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The Use of Different Proteins as a Carrier Protein to Obtaining Morphine-Protein Conjugates for ELISA Diagnosis of Drug Addicts

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Drug addiction is one of the biggest problems of medicine because diagnosis and treatment of drug addiction are difficult compared with some other socially significant diseases. In this study, synthesis and evaluation of four carrier protein-morphine conjugates were experimented. These conjugates were evaluated based on ELISA; soybean protein-based conjugate was selected for further analysis. The total soybean protein was isolated from the local soybean variety and; it was fractioned by the gel-filtration method and their amino acids compositions were studied. After that, the ELISA drug addicts were conducted based on soybean protein-morphine conjugates synthesized with soybean protein fractions. The high molecular weight soybean protein- morphine conjugate showed the highest quality.

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1. INTRODUCTION

Drug addiction is one of the most prevalent problems in the world of medicine. According to the World Health Organization, currently, 275 million people, or 5.6% of the world's population, between the ages of 15 and 64, use drugs, which cause 500,000 deaths each year. Opiates are the most dangerous drug, accounting for 76% of drug-related deaths [1,2]. This current situation requires the development of new accurate and sensitive analysis methods to identify latent addicts.

All over the world, the physicochemical methods are widely used to the detection of a drug or its metabolites in the practice of drug diagnosis. These methods are based on the detection of a drug or its metabolic products used in human biological fluids [3-6]. Such an approach to the diagnosis of drug addicts has significant drawbacks, as these methods allow detection within 24-48 hours from the time of drug acceptance [7]. This is due to the fact that the drugs taken and their derivatives are eliminated from the body in a short period of time. In addition, the detection of addicts using physicochemical methods requires a lot of time, highly qualified personnel and expensive equipment [3]. However, it is known that the human body produces antibodies against accepted opioids [8]. Therefore, the ELISA method, which is based on the detection of specific antibodies formed against accepted drugs, is an effective method in identifying individuals who have admitted drugs (opiates). This method allows the detection of antibodies against opiates even within 2-3 months after drug administration. The basis for the development of this type of ELISA test kits are hapten-protein conjugate absorbed immunosorbents. Because hapten-protein conjugates provide the specificity and sensitivity of the ELISA test kits [9-12].

To date, various carrier proteins have been used by scientists to obtain morphine-protein conjugates. For example, to obtain morphine-protein conjugates tetanus anatoxin, human serum albumin, bovine serum albumin, ovalbumin, lysozyme, thyroglobulin, and fibrinogen were used by authors. [13-19].

The aim of this study was to evaluate the usage of different morphine-protein conjugates in the ELISA diagnosis of drug addictions.

2. MATERIALS AND METHODS

2.1 Synthesis of Morphine-Carrier Protein Conjugates

First, morphine-hemi-succinate was synthesized to create a reactive carboxyl group as described in the previous work [20]. After that, morphine/carrier protein conjugates were obtained with 4 different carrier proteins (Bovine serum albumin (BSA), human serum albumin (HSA), ovalbumin and soybean protein) on the following condition: a solution of 25 mg of protein in 2.5 ml of distilled water was mixed with 1 ml of dimethylformamide containing 7.5 mg of morphine 6-hemisuccinate and 5 mg of water-soluble carbodiimide in 1.5 ml of distilled water. The reaction mixture was incubated for 5 hours at 4 °C. After the reaction, obtained conjugate was dialyzed against 0.02 M carbonate buffer pH 9.5. The concentration of conjugates was determined by Lowry method [21].

2.2 ELISA of drug Addictions Blood Serum

Obtained morphine hemi-succinate-carrier protein conjugates were adsorbed to the 96-well clear flat bottom polystyrene high binding ELISA microplate (Costar, USA) in 100 µl/4 µg to each well and incubated overnight at 4 ^{o}C. The plate was then blocked 24 hours at 4 ^{o}C with a 1% solution of four proteins respectively to each conjugate. The plate was washed 2 times with 300 µL of washing buffer (0.1 M PBS pH 7,4; 0,05 % Tween-20) (60 seconds) and a 1:100 diluted plasma in PBS-T was added to each well and incubated for 60 minutes at 37˚C. After incubation of the serum, the plate was washed 5 times with 300 µL washing buffer (60 seconds) and secondary antibody conjugate (Anti-Human IgG (µ-chain cpecific)-Peroxidase antibody produced in goat, Sigma, USA) was added to 100 µl to ELISA plate holes and incubated at 37 ^{o}C for 30 minutes. ELISA plate was washed again 5 times with a washing buffer and then 3,3′,5,5′-tetramethylbenzidine (TMB) in 0.05 M phosphate-citrate buffer and H_2O_2 into 100 µl were added as the peroxides' substrate. The reaction was stopped after 15 minutes by the

addition 50 µl of 2 M sulphuric acid to reaction mixture and result of ELISA was determined at 450nm with a plate reader (ELx800 Universal Microplate Reader, Bio-Tek Instruments Belgium).

2.3 Extraction and Isolation of Soybean Protein

Seeds of Uzbek-6 soybean variety were homogenized and defatted with acetone (10:1) for one hour. The defatted homogenate was extracted with 10/1 ratio of 0.2 M extraction buffer (0,5 M tris-OH pH 7,4; 10% sodium dodecyl sulfate (SDS); 0,5 M ethylenediaminetetraacetic acid) for 2 hours at room temperature. Then the extract was centrifuged for 30 minutes at 6000 rpm and the supernatant was dialyzed for 12 hours against distilled water. After dialysis, the protein content in the samples was determined by the Lowry method [21] and lyophilized.

The isolated total was divided into fractions by gel-filtration method. The gel-filtration was performed on a column (2.5 x 70 cm) and Sephadex G-75 as sorbent and phosphate buffer (0,2 M, pH 7.4.) was used for elution with 1 ml/min flow rate.

Electrophoretic analysis of the fractionated proteins was performed by 10% SDS-Polyacrylamide gel according to the Laemmli method [22].

2.4 Determination of the Amino Acid Composition

The hydrolysis of samples containing protein fractions was carried out using 5.7 N HCl in a vacuum for 24 h at 110°C.

Synthesis and determination of phenylthiocarbonyl (PTC) amino acid derivatives was carried out according to the method of Steven A., Cohen D [20]. The identification of PTC-amino acids was carried out on an Agilent Technologies 1200 chromatograph on a 75x4.6 Discovery HS C18 column. Solution A: 0.14 M CH3COONa + 0.05% TEA, pH 6.4; B: CH3CN. Flow rate 1.2 ml / min, absorption 269 nm. Gradient% B / min: 1-6% / 0-2.5 min; 6-30% / 2.51-40 min; 30-60% / 40.1-45 min; 60-60% / 45.1-50 min; 60-0% / 50.1-55 min.

2.5 Statistical Analysis

Statistical analysis was performed using Origin 8.6 software (Microcal Software Inc., Northampton, MA). Results were expressed as mean ± S.E.M. To determine the statistical significance of the results One-Way ANOVA and two-tailed t-test were performed.

3. RESULT AND DISCUSSION

It is known that the human body produces antibodies against opioids (morphine, heroin) [23-28]. Based on these data, we obtained morphine-protein conjugates using 4 different proteins in order to using ELISA for detect antibodies against opioids in the serum (Fig. 1).

After that, the serum of opioid addicts was analyzed by ELISA to determine the activity of 4 different conjugates. For this purpose, serum samples of 25 opioid addicts carried from the Republican Narcology Center were tested and the serum samples of 25 healthy people were used as a negative control. In this case, each serum sample was tested 3 times and their average optic density was used as a result. The ELISA analysis using morphine-protein conjugates based on the 4 different proteins listed above was performed under the same conditions and in parallel using the same blood serum samples. However, the obtained results varied significantly (Fig. 2).

For evaluating ELISA results, the critical point of the optical density (Cut-off) of the analysis was calculated using the following formula:

$$OD_{Cut\text{-}off} = OD(N^-)_{average} \times 2{,}1$$

Where $OD(N^-)_{average}$ is the average arithmetic optical density of the tested samples.

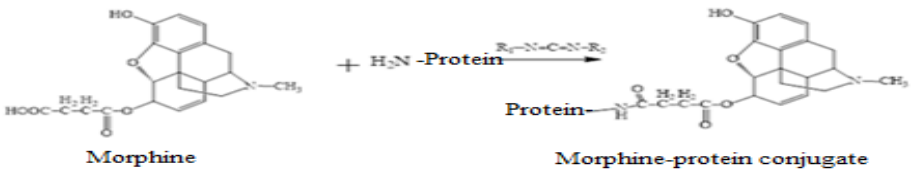

Fig. 1. Reaction of morphine-protein conjugation

Based on the ELISA results of the examined serum samples of 25 drug addicts and healthy people, the specificity and sensitivity of the four conjugates used in this analysis were determined using the following formulas:

$$\text{Specificity} = \frac{RNR}{RNR + FPR} \times 100\ \%$$

RNR (real negative results) – number of negative results
FPR (false positive results) – number of false positive results

$$\text{Sensitivity} = \frac{RPR}{RPR + FNR} \times 100\ \%$$

RPR (real positive results) – number of positive results
FNR (false negative results) – number of false negative results

The specificity and sensitivity of ELISA conducted based on morphine-soybean protein conjugate were 92% and 91% respectively. The levels of specificity and sensitivity of other conjugates did not show sufficient results for using preparation of ELISA test kits designed to diagnosis of drug addicts. For example, the results of the analysis based on morphine-HSA conjugates, the analysis showed the highest level of sensitivity and, at the same time, the lowest level of specificity. This is depended to the nonspecific attachment of antibodies in the samples to the conjugate. The low specificity of ELISA test kits leads to false-positive results and low sensitivity gives to false-negative results [29-31].

Based on the ELISA results, the main focus was on and the morphine-soybean protein conjugate. Therefore, the total proteins of Uzbek-6 variety of soybean were isolated. Then the isolated protein was separated by gel filtration into low and high molecular weight fractions, which were analyzed by gel electrophoresis in PAGE (Fig. 3).

According to the Fig. 3, the high molecular weight fraction contains proteins with molecular weights from 40 to 135 kDa and the low molecular weight fraction - below from 35 kDa.

Undoubtedly, excess free amino groups in the protein that can interact with the carboxyl groups of morphine hemi-succinate, the more moles of morphine will bind to one mole of the carrier protein. In this regard, the study of the amino acid composition of soy protein fractions used to obtain conjugates is of great importance. A study of the amino acid composition of soybean protein fractions showed that; the largest amount of ε-amino acids (lysine, arginine) was contained in the high molecular weight fraction of soybean protein (Table 1). It is obvious that the higher amount of the ε-amino acids in proteins increases the efficiency of obtaining hapten-protein conjugates.

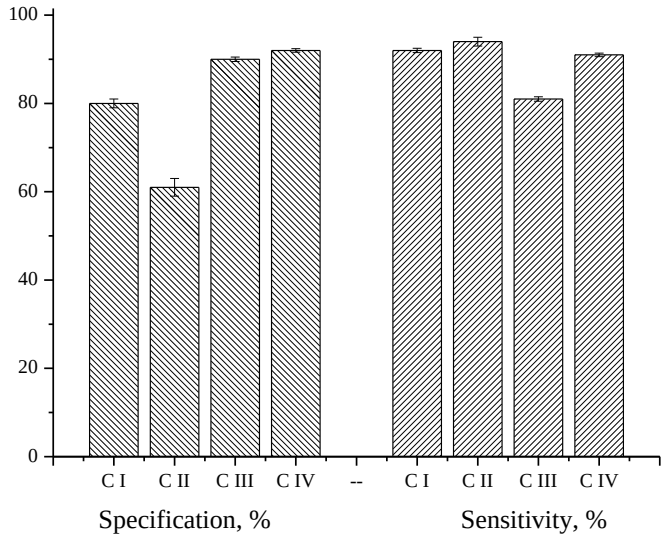

Fig. 2. The specificity and sensitivity of the four conjugates used in ELISA

Note. C I – morphine-BSA conjugate; C II – morphine-HSA conjugate; C III – morphine-ovalbumin conjugate; C IV – morphine-soybean protein conjugate

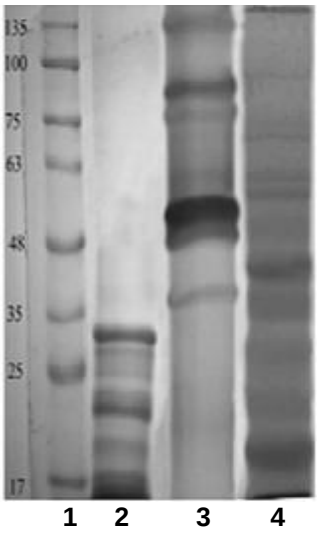

Fig. 3. Gel-electrophorogram of soybean protein fractions in 10% SDS PAGE; 1-marker; 2-second fraction; 3- first fraction; 4- total soybean protein

Table 1. Amino acids contents of isolated soybean protein fractions, in %

Amino acids	High molecular weight fraction	Low molecular weight fraction	Amino acids	High molecular weight fraction	Low molecular weight fraction
Asp	4,943	7,986	*Pro*	2,691	1,818
Glu	0,000	10,73	*Tyr*	2,159	1,329
Ser	3,284	2,710	*Val*	3,909	2,000
Gly	3,923	2,402	*Met*	0,000	0,294
Asn	*0,000*	0,000	*Ile*	5,502	3,146
Gln	*0,000*	0,000	*Leu*	6,743	3,112
Sys	0,501	0,732	*His*	0,000	0,000
Thr	2,259	1,632	*Trp*	0,000	0,000
Arg	6,878	3,621	*Phe*	2,403	1,340
Ala	2,999	1,939	*Lys*	1,298	0,704

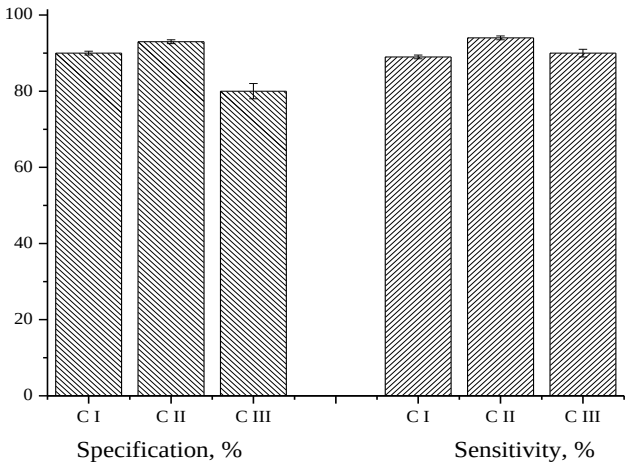

Fig. 4. The specificity and sensitivity levels of ELISA conducted based on morphine-protein conjugates obtained with different soybean protein fractions

Note. C I – morphine-protein conjugate with the total soybean protein; C II – morphine-protein conjugate with high molecular weight fraction; C III – morphine -protein conjugate with low molecular weight fraction;

After that, the morphine hemi-succinate conjugates were obtained with the total soy protein, the high molecular weight, and the low molecular weight fractions of soybean protein for comparison of their possibilities on hapten-protein conjugate development for using ELISA tests. The obtained conjugates were adsorbed on the surface of ELISA plate and the serum samples of 25 drug addicts and the serum of 25 healthy people were tested using the plate (Fig. 4) in order to evaluate the conjugates.

According to results of ELISA, the morphine-protein conjugate synthesized with the high molecular fraction of soy protein showed the highest level of specificity (93%) and sensitivity (95%). It was found that the morphine-protein conjugate obtained with the high molecular fraction of soy protein was the most efficient conjugate among the studied conjugates for using the ELISA diagnosis of hidden drug addicts.

4. CONCLUSION

The morphine hemi-succinate-soybean conjugated protein-based ELISA of drug addicts demonstrated higher specificity and sensitivity than other conjugates-based analyses. Therefore, total proteins of Uzbek-6 soybean variety were isolated, fractioned to two fractions and their amino acids contend was studied. The morphine conjugates were obtained with protein fractions of total soy proteins and among them, the morphine-protein conjugate obtained with the high molecular fraction of soybean protein was preferred over others. These results showed that this conjugate can be used to develop ELISA test kits, designed for the diagnosis of drug (opiates) addicts. Diagnosis of drug addicts in this way allows early detection of latent drug addicts.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by the personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard written ethical approval has been collected and preserved by the author(s).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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