Original Article

Comparison of Methadone Level Measurement by Enzyme Immunoassay with Gas Chromatography–Mass Spectrometry

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ABSTRACT

Determination of poisoning and its type in drug poisoning is one of the important challenges of the forensic laboratory, given that drugs have therapeutic, toxic and pathogenic doses. In this study, we tried to measure the blood level of methadone with enzyme immunoassay and compare it with *Gas chromatography–mass spectrometry* method. Samples were developed in the methadone treatment center for addiction treatment. The ELISA kit was purchased from RANDOX Company and performed according to the protocol set forth on the respective test kit, and then the results were obtained. Methadone's analytical standard was provided by Sigma-Aldrich. Serum samples were extracted by alkaline extraction with ethyl acetate. The results were analyzed statistically. The result of the relative error in the accuracy of one day ELISA method was 3. 3% and the GC / MS method was 4/4% and the result of the relative error in the accuracy in the ELISA method was 4.1% and in the GC/MS method was 2.3%. The GC/MS method was linear from 30 ng to 10 µg, but the ELISA method was linear from 1.2 to 100 ng. And both methods did not interfere with the materials contained in the matrix and with drugs similar to methadone. The proposed GC/MS method is preferable to RANDOX's ELISA kit, given its high ability in methadone analysis at different concentrations. But given the time consuming, the great effort, high cost, hazards of chemical solvents and complexity of the device, it is not possible to use it everywhere and in large measure with many samples. But ELISA, with a high sensitivity and high speed, and relatively simple to use, compared to GC/MS, can analyze the number of samples in a very short time. If the methadone is positive by *Gas chromatography–mass spectrometry*, the ELISA method can be used to determine the blood level for a precise decision.

Keywords: Blood level of Methadone, enzyme immunoassay, Gas chromatography–mass spectrometry.

Introduction

Substance abuse disorders are one of the most common psychiatric problems that arise from the interaction of genetic and environmental factors, such as growth abnormalities and psychosocial disadvantages. Symptoms of substance dependence include a set of physiological, behavioral, and cognitive symptoms, according to which the person continues to consume despite substance-related disorders. In such cases, there is an open consumption pattern that usually results in tolerance, deprivation, and forced behavior for consumption. According to this definition, substance abuse results in destruction of mental or physical functions that the person has compulsorily and uncontrollably consumed, and shows the absence of withdrawal symptoms. There are different treatments for drug dependence treatment, which are the most commonly used for treatment for detoxification or maintenance of opioid dependence. In these methods, a variety of opiate drugs and non-opioid drugs are used. Methadone is one of the most widely used drugs in the field. It also has a physiological and psychological dependence properties, has a significant liver metabolism, and its side effects include effects on the rhythm of the heart (change in QT intervals), sexual dysfunction, night-time sleep disturbances, daily sleepiness, dizziness, sweating, etc. that they are often dose-dependent [1]. Also, high doses cause respiratory suppression, apnea and even death. Currently, in some forensic labs, the results of the tests are reported qualitatively and the concentration or blood level

Access this article online	
Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Mehdi Forouzesh, Rooholah Valipour, Ahmad Shekari, Abdolrazagh Barzegar, Mehrdad Setareh. Comparison of methadone level measurement by enzyme immunoassay with Gas chromatography–mass spectrometry. J Adv Pharm Edu Res 2019; 9(4):105-108.
Source of Support: Nil, Conflict of Interest: None declared.

of the drug is not measured. This would confuse the relevant medical practitioner to determine the cause of death, and he asks whether the drug is found to be at treatment level or poisoning, or even at a fatal level. Therefore, it is the responsibility of the laboratory to identify the blood level of drugs with specific and sensitive methods. ELISA is a method that requires a very low sample (generally less than 100 microliters). Also, given that many samples can be tested in less than 2 or 3 hours, and the equipment and consumables are much cheaper than chromatographic methods, and also have a specificity and sensitivity, it can be used as a suitable method for legal toxicology. By this study, we can compare the results of the two methods mentioned above in measuring methadone levels and identify the disadvantages and advantages of both methods in the legal toxicology lab. Methadone is a solid drug with a chemical formula of C21H27NO and a molecular weight of 309.4 which has a melting temperature of 78 degrees Celsius and is soluble in water. Methadone is rapidly absorbed after oral ingestion and is widely distributed in tissues that have high concentrations in liver, lungs, kidneys, and blood. It passes through the pair and also secretes in milk. Its metabolism is catalyzed by the CYO3A4 iso-enzyme, Citro Chrome P450.

Table 1: Methadone concentrations in different tissues

Tissue	Methadone mg/L (μg/g)	EDDP mg/L (μg/g)
Blood	0–3 (1. 2, 21)	0–0. 4 (0. 1, 10)
Bile	1. 1–75 (13, 18)	0. 2–315 (41, 18)
Brain	0. 23–2. 2 (0. 9, 7)	—
Kidney	0. 51–18. 3 (3. 5, 19)	0. 5–3. 1 (1. 2, 14)
Liver	0. 05–49. 5 (6, 23)	0. 02–2. 7 (0. 6, 16)
Lung	1. 6–110 (16, 17)	0. 01–0. 98 (0. 2, 10)
Spleen	1. 6–20. 9 (5. 3, 7)	0–0. 98 (0. 7, 4)
Urine	0. 52–76. 2 (21, 22)	0. 4–46. 2 (11, 17)

An ELISA test, called the Enzyme-Linked Immunosorbent Assay, is a simple biochemical laboratory method with high sensitivity that can analyze a large number of samples simultaneously. This method is used in immunology to detect the presence of an antibody or antigen in a test sample that is commonly used as a diagnostic tool in medicine and pathology as well as in quality control tests in many industries. ELISA is based on the measurement of the antigen and antibody color scheme [2]. One of the most advanced devices used in iinstrumental analysis is to separate compounds via gas chromatography and then detect them via a mass spectrometer. In fact, gas chromatography mass spectrometry (GC-MS) is a device that classifies the pregnant gas molecules based on their mass, separates them in terms of their mass, and measures their amount in the solution [3]. These devices are widely used in identifying toxins and measuring their residues in foodstuffs, in the pharmaceutical, petrochemical, etc. industries for the isolation and identification of unknown compounds with low boiling point. In simple terms, a mass spectrometer performs three operations: first, it evaporates objects with different evaporation powers. Second, it converts the molecules of vapor into ions, and eventually separates the ions by mass to charge ratio (m/z). Since ions that have some positive charge are rarely formed than ions which have one positive charge, z is usually one, so m/z gives ion mass. Hence, a mass spectrometer is a device for the production and determination of mass of ions. When the generated ions are isolated and recorded, the resulting spectrum is called the mass spectrum. In this spectrum, the frequency of each ion is plotted against m/z [4].

Research Method

This study was performed to compare serum methadone levels by enzyme immunoassay with Gas chromatography–mass spectrometry. Blanc serum samples were collected for the purpose of the methodology of volunteers who were employed as a staff member in the legal medicine laboratory of Zanjan province, who had no history of consuming any substances or drugs for at least one month before the sampling. 50 serum samples were taken from methadone-treated patients in one of the methadone-treated drug addiction treatment centers. Serum Samples of 5 ml, without protective material, were collected in standard plastic containers and stored until analysis at refrigerated temperature. In this project, the Elisa method was repeatedly investigated by Randox Company Elisa and extraction method and gas chromatography method is briefly referred to GC/MS.

Research Findings

All data were analyzed using MS-Excel 2013 software and charts were drawn using this software. Blood samples will be used directly in a volume of 100 μl, due to the fact that they do not need sample preparation in the ELISA method, and will be bound to wells tested with pre-blotted antibodies. After binding, the extra material in the sample will be removed from the environment with a Tween 20 solvent. Then, the specimen sampled in the wells will react with the anti-hydrogen antibody indicated by the hydrogen peroxidase enzyme, and again the excess material in the reaction medium will be removed from the rinse aid. Then, a certain volume of H2O2 reactive substance will be added to the reaction medium and will be terminated by reaction with Hcl after 15 minutes. And optical absorption of the specimens will be read by the Elisa photometric apparatus against the standard curve. Appropriate quality control standards will be used to validate the method. The technical specifications included in the Randox company kit are as follows.

Table 2: The limit of detection of the kit

Analyte	Matrix	Limit of Detection
Methadone	Blood	0. 18 ng/ml

Table 3: Kit specificity

Compound	% Cross-Reactivity
Methadone	100
EDDP	<1
EMDP	<1
LAAM	<1
Other drugs	0

To check the accuracy of one day, three standard samples (with methadone spiked to the sample) were prepared at three levels of control one (50 ng/ml), control two (100 ng/ml) and control three (200 ng/ml), and each of the specimens were divided into five equal parts, which were injected into the machine after extraction in one day.

Table 4: Results of accuracy on a GC/MS method day

	Added Concentration (ng/mL)	Run	Measured Concentration (ng/ml)	Mean	SD	RE%
Sample1	50	1	60	54	6. 3	-8
		2	56			
		3	44			
		4	52			
		5	58			
Sample 2	100			96. 2	9. 5	3. 8
		1	107			
		2	90			
		3	85			
		4	105			
		5	94			
Sample 3	200	1	215	202. 8	14. 6	-1. 4
		2	220			
		3	193			
		4	185			
		5	201			

To check the accuracy of one day, two standard controls were prepared at two levels of control one (50 ng / ml) and control two (100 ng / ml), and each of the controls were divided into five equal parts.

Table 5: Results of accuracy in one day ELISA method

	Added Concentration (ng/mL)	Run	Measured Concentration (ng/ml)	Mean	SD	RE%
Sample1	50	1	53	52. 2	8. 8	4. 4
		2	52			
		3	51			
		4	53			
		5	52			
Sample 2	100	1	98	97. 8	2. 8	-2. 2
		2	97			
		3	98			
		4	102			
		5	94			

In order to check the accuracy of the method in a few days, three standard urine specimens (with methadone spiked to blanc urine sample) at three levels of control one (50 ng / ml), control two (100 ng / ml) and control three (200 ng / ml) were prepared and each specimen was divided into five equal parts, which were injected into the machine after extraction for five consecutive days.

Table 6: Results of the accuracy of the GC-MS method in a

	Added Concentration	Run	Measured Concentration	Mean	SD	RE%
Sample 1	50	1	55	54. 8	6. 1	9. 6
		2	61			
		3	59			
		4	54			
		5	45			
Sample 2	100	1	109	96. 8	10. 7	3. 2
		2	93			
		3	107			
		4	91			
		5	84			
Sample 3	200	1	205	197. 8	6. 8	-1. 1
		2	195			
		3	198			
		4	203			
		5	188			

The results of the accuracy of ELISA method are summarized in Table 7.

Table 7: Results of accuracy by ELISA method

Concentration (ng/ml)	Concentration	mean	Standard deviation	Relative error %
50	50	51. 6	1. 5	3. 3
	52			
	53			
100	108	105	3	5
	102			
	105			

Conclusion

In a study on accuracy related to the GC/MS and ELISA methods in one day, the mean magnitude of relative error in the GC/MS method was 4.4% and the ELISA method was 3.3%, and in the accuracy check in a few days, 4.6% for GC/MS method and 4.2% for ELISA method, which indicates high accuracy in ELISA method. However, the GC/MS method was more stable over time and did not require re-calibration, and was used for high-precision specificity of analysis of narcotics and other drugs [5]. On the other hand, in the ELISA method, there are no sample preparation processes.

The error in this section was deleted and the results were more precise, and Cooper et al. and Pougwell et al. concluded the same [3, 6]. In the study of the accuracy of the methods, the mean absolute magnitude of the relative error in different concentrations in the GC/MS method was 2.3% and in the ELISA method it was 4.1%, which showed a higher accuracy of the GC/MS method but in general, both methods were acceptable, which had been concluded by Aguiliss et al [7]. In the linearity study, the GC/MS method was linear in range from 30 ng to 10 μg. In the ELISA method, the analysis range was from 1.2 ng to 100 ng, that the GC/MS method analysis was much more than the ELISA method. But concentrations lower than detection limit and measurement limit of GC/MS method with extraction method under study were measured via ELISA method. And at levels above 100 ng it should be analyzed after dilution of the sample. And the treatment and methadone toxicity is higher than 100 ng per cc of blood. In the study of the specificity of both methods after verifying the non-interference of the Blanc sample matrix in the analytical methods, 50 samples of individuals whose methadone were not positive were tested in the design .None of the drugs containing tramadol, morphine, codeine, and methamphetamine, nicotine and pethidine have been interfered with in both ways. In general, GC/MS is used to determine the chemical properties and qualitative and quantitative analysis of the samples as a major factor in drug analysis [8]. And compared to the ELISA method provided by Randox, it is more capable of analyzing a wide range of serum concentrations of methadone over a long period of time, which simultaneously analyzes various analytes. But the method is time consuming and expensive, and the complexity of the device is high. However, the ELISA method with high sensitivity and high speed can analyze the number of samples in a short time, and also does not require much basic tools and hazardous chemical solvents.

Suggestions

Given that in some forensic labs, blood levels of drugs are not monitored, blood levels can be found in many cases along with other documents and documentation. In case of confirmation of the presence of dangerous and prevalent drugs in poisoning by ELISA method, their blood level is determined.

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