Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2014, 6(6):1709-1715

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Microanalysis of carbon monoxide in decomposed blood and hepatic tissues by headspace gas chromatography and mass spectrometry

Hongxia Hao[1,*], Hong Zhou[2], Ling Zeng[1] and Zhongshan Yu[2]

[1]*Key Laboratory of Evidence Science, China University of Political Science and Law, Ministry of Education, Beijing, China*
[2]*Institute of Forensic Science Ministry of Public Security, Beijing, China*

ABSTRACT

Head-Space Gas Chromatography-Mass Spectrometry (HS-GC/MS) was used to determine blood carboxyhemoglobin (COHb%), especially in cases involving blood decomposition or death, when blood extraction is impossible. Until now, the problem eluded complete solution using forensic toxicology. Therefore a method was developed using Headspace Gas Chromatography-Mass Spectrometry (HSGC/MS), which should enable the determination of COHb in decomposed blood or hepatic tissues. The technique has a clear advantage over other methods such as UV spectrophotometry. COHb% in hepatic samples were determined, which were stored at different temperatures (-20°C for 1-2 years, 0°C, and 18°C for two months). Using a column packed with molecular sieve, CO levels were quantified down to 0.01% in the air and COHb levels down to 0.2% in small blood samples quickly. The 3-min procedure requires only 0.25ml of blood sample or 1g of hepatic tissue, each time. It was shown that the method has good reproducibility with RSD of the COHb <1%. The method was accurate and reliable to investigate deaths related to CO exposure, by determining COHb and CO levels.

Keywords: carbon monoxide poisoning; COHb; decomposed hepatic tissues; Headspace Gas Chromatography/Mass spectrometry (HS-GC /MS)

INTRODUCTION

Carbon monoxide exposure in humans is impossible to detect as it is colorless, tasteless, odorless, and nonirritating. When inhaled, CO is readily absorbed from the lungs into the bloodstream, where it forms a tight but slowly reversible complex with hemoglobin (Hb) known as carboxyhemoglobin (COHb). The presence of COHb in the blood decreases its oxygen-carrying capacity, reducing the availability of oxygen to body tissues, resulting in tissue hypoxia. A reduced oxygen delivery associated with elevated COHb level, exacerbated by impaired perfusion resulting from hypoxic cardiac dysfunction, potentially will impair cellular oxidative metabolism. In China, death due to CO poisoning was identified by elevated COHb levels (> 40%) in postmortem blood.

During the past twenty years, several methods for the determination of COHb levels in postmortem specimens have been published. These methods include UV and FTIR spectrophotometry [1-3], CO-oximetry [2,4], and Capillary Electrophoresis (CE) [5,6]. Although gas chromatographic techniques are more suitable for forensic materials [3,7,8], they are complicated, time-consuming and require larger samples, when compared with CO-oximetry and GC/MS. However, CO-oximeters cannot detect COHb less than 10% and COHb content in putrid blood [4]. In China, several postmortem samples collected in suspected carbon monoxide (CO) poisoning cases are decomposed and devoid of blood, as the deceased individuals were discovered days or months following death. In such cases, the quantification of COHb may be difficult for a toxicologist. In this paper, the HS-GC/MS method was describe, for the determination of COHb in decomposed blood and examine the CO of the hepatic tissues extracted from dead bodies in order to provide an experimental basis for the analysis CO poisoning among dead humans.

A study of 22 deaths resulting from unvented gas heaters revealed a mean COHb level of 49.5% in the victims' blood, with a minimum value of about 30% and a maximum of 75% for COHb [9]. Another study examined the distribution of COHb among survivors (mean = 28.1%, n = 159) and fatalities (mean 62.3%, n = 101); the 50% survival probability was associated with approximately 50% of COHb [10].

Liver is the biggest gland in human body, and plays an important role in metabolism. It has complex functions, including: manufacturing bile, glucose metabolism, protein metabolism, fat metabolism, biotransformation, etc. Liver can store blood and regulate blood volume. At rest or in emotional stability, massive blood is stored in the liver. At work or when excited, the hematic quantity rises, and massive blood supplies are released from the liver to the body. Of all the organs, liver and spleen have the largest blood volume, approximately 0.2-0.3ml/g.

EXPERIMENTAL SECTION

The protocols in this study were approved by institutional review board and the Animal Care and Use Committee of China University of Political Science and Law, Ministry of Education (Beijing, China).

2.1 Experimental design or liver (1g)

The static head-space technique is an indirect method for analysis of volatile compounds in liquid or solid samples. Measurement of CO levels in the present study relied upon addition of lactic acid to blood samples in order to release CO. Samples were prepared as follows: blood (0.25 mL per vial) was placed into head-space vials and 0.25 mL of lactic acid(25%) was added to each vial to release CO. The vials were then heated on a heating block for 30 min at 50˚C, following which 200 μL of head-space gas (the vapor phase, in thermodynamic equilibrium with the sample) was extracted with a syringe for rapid analysis using gas chromatography-mass spectrometry (GC/MS). The combination of GC with MS provides a powerful analytical tool for separation and identification of the individual components of an organic solution and determination of the individual quantities of each of these components.

When the vapor pressure of a sample is relatively low, the chromatographic peak area (A_i) of a volatile component changes proportionally with its vapor pressure (P_i):

$A_i = S_i P_i$

S_i is the sensitivity constant of component i. The partial vapor pressure may be expressed as:

$P_i = P_i^0 X_i$

Where P_i^0 is the vapor pressure of component i and X_i the mole fraction of the component in the sample. The chromatographic peak area A_i can then be rewritten as:

$A_i = S_i P_i^0 X_i = P X_i$

Then the percentage saturation of blood with CO was calculated by the ratio of the peak areas obtained from untreated blood (A_c) and CO saturated blood (A_s):

$A_c/A_s \times 100\% = COHb\%$ [8]

A_c = Area of CO peak from untreated blood
A_s = Area of CO peak from CO-saturated blood

$$\text{CO in liver } (\mu l) = \frac{peakareaof \quad hepatictis \quad sues}{addictivep \quad eakareaofb \quad lanklever} \times 100$$

As the blood volume in liver is constant, it may be inferred that CO content in the liver tissues and blood are correlated.

When dealing with the putrid blood in CO poisoning cases, in order to saturate hemoglobin CO was bubbled though the same blood from the deceased. This excludes the effects of variable Hb concentration when dealing with putrid samples which maybe showing low or high Hb concentrations due to unequal setting of blood. [13]

2.2 Materials

10 rabbits were purchased from Shanghai Slack Corporation (Shanghai, China). Blood was obtained from the Blood Center of the Beijing Red Cross Society. Formic acid, CO gas (99.9%), sulfuric acid, and sodium hydroxide (to absorb gases produced from secondary reactions, such as SO_2 and SO_3) were purchased from Sinopharm Chemical Reagent Corporation (Shanghai, China).

2.3 Blood sample preparation and determination

2.3.1 Saturated blood preparation

Fresh blood (150 mL) was placed into a conical flask (de-bubbling by n-butanol), and CO gas was gently bubbled through the blood for varying periods of time in order to saturate hemoglobin with CO.30 min turned out to be the minimum time required to achieve saturation. Following the study of optimal N_2 aeration time, a stream of nitrogen gas was passed through the blood for 5 min, at a rate of 6-10 mL/min, to remove the free CO in blood. The flask was then covered with a tight-fitting lid and shaken slowly for 30 min, following which the sample was preserved at 4˚C in an airtight container. Blood samples prepared as described above were considered to have a COHb saturation of 100% [7,8]

2.3.2 Blank blood preparation

Air was bubbled through 100 mL of fresh blood for 30 min, followed by a stream of nitrogen gas for 5 min. The blank blood was then preserved at 4˚C in an airtight container.

2.3.3 Preparation of a concentration series of COHb blood

Mix appropriate volumes of COHb-saturated blood and blank blood allowed by the preparation of a series blood samples containing various COHb concentrations: 0% (blank blood), 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. These samples were preserved in sealed syringes and stored in airtight containers at 4°C, 17°C, and -20°C, respectively.

2.3.4 Headspace sample preparation

A 0.25 mL aliquot of blood was placed into a 10 mL head-space vial. To this was added 0.5 mL of distilled water and 0.25 mL of 25% lactic acid and the vial was then covered immediately with an aluminum cap containing a rubber septum. The vial was heated on a heating block for 30 min at 50˚C and 200 µL of gas was then extracted for GC/MS analysis.

2.3.5 Determination the COHb linear range of blood

Head-space samples were prepared as above using 0.25 mL of blood from each of the following standard concentrations of COHb in blood: 0% (blank blood), 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. GC/MS analysis of each of these samples enabled the construction of a standard curve for COHb%.

2.4 Determination of CO in hepatic tissues

2.4.1 Experimental animal hepatic tissue

10 healthy male rabbits with similar weight were taken, NO.1 and NO.10 rabbits were killed by intravenous injection of air into ear margin; another 8 died of CO poisoning. The experiment was so designed that male rabbits inhaled CO (CO >1%, lasting for 30mins) in an exposure apparatus, i.e. a homemade glass box with CO gas inlet and outlet. Immediately after death, the blood and hepatic tissue samples were collected and analyzed for CO and COHb with HS-GC/MS. Finally, hepatic tissues were extracted (Fig. 2).

It is showed that exposure to CO at 4600 to 5000 ppm and COHb levels greater than 60% for 30 min causes death of rabbits within minutes of exposure, the rabbits started to twitch all around their eyes, with their lips turning red. Ten minutes later, the rabbits began to give blood-curdling screams and 30 minutes later, the rabbits died one by one, with their lips and teeth open, full of feces and gatism. After the dissection, 10-15 ml of the cherry-red cardiac blood was transferred into an airtight cuvette. Other organs were transferred into sealed bags. Livers were used in this experimental study, and other organs stored as samples for use in later experiments.

2.4.2 Determination of CO in hepatic tissues

1g of hepatic tissues was isolated, which were subjected to similar analysis as described in the previous section 2.4.1. The determination of CO content in putrid liver tissues was as follows: the hepatic tissues prepared as in section 2.4.1 above were stored in sealed plastic bag respectively, at room temperature (15~17°C) until the 8th day (blood on the liver surface turning darker) and the 35th day (when the liver turned into mud with a terrible smell). Sample preparation and CO analysis was as described in the previous section. Preparation of saturated hepatic tissue entailed transfer of 1 gram blank liver into the hepatic tissue as described previously. 100µl sterling CO was extracted by a sample injector, and transferred into a headspace bottle, and 10 minutes later, measured again and compared with

100µl sterling CO.

2.4.3 Stability of bloods COHb%
Three portions of 0.25mL of four different concentrations of COHb blood(n = 3 for each temperature) were stored under airtight conditions at 4°C, 17°C, -20°C, respectively (Table 1).

2.5 GC/MS analysis
A Shimadzu GC/MS System Model QP 5000 was used as follows. (1) GC parameters: fused silica capillary column with Hp-plot, 30m×0.25mm×12µm 5A Molecular sieve(Shimadzu); column temperature, heating from 50 to 250°C; injection temperature , 250°C; carrier gas, He flow, 1.5L /min, splitting ratio 10: 1; head-space injection. (2) MS parameters: mass range 10~60; scanning interval 0.5s; acquisition time 0.5~3min; source temperature 200°C Multiplier voltage 1.35kV; electronic energy 70eV.

Samples were prepared as follows: Blood (0.25ml each) and hepatic tissues (1g each) were transferred into headspace vials separately, which released CO after addition of 25% lactic acid. Then, the vials were heated on heating blocks for 30 min at 50°C, from which 200µl headspace gas was extracted for GC/MS analysis accurately and rapidly. The samples were then stored in test tubes with plugs at different temperatures (-20°C, 0°C, 18°C) and COHb was determined after 7 days, 14 days, 30 days and 40 days.

In each experiment, three sample were parallel prepared at the same time (n = 3). Each sample was analyzed 5 times; furthermore, care was taken to ensure that each 200 µL extraction of head-space gas was carried out accurately, and was used rapidly for GC/MS analysis.

2.6 Comparison with UV method
An aliquot of 5 ml sodium hyposulfate solution was taken in a test tube, and 1 drop of blood samples was added, and gently shaken to perform the UV analysis (Table 2).

RESULTS AND DISCUSSION

In this paper, a protocol was established for the determination of CO content in liver tissues. The stability of CO in blood was investigated and the experimental basis for these cases of CO poisoning determined.

3.1. CO stability in blood
In 2000, Kunsman [11] reported that COHb levels in blood were stable for two years. In reality, the blood in CO poisoning cases is left outside for extended periods of time, before sending it for laboratory analysis. Our research emphasizes the stability of COHb under ambient conditions or low temperature (for instance, 0~4°C or -20°C). Standard CO blood was prepared at different concentrations of 10%, 30%, 50%, and 70%, stored at room temperature (17~19°C), in cold storage at 0~4°C, or refrigerated at -20°C. Table 1 shows the experimental results.

Based on the data shown in Table 1, it is concluded that both time and temperature affect the determination of COHb, with 4°C being the most suitable temperature for storage. Under prolonged conditions of freezing, COHb in the blood sample appears to rise. The results may be affected by unstable or low temperature. With freezing temperatures below -30°C, the level of blood met-hemoglobin (MetHb) increases. When it does not combine with CO; the high levels of MetHb diminish the ability of CO combination resulting in elevated levels of COHb [12].

The HS-GC/MS method offers a clear advantage over the UV method as seen from the data in Table 1.When the specimens were stored at room temperature, the COHb levels were reduced by about half: the original 70% COHb levels fall to 45.1%, which implies that it was not a case of CO-poisoning death, contrary to the facts.

3.2. Determination of CO content in liver tissues
The NO.1 and NO.10 rabbits died of intravenous injection of air into ear margin. Results from animal experiments of CO-poisoning death are shown in chart 2, which reveals that the content of CO is sharply reduced with increased storage time of liver tissue. The reduced ratio needs further study.

The hepatic tissues were isolated on the day of the rabbit death. The samples from rabbits NO.1-5 were placed at room temperature (15-17°C); From rabbits NO.6, 7, 10 under freezing temperature; and those from rabbits NO.8, 9 cryopreserved. All blood samples were in good condition at the baseline. They were cherry-red, and delaminated after stratified. After storage at normal temperature for 2 days, the blood darkened and the sample was hemolyzed. The blood turned into dark red after 45 days of preservation. When the blood was stored at 4°C, no obvious change occurred in the first 8 days, the color turned dark after one month, and the sample became moderately hemolyzed 35

days later. When the blood was preserved at - 20°C, no obvious change was seen within one month, however, it turned dark after one month, and the sample was hemolyzed after 35 days. When the hepatic tissue was stored at normal temperature, the blood agglutinated to the surface, the tissue started to decompose, and the color became garnet. The liver became highly decomposed as mush after 35 days, turning greyish-green and malodorous (Fig. 3, Table 2).

3.3. CO poisoning

After two people died of CO poisoning, their dead bodies were found four days later. Their blood samples were stored at room temperature for 15 days, and then transferred to our lab. The blood turned black red, and no results were available when they were detected by UV method. However, using the method we established, the COHb was detected as showed in Table 3 and Fig. 4.

Analysis of blood sample from CO poisoning death revealed 54% COHb. The sample was preserved at 4°C. It was analyzed again by HS-GC/MS method after it was cryopreserved for two years (Table 4).

In contrast, use of UV spectrometry provided values of COHb% that were well below 30%, and in the first case not detectable, which would not have supported CO poisoning as the cause of death in all individuals. The UV method is often used to detect CO concentrations in fresh blood samples, but may not be suitable for decomposed blood, where it may give values lower than the true levels. Thus, the HS/GC/MS technique may have significant advantages over the UV method, particularly when blood samples are not fresh.

Table 1 Stability of COHb under different storage conditions

Temperature	Time	Measured values of COHb in blood (mean)							
		10%		30%		50%		70%	
		HS-GC/MS	UV	HS-GC/MS	UV	HS-GC/MS	UV	HS-GC/MS	UV
4°C	1	10.0	10.0	30.1	30.3	49.7	50.7	70.0	70.0
	4	10.5	9.6	29.6	28.1	49.8	46.6	70.1	62.8
	14	9.6	---	28.1	28.8	48.6	48.1	68.8	33.6
	45	9.6	--	28.9	---	47.7	---	68.9	32.7
17°C	1	10.0	8.4	31.2	25.8	50.7	49.9	69.4	66.3
	4	10.7	---	29.9	---	49.5	35.5	68.4	28.6
	14	9.4	--	29.8	10.8	48.9	22.3	69.7	--
	45	9.8	--	28.5	--	49.1	--	69.5	--
-20°C	1	10.1	10.0	31.1	30.0	52.6	50.7	71.4	72.9
	4	10.4	10.0	30.9	30.3	51.1	50.7	69.2	60.0
	14	10.0	9.6	29.7	28.1	49.7	51.7	68.3	62.8
	45	9.1	---	32.5	---	54.6	56.6	72.9	33.6

*-- *not detectable*

Table 2 Comparison of the saturation levels at room temperature

Rabbits	COHb% (HS-GC/MS)			COHb% (UV method)
	0d	8d	35d	35d
1(blank)	0.2	0.2	2.3	--
2	62.1	61.2	57.0	--
3	68.8	64.0	64.3	28
4	63.4	63.4	64.6	--
5	68.3	62.4	60.0	--
6	73.2	70.7	69.1	29
7	67.5	62.0	65.7	--
8	64.5	64.5	64.5	--
9	64.6	65.4	63.6	--
10(blank)	0.6	0.6	0.5	--

*-- *not detectable*

Table 3 Specimen analysis

	Area of blood	Area of CO-saturated blood	Area of hepatic issues	Area of CO- saturated hepatic issues	Volume of CO in hepatic issues (μL)	COHb%	COHb% UV method
A	249518	366275	219258	478256	49.8	65.1%	--
B	337065	513052	428105	956339	58.0	68.7%	--

*-- *not detectable*

Table 4 Results of CO poisoning specimens after 2 years of cryopreservation

Area of blood	Area of CO-saturated blood	Area of hepatic issues	Area of CO-saturated hepatic issues	COHb%	Volume of CO in hepatic issues (μL)	COHb% (UV method)
310603	586305	996706	4010199	52.9%	33.1	28.1%

*-- *not detectable*

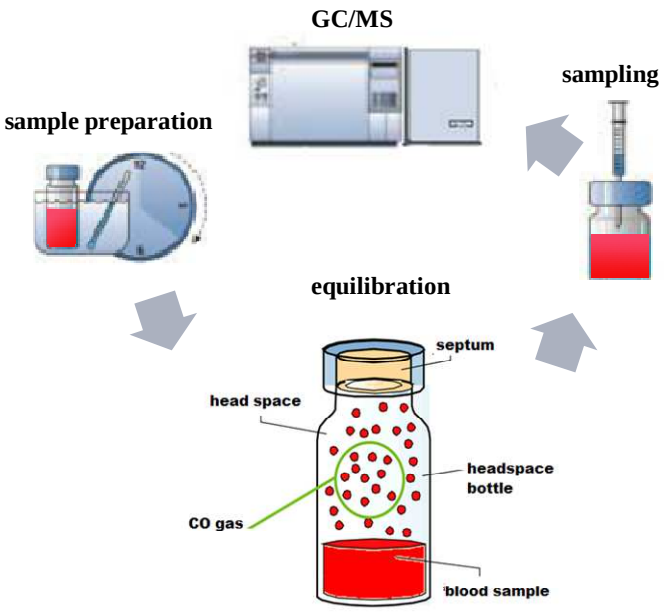

Fig.1. Experimental design and detecting process

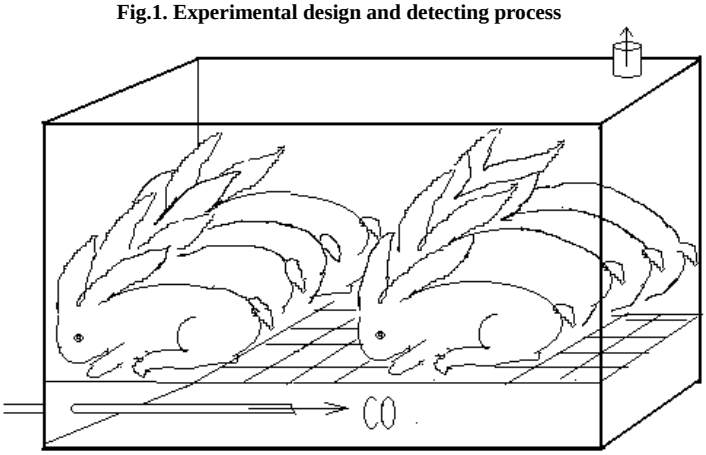

Fig.2. test equipment of CO poisoning of animals

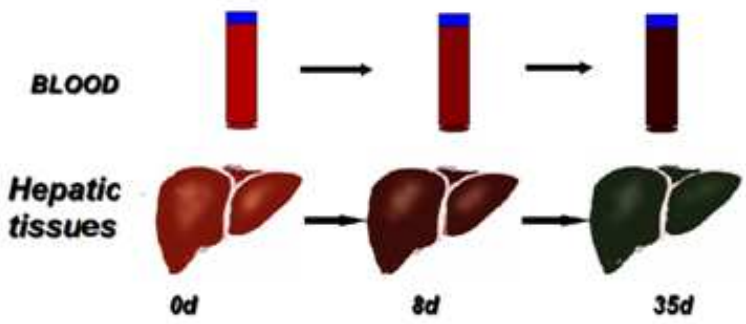

Fig.3. the change of samples in room temperature

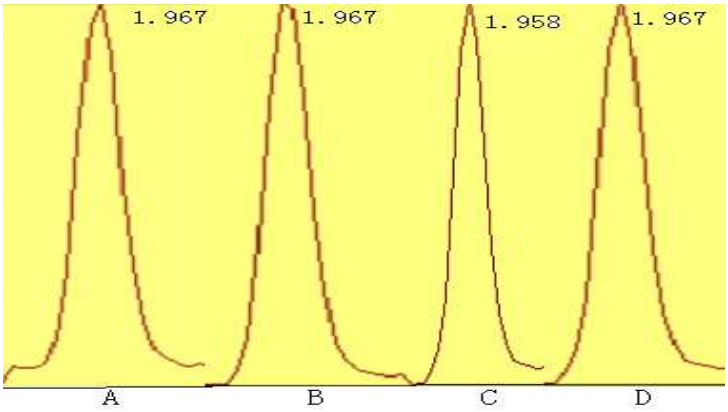

Fig.4. Mass chromatograms for the blood and hepatic tissues specimens of five deaths（A,B：the peak corresponding to CO in the blood and hepatic tissues in person A；C,D：the peak corresponding to CO in the blood and hepatic tissues in person B）

CONCLUSION

HS/GC/MS is associated with high sensitivity regardless of the sample condition— fresh or highly decomposed. It requires fewer samples and sample time compared with other methods. Our method showed that we can disregard the strong effect of storage temperature on the COHb determination. The COHb levels in the decomposed blood samples were consistent with the COHb levels determined with the fresh blood on the day of exposure to CO gases (RSD<5%). The equipment is easily available since most labs already have gas chromatography and mass spectrometry instrumentation. In fact, manual injection may substitute for the absence of a headspace automatic sampler. The method has a much higher sensitivity than the commonly used techniques. Using a packed molecular sieve column, CO levels were able to be quantitating down to 0.01% in the air and COHb levels down to 0.2% in small blood (0.25ml) and liver (1g) samples.

Acknowledgements
We are grateful to The Program for Young Innovative Research Team in China University of Political Science and Law (1000-10814344), Program for Changjiang Scholars and Innovative Research Team in University (IRT0956), China Scholarship Council Fund, and Academician Foundation of the Ministry of Public Security (No.2011-23210044, 2011-23211119, 23212052) for their financial supports.

REFERENCES

[1] J. Van Dam, P. Daenens, *Journal of forensic science*s 39 (**1994**) 473-478.
[2] S. Oritani, B.L. Zhu, K. Ishida, K. Shimotouge, L. Quan, M.Q. Fujita, H. Maeda, F*orensic science international* 113 (**2000**) 375-379.
[3] R.J. Lewis, R.D. Johnson, D.V. Canfield, *Journal of analytical toxicology* 28 (**2004**) 59-62.
[4] C. Brehmer, P.X. Iten, *Forensic science international* 133 (**2003**) 179-181.
[5] G.G. Fechner, D.J. Gee, *Forensic science internationa*l 40 (**1989**) 63-67.
[6] A.K. Chaturvedi, D.R. Smith, D.V. Canfield, *Forensic science international* 121 (**2001**) 183-188.
[7] H.A. Collison, F.L. Rodkey, J.D. O'Neal, *Clin Chem* 14 (**1968**) 162-171.
[8] K.L. Wallace, S.C. Curry, *Journal of toxicology. Clinical toxicology* 40 (**2002**) 91-94.
[9] E.A. Tyrrell, D.A. Kale, memorandum (**1979**).
[10] J. Pach, L. Cholewa, Z. Marek, M. Bogusz, B. Groszek, *Veterinary and human toxicology 2*1 Suppl (**1979**) 158-159.
[11] G.W. Kunsman, C.L. Presses, P. Rodriguez, *Journal of analytical toxicology* 24 (**2000**) 572-578.
[12] R.O. Wright, W.J. Lewander, A.D. Woolf, *Annals of emergency medicine* 34 (**1999**) 646-656.
[13] Zhongshan Yu. *Journal of Chinese Forensic Medicine,* **1992**,7 (3): 12.