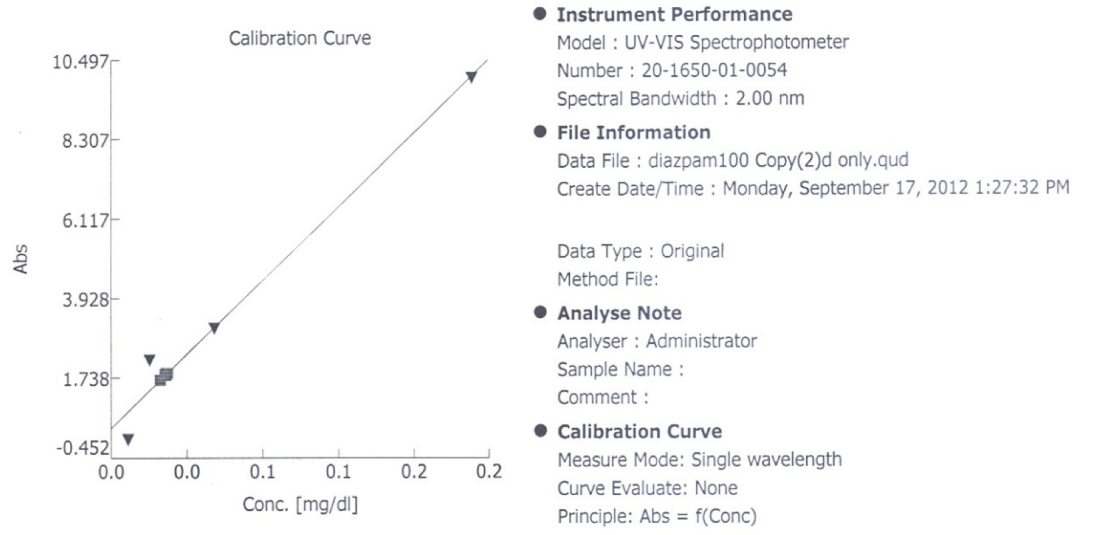

Figure (4) : Diazepam liver tissue concentrations in rats received lethal dose of diazepam only immediately after death on diazepam calibration curve.

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تأثيرالتعفن الرمى على الكشف عن كل من الإيثانول والديازيبام بعد الوفاة (دراســـة تجريبيـة)

المشتركون فى البحث

د. صفاء عبدالظاهر أمين د. أميره محمد الصعيدى

قسم الطب الشرعي والسموم الإكلينيكية – كلية الطب – جامعة المنوفية

تستخدم تركيزات الأدوية والكحول كدليل فى الدعاوى الجنائية والمدنية. وقد يطلب من خبراء الطب الشرعى والسموم فى كثير من الأحيان إبداء الرأى خلال العمل الروتينى وقد يكون التفسير حاسما بالنسبة للتحقيقات الدقيقة لأنواع مختلفة من الحالات الطبية الشرعية سواء قبل الوفاة أو بعدها. لذا، فإن الهدف من هذا العمل هو دراسة تأثير التعفن الرمى على الكشف عن كل من الإيثانول والديازيبام بعد الوفاة.

وقد أجريت الدراســة على ٩٦ من ذكـور الجــرذان البيضــاء البالغين تراوحت أوزانها بين ١٥٠ – ٢٠٠ جرام، والتى قسمت إلى أربع مجموعات (٢٤ جرذا لكل مجموعة) قسمت بدورها إلى ٤ مجموعات فرعية أ، ب، ج، د (٦ جرذان لكل مجموعة فرعية).

المجموعة الأولى: مجموعة ضابطة. المجموعة الثانية: أعطيت جرعة مميتة من الايثانول. المجموعة الثالثة: أعطيت جرعة مميتة من الديازيبام. المجموعة الرابعة تم إعطاءها جرعة مميتة من كل من الإيثانول والديازيبام. بعد حدوث الوفاة مباشرة تم تشريح كل من المجموعات المعالجة الفرعية " أ" والمجموعة الضابطة فى نفس الوقت وباقى جرذان المجموعات المعالجة تركت فى درجة حرارة الغرفة بعد ١٨ ساعة من الوفاة ثم تم تشريح المجموعات المعالجة الفرعية "ب" وتركت المجموعة المعالجة الفرعية "ج" ليتم تشريحها بعد ٢٤ ساعة أما المجموعة المعالجة الفرعية "د" فقد تم تشريحها بعد ٤٨ ساعة. بعد التشريح تم أخذ أوزان متساوية من أكباد الجرذان وإخضاعها لعمليات الاستخلاص والكشف عن كل من الايثانول والديازيبام.

أظهرت المجموعــة الضابطــة زيـادة الايثانــول فى أنسجة كبــد المجموعتــان الفرعيتــان "ج" و "د".

وأظهـرت الدراســة الارتفـاع التدريجى لمسـتوى الإيثانـول فى أنسجة كبـد جـرذان كل من المجموعـة الثانيــة والرابعـة بزيــادة الفاصل الزمني بين الوفـاة والتشريح.

أما بالنسبة لتغير مستوى الديازيبام فى أنسجة كبد جرذان المجموعة الثالثـة أوضحـت الدراســة أنه ليس له دلالــة احصائيــة. بالنظـر إلى مستوى الديازيبام فى أنسجة كبد جـرذان المجموعـة الرابعـة أوضحت الدراســة حـدوث تغيير أيضـا بـدون دلالة احصائية في مجموعـاتها الفرعية "ب" و "ج" و "د" بالمقارنة بالمجموعة الفرعية "أ".

ويمكن أن نستخلص من هذه الدراسة أن إمكانية إنتاج الإيثانول بعد الوفاة يؤدى إلى صعوبة الجــزم بصحة وجـود الإيثانول في عـينات التشريح الشرعي، كما أن مستوى الديازيبام يرتفع بزيادة الفاصل الزمنى بين حدوث الوفاة و واجراء التشريح، كما يرتفع مستوى كل من الإيثانـول والديازيبــام بالتعفن الرمــى.