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ANALYSIS OF INSULIN IN PHARMACEUTICAL PREPARATIONS PRESENT IN LIBYAN COMMUNITY PHARMACIES

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ABSTRACT

Background: Insulin is an anti-diabetic drug which is used for the treatment of diabetes type I. There are many methods for analyzing insulin starting from ELIZA to HPLC methods. There are many brands for insulin preparations that are imported by private companies in addition to those provided by the National Hospital of Diabetes. Aim: This study aims to analyze random samples of insulin preparations collected from different community pharmacies in Tripoli-Libya in an attempt to check if these brands have adequate insulin concentrations for diabetic patients. **Method:** In this study, random samples were collected for insulin preparations in the form of insulin pens. They were analyzed using spectroscopic method. An absorption maximum was found to be at 276 nm using 0.01M HCl as a solvent. The results were compared to that of similar samples obtained from the national hospital of diabetes. **Results:** The decrease in amount of insulin in the later two brands reached 10% or more making them inappropriate to deliver the needed amount of insulin at different doses. This is very vital because it will lead to serious problems on short as well as long terms to diabetic patients.

KEY WORDS: Insulin, spectrophotometric analysis, absorbance and dose.

INTRODUCTION

Insulin is an important polypeptide hormone that regulates carbohydrate metabolism. Abnormal levels of insulin are associated with diabetes mellitus that is characterized by chronic hyperglycemia. Insulin is a protein comprising of 2 polypeptide chains A (with 21 amino acid residues) and B (with 30 amino acid residues) (Fig. 1). Chains A and B are linked by disulphide bridges. In addition A-chain contains an intra-chain disulphide bridge linking residue 6 and 11. The structure of insulin is shown in the figure below. C-chain, which connects A and B chains is liberated along with insulin after breakdown of proinsulin. Insulin monomers aggregate to form dimers and hexamers.[1]

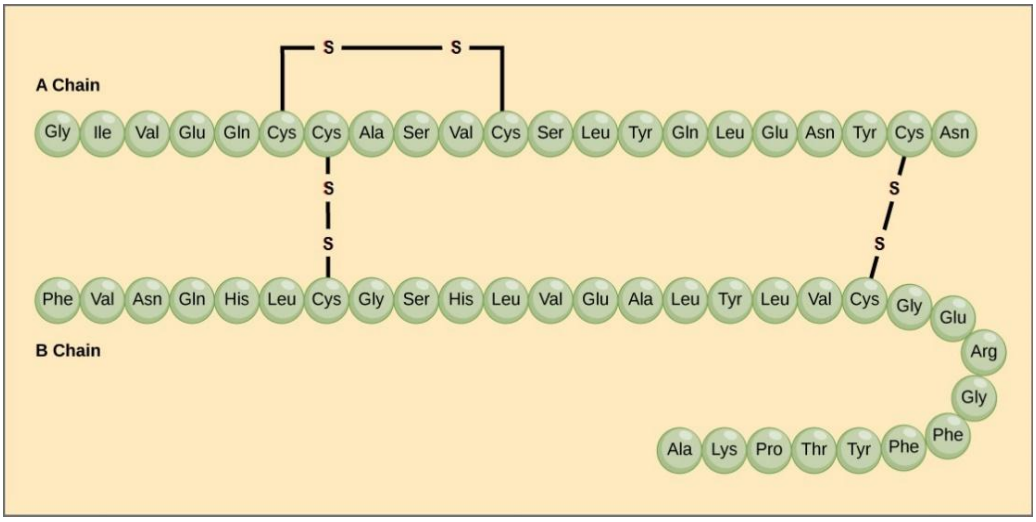

Figure 1: Insulin structure.

Insulin is secreted from the beta cells (also called B-cells) of the islets of Langerhans in the pancreas. It is secreted as a response to elevated levels of blood glucose, arginine, and sulphonyl ureas. Various neural, endocrine and pharmacological agents can also similar response. It is secreted together with inactive C-peptide, which gives a useful index of insulin secretion as its plasma concentration is low or absent in patients with type 1 diabetes. Diabetes mellitus (fasting blood glucose concentration of ≥7 mmol/L) is caused by an absolute or relative lack of insulin. Such patients are usually young and non-obese at presentation. In type 2 diabetes, there is a partial lack of insulin secretion, coupled with marked resistance to its action.[2] Therefore the circulating concentration of immunoreactive insulin may be normal or even increased. Insulin is used for treatment of type 1 diabetes where it is usually administered by subcutaneous injection while sulfonylureas and biguanide drug such as metformin, is used for treatment of type 2 diabetes.[3]

Potential sources of pharmaceutical insulin:

- **Non-recombinant sources**
  Porcine pancreas, bovine pancreas, peptide synthesis, and enzymatically converted human insulin.
- **Recombinant sources**
  Escherichia coli, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Arabidopsis thaliana* seeds, tobacco and *Lettuce chloroplasts*, and transgenic murine milk.[4]

Types of insulin

1. **Rapid-acting** – **Aspart** Novorapid®, **Lispro** Humalog ®, **Glulisine**Apidra®.
2. **Short-acting**– **Regular** Actrapid®, Insulin R®, Human insulin®.
3. **Intermediate** – **NPH** (neutral protamine hagedorn), Insulatard HM®, insulin®.
4. **Long-acting- Glargine** Lantus®, **Detemir** Levemir®
5. **Premixed -**is a combination between rapid or short acting with intermediate acting mixtard®.[5]

Physical and chemical properties of insulin

- Regular human insulin is crystalline zinc insulin dissolved in a clear solution. It may be administered by any parenteral route: subcutaneous, intramuscular, or intravenous.[6]
- Insulin aspart, glulisine and lispro are also soluble crystalline zinc insulin, but are intended for subcutaneous (SQ) injection. When administered intravenously, the action of these rapid-acting insulin analogs is identical to that of regular insulin. NPH, or neutral protamine hagedorn, is a suspension of regular insulin complexed with protamine that delays its absorption. Insulin suspensions should not be administered intravenously. All insulins, except insulin glargine, are formulated to a neutral pH.[6]
- Long-acting insulin glargine is a soluble, clear insulin, with a pH of 4.0 which affects its SQ absorption characteristics, discussed further in the pharmacokinetics section. Insulin glargine should not be mixed with other insulins, and should only be administered subcutaneously.[7] Insulin detemir is an insulin analog coupled to an 18-chain fatty acid that binds to albumin in the SQ tissue. This results in delayed absorption and a prolonged duration of action. Insulin degludec is an ultra-long insulin analog that breaks down into monomers from dissociating zinc molecules after administration.[8] Insulins detemir and degludec should also not be mixed with other insulins and are intended only for subcutaneous use.[9]

Analytical methods for insulin

1. **Immunoassays: the enzyme-linked immunosorbent assay (ELISA).**

The purpose of ELISA is to detect whether a target antigen is present in the sample.[10] Sandwich type ELISA is the most popular for insulin determination and contains two antibodies: a capture antibody (anti-insulin antibody)[11] and a detection antibody (peroxidase labeled anti-insulin antibodies). During incubation, the insulin in the sample reacts with the anti-insulin antibodies that are immobilized on the plate.[12] The unbound antibody is then washed using a washing solution and 3,3,5,5-tetramethylbenzidine (TMB) is added as a chromogenic substrate to react with the insulin bound conjugate. After the reaction is stopped by adding an acid, the colored products are measured at 450 nm by using a spectrophotometer and the measured signal is correlated with the analytic concentration (fig. 2).[12]

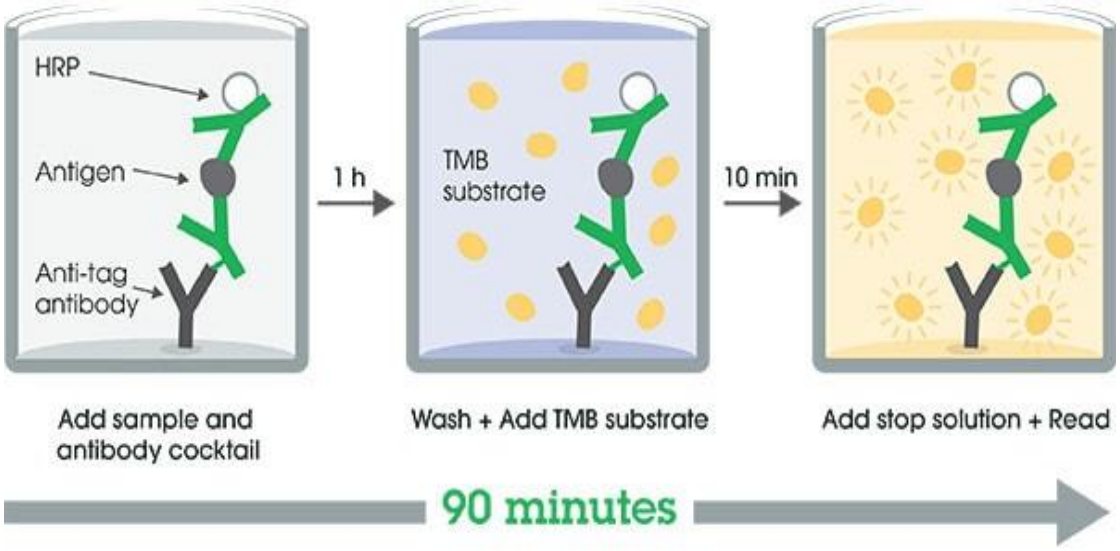

Figure 2: Direct sandwich ELISA for insulin determination.

2. Chromatographic methods

With the increasing demand for studies on insulin and its analogues or metabolites, chromatographic methods have been developed to achieve adequate resolution between insulin and its major degradation products or its analogue drugs. The mechanisms of chromatographic methods are based on column separation assisted by high performance liquid chromatography (HPLC) or electrophoresis and identification using an ultraviolet (UV) detector and mass spectrometry (MS). Different sample extraction procedures prior to analysis also provide different selectivity and sensitivity for insulin determination.[13]

HPLC-UV

HPLC-UV is a highly effective screening method based on column separation and detection under the maximum UV absorption of an analyte.[14] Liquid–liquid extraction (LLE) is a common method used for insulin extraction. Generally, dichloromethane is added to the insulin standard solution or biological samples, vortexed and centrifuged. The organic phase is transferred to a new tube and 0.05 M HCl is added and vortexed for injection.[14] In the study by Ravi et al. (2007), the developed HPLC system consisted of a Gilson delivery pump equipped with a 6-valve sample injection port with a UV–Vis detector and a chromato-integrator.[15] Based on the different chemical structures and affinities of insulin and its analogues with the chromatographic column, HPLC-UV is able to achieve complete separation and quantitation. Also, the general running time of the HPLC-UV method was approximately 10–20 min, which is much faster than the running time of other immunoassays. However, most of the reported HPLC-UV methods have been validated only by using insulin standards in a Phosphate-buffered saline PBS buffer instead of real biological samples.

3. HPLC / Tandem Mass spectrometry (LC-MS/MS)

The LC-MS/MS is superior in that it has high specificity and selectivity, and wide dynamic range and is less susceptible to interference. The samples need to be purified using SPE (solid phase extraction) prior the analysis by LC-MS/MS. The insulin database needed to be created with pure standard solutions for each analogue. Stock standard solutions (0.3 mg/mL) were made in pure water and working solutions (0.05 mg/mL) were made in 1% formic acid in water for each substance. Eventually the database contained theoretical monoisotopic exactmasses, retention time, MS spectrum, MS/MS spectrum and di-agnostic ions for each insulin. These collected information of standard insulin is compared to samples.[16]

4. UV-Visible absorption spectrophotometry

A variety of detectors are available for UV-Visible measurements. Very few reactions are specific for a particular substance, but many give colors for a small group of related substances only, that is they are selective. By altering and controlling of pH, close approximation to specificity is obtained. It is desirable that the system follows Beer's law even when photo electric colorimeters are used. The color procedure should be sufficiently stable to permit an accurate reading to be taken. Calibration is one of the most important steps in spectrophotomertic analysis. Good precision and accuracy can only be obtained when a good calibration procedure is used.[17]

5. Electrochemical biosensor

An electrical biosensor is defined as a device that relies on the measurement of current and/or voltage to detect the binding of the analyte to the recognition element in the biosensor**.** The electrochemical sensor is fabricated with carbon nanotubes (CNT) to which the

insulinaptamer is covalently attached. The fabricated insulin-aptamer biosensor functions as the working electrode in a three-electrode system, with Ag/AgCl as the reference electrode and a platinum wire as the counter electrode. Then, the signal transducer converts the recognition event into a detectable and readable signal. The electrochemical signal of insulin was measured by cyclic voltammetry with a potential window of −1 to 1 V and a scan rate of 0.1 v s−1. Based on the different coating materials on the carbon nanotubes, the detection limit and linearity range varies.[18]

Experimental analysis of insulin formulations by UV

It is based on light absorption according to the solubility characters of basic insulin. Three samples were collected for analysis:

1. human insulin (actirapid®)
2. aspart insulin(novorapid®)
3. glargine insulin(lantus®)

The differences between the three formulations are shown in the figure 3 below. Light absorption was determined according to Indian PH 1996 using zero order spectrophotometric method: absorbance of a 0.05% w/v solution in 0.01M hydrochloric acid at the maximum at about 276 nm was 0.48 to 0.56 ; where:

1 unit from human insulin and aspart equals to 0.03846 mg

1 unit from glargine equals to 0.0364.

The content of samples must lie within the range of 90-110% as stated in Indian Pharmacopoeia 1996.

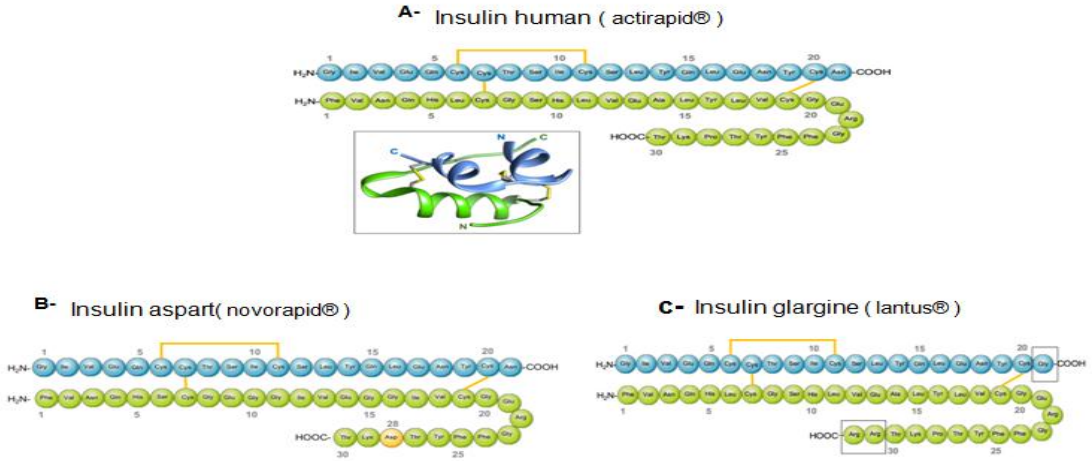

Figure 3: Differences between insulin preparations in samples analyzed.

Materials and equipments

Hydrochloric acid (analytical grade) and samples were measured using UV/Vis Specord spectrophotometer (Analytikjena, Germany).

Procedure followed for samples analysis

0.01M HCl was prepared. (0.1-1 ml) from insulin preparation were placed in clean and dry closed test tubes and volume was completed to 6 ml by 0.01M HCL using a sterile syringe. Test tubes were gently shaken and left to stand for 10 min. 1 ml from each test tube was transferred using sterile syringe to clean and dry test tube and complete volume to 10 ml from 0.01M HCL. Tubes were gently shaked and left it to stand 5 min. Absorbance at 276 nm was measured taking 0.01M HCl as background measurement. Quartz cuvette was used and average absorbance for each sample preparation was collected.

Table 1: Concentration (mg) of insulin per volume (ml) from India pharmacopeia 1996 (human insulin and aspart insulin).

Taken Volume of sample (ml)	Contents of insulin (mg) / unit	Contents of insulin (units) / ml (in 6 ml final solution)*	Contents of insulin (units) / ml (in 10 ml final solution)*
0.1 ml (1 unit)	0.03846	0.00641	0.000641
0.2 ml (2 units)	0.07692	0.01282	0.001282
0.3 ml (3 units)	0.11538	0.01923	0.001923
04 ml (4 units)	0.15384	0.02564	0.002564
0.5 ml (5 units)	0.19230	0.03205	0.003205
0.6 ml (6 units)	0.23076	0.03846	0.003846
0.7 ml (7 units)	0.26922	0.04487	0.004487
0.8 ml (8 units)	0.30768	0.05128	0.005128
0.9 ml (9 units)	0.34614	0.05769	0.005769
1.0 ml (10 units)	0.3846	0.06410	0.006410

***theoretical concentration of insulin for 6 ml and 10 ml preparations

Table 2: Concentration (mg) of insulin per volume (ml) from India pharmacopeia 1996 (glargine insulin).

Taken Volume of sample (ml)	Contents of insulin (mg) / unit	Contents of insulin (units) / ml (in 6 ml final solution)*	Contents of insulin (units) / ml (in 10 ml final solution)*
0.1 ml (1 unit)	0.0364	0.00607	0.000607
0.2 ml (2 units)	0.0728	0.01213	0.001213
0.3 ml (3 units)	0.1092	0.0182	0.00182
04 ml (4 units)	0.1456	0.02427	0.002427
0.5 ml (5 units)	0.1820	0.03033	0.003033
0.6 ml (6 units)	0.2184	0.0364	0.00364
0.7 ml (7 units)	0.2548	0.04247	0.004247
0.8 ml (8 units)	0.2912	0.04853	0.004853
0.9 ml (9 units)	0.3276	0.05460	0.005460
1.0 ml (10 units)	0.3640	0.06067	0.006067

*theoretical concentration of insulin for 6 ml and 10 ml preparations.

RESULTS

A- Standard of Human Insulin (Actirapid®)

Table 3: Average absorption of insulin standard preparation and theoretical concentration.

Unit	Concentration mg/ml	Absorption
1	0.00064	0.08373
2	0.00128	0.16293
3	0.00192	0.23300
4	0.00256	0.29430
5	0.00321	0.35920
6	0.00385	0.42587
7	0.00449	0.49370
8	0.00513	0.57010
9	0.00577	0.63457
10	0.00641	0.71197

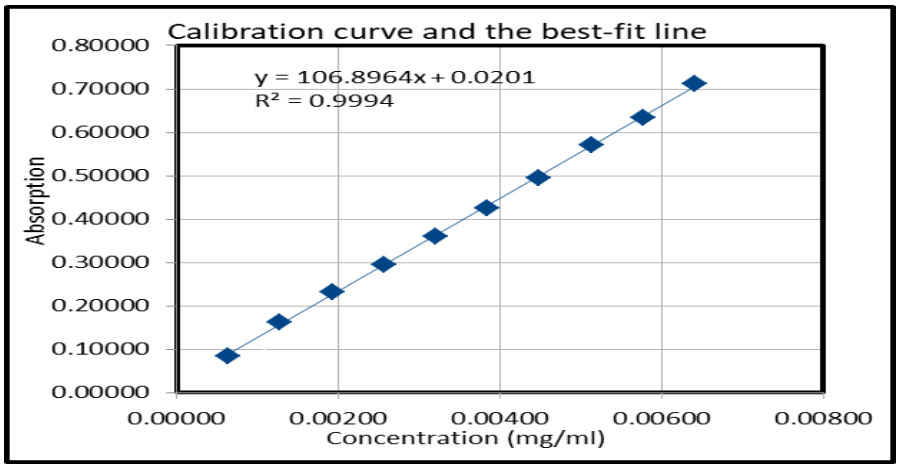

Figure 4: Calibration curve of human insulin (Actirapid®).

Table 4): Content result calculation of insulin test preparation sample (Actirapid®, imported from Egypt).

Sample ID	Readings of the unknowns	Calculated concentration (mg/ml)	%content
1 U	0.08770	0.00063	98.70
2 U	0.14070	0.00113	88.02
3 U	0.22450	0.00191	99.45
4 U	0.27120	0.00235	91.62
5 U	0.34490	0.00304	94.81
6 U	0.41260	0.00367	95.48
7 U	0.48660	0.00436	97.27
8 U	0.52460	0.00472	92.04
9 U	0.60550	0.00548	94.93
10 U	0.62560	0.00566	88.37

B- Insulin aspart (novorapid®)

Table 5: Average absorption of insulin standard preparation and theoretical concentration.

Unit	Concentration mg/ml	Absorption
1	0.00064	0.07583
2	0.00128	0.16193
3	0.00192	0.22860
4	0.00256	0.27600
5	0.00321	0.37077
6	0.00385	0.43893
7	0.00449	0.49993
8	0.00513	0.57580
9	0.00577	0.64047
10	0.00641	0.76607

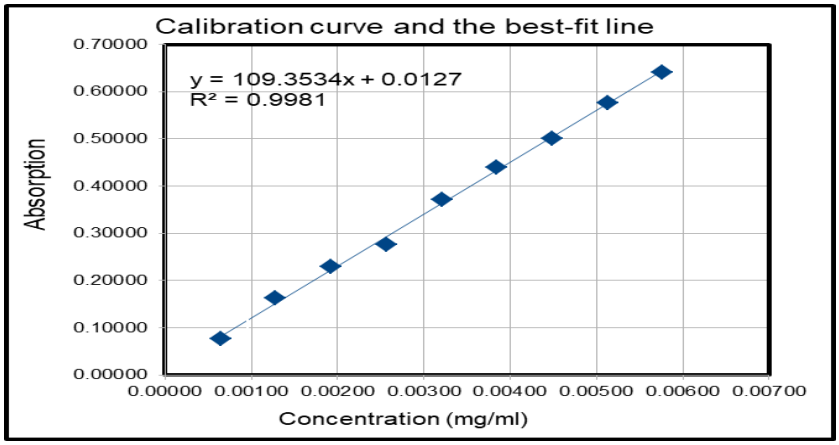

Figure 5: Calibration curve of Insulin aspart (Novorapid®)

Table 6: Content result calculation of insulin test preparation sample.

Novorapid Pen / TURKEY	Spl ID	Readings of the unknowns	Calculated concentration (mg/ml)	%content
	1 U	0.08280	0.00059	91.54%
	3 U	0.22570	0.00192	100.03%
	5 U	0.37640	0.00333	130.01%
	7 U	0.51530	0.00463	103.25%
	9 U	0.67370	0.00611	105.99%
Novorapid Pen / FRANCE / Imported from EGYPT	1 U	0.08270	0.00059	91.40%
	2 U	0.14990	0.00121	94.74%
	3 U	0.22720	0.00194	100.76%
	4 U	0.29740	0.00259	101.18%
	5 U	0.36620	0.00324	101.03%
	7 U	0.49090	0.00440	98.16%
	9 U	0.63140	0.00572	99.13%

C- Insulin glargine (Lantus®).

Table 7: Average absorption of insulin standard preparation and theoretical concentration.

Unit	Concentration mg/ml	Absorption
1	0.000607	0.06973
2	0.001213	0.11520
3	0.001820	0.17833
4	0.002427	0.24747
5	0.003033	0.28243
6	0.003640	0.34853
7	0.004247	0.42820
8	0.004853	0.48047
9	0.005460	0.55487
10	0.006067	0.60010

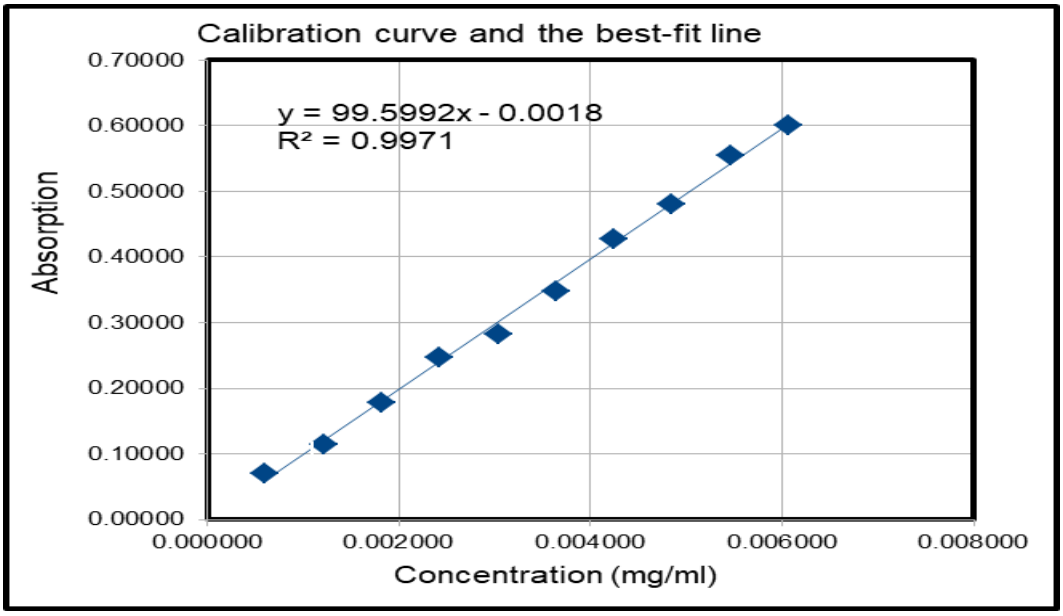

Figure 6: Calibration curve of insulin glargine (Lantus®).

Table 8: Content result calculation of insulin test preparation samples.

Lantus solostar Pen /Imported from EGYPT	Spl ID	Readings of the unknowns	Calculated concentration unit	%content
	1 U	0.06430	0.00041	68.16%
	3 U	0.16720	0.00138	75.62%
	5 U	0.28290	0.00246	81.07%
	7 U	0.40590	0.00361	84.99%
	9 U	0.49220	0.00442	80.89%
Lantus solostar Pen / TURKEY	1 U	0.06790	0.00045	73.71%
	2 U	0.12490	0.00098	80.84%
	3 U	0.18230	0.00152	83.38%
	4 U	0.23980	0.00206	84.69%
	5 U	0.30570	0.00267	88.10%
	7 U	0.41760	0.00372	87.56%

DISCUSSION OF RESULTS

A- Discussion of insulin human sample (Actirapid®)

All samples contents lied within the accepted range (90-110%) except for 2U and 10U, although the deviation was by 2% or less. Percentage of error was not proportional with sample size (not constant as well), so it is decreasing as sample size increases which is preferred.

B- Discussion of insulin aspart (Novorapid®)

Only one sample (5U) was out of accepted range (90-110%), which may be due to personal error whereas other samples were all having accepted content. Values for percentage of error were all decreasing as sample size increases making them less significant (< 5%).

C- Discussion of insulin glargine (Lantus®)

For the two brands, all samples showed to have unacceptable content (< 90%) making them having inadequate amount of insulin at different dose units. In addition, values for percentage of error were higher compared to the two previous types especially at lower units, 1U and 2U, where it reached 8.53% and 7.90% for Egyptian and Turkish brands respectively. However, these values for % error were decreasing as sample size decreased (not proportional with sample size).

CONCLUSION

The imported insulin to the National Hospital of Diabetes found to have accepted contents compared to brands manufactured in Turkey and transportation from Egypt. The decrease in amount of insulin in the later two brands reached 10% or more making them inappropriate to deliver the needed amount of insulin at different doses. This is very vital because it will lead to serious problems in case of a vital drug like insulin. These inadequate contents would make the dose difficult to be accurately measured. Therefore, it is recommended for authorized bodies in the local quality control system to pay more attention in evaluating different brands of insulin in local pharmacies. This will ensure better health care for diabetic patients who are vulnerable to many

irreversible complications if not received optimum doses of insulin.

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