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HORMONAL IMMUNOASSAYS;
COMPARISON USING ECL & ELFA

Bashir Elfatih Abdalla[1], Abedelmula M. Abdealla[2]

1. Clinical Chemistry Department Medical Laboratory Sciences Al-Neelain University-Khartoum Sudan.
2. Medical Laboratory Faculty of Medical Technology (Sirte) University of Sirte - Libya

Correspondence Address:
Abdelmula M. Abdella
mula200099@yahoo.co.uk
Bashir Elfatih Abdalla
abbaru14@yahoo.com

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ABSTRACT… Objectives: several improvements have been made in the design of immunoassays such as method of antibody production, labeling, automation and detection technology. The aim of the present study was to compare the accuracy and precision of enzyme linked immunofloriscence assay (ELFA) and electrochemiluminescence assay (ECL), with Elisa for determination of serum TSH levels. **Period:** Feb 2014 to Nov 2014. **Setting:** College of postgraduate studies, University of Al-Neelain, Khartoum, Sudan. **Material and Methods:** Three commercial control materials low, normal and high levels of TSH, were used for imprecision studies of immunofloriscence assay (ELFA) and electrochemiluminescence assay (ECL) methods and 120 patients samples including low (20%), normal (50%), and high (30%) TSH levels, were measured by the two methods, and used for methods comparison. In addition to six assigned prepared pool serum used for linearity evaluation of the two methods. **Results:** Inter- and intra-assay CV% for ECL and ELFA was significantly low compared with the required by the manufacture. (Intraassay CV% for ECL was 2.9%, 2.74%, and 2.55% for low, normal, and high respectively of TSH levels of the control sera. Intraassay CV% for ELFA was 3.95%, 3.75%, and 5.73% for low, normal, and high respectively of TSH levels of the control sera. Interassay CV% for ECL was 3.0%, 2.75%, and 2.81% for low, normal, and high respectively of TSH levels of the control sera. Intraassay CV% for ELFA was 4.26%, 4.0% , and 5.75% for low, normal , and high respectively of TSH levels of the control sera. Although the mean TSH levels of the three levels of the control sera measured by ECL & ELFA methods, is significantly difference from assigned TSH mean values(low 0.488+/-0.078,normal 6.016+/- 0.952,high 33.651+/-5.39) , but the measured values is within the mean range of the assigned means values. ECL; low (0.611 +/- 0.018. $p \leq 0.001$), normal (6.6785 +/- 0.183. $p \leq 0.00$), high (35.0485 +/- 0.894. $p \leq 0.02$). ELFA low (0.50545 +/- 0.020. $p \leq 0.00$) , normal (6.5395 +/- 0.244. $p \leq 0.00$), high (31.0350 +/- 1.779. $p \leq 0.001$).The mean TSH levels of the 120 patients samples measured by ECL & ELFA , is significantly difference , for ECL (15.74+/- 1.181 . $p \leq 0.00$) when compared with the mean TSH value (14.56 +/- 1.65) of the patients samples. For ELFA method also there is significant difference (13.76 +/- 1.59 , $p \leq 0.00$) when compared with mean of the assigned TSH values(14.56 +/- 1.65) of the patients samples, but within the target values of the means .The study showed strong relationship between the two TSH levels measured by ECL(mean 15.74 mIU/L, slope 0.67 , correlation coefficients 0.991, $p \leq 0.00$) and by ELFA (mean 13.76 mIU/L, slope 0.54, correlation coefficients 0.995, $p \leq 0.00$) with the assigned values (14.56) of 120 patients sample .The results illustrates no significant difference of TSH mean level in six prepared pool samples measured by ECL(22.63 mIU/l +/- 1.12 , $p \leq 0.1$) and ELFA(19.87mIU/l +/- 1.15 , $p \leq 0.11$) when compared with the TSH assigned values(22.54 mIU/l +/-0.96), and with strong correlation between the two TSH levels measured by ECL(22.63 mIU/l, slope 0.79, correlation coefficients 0.999, $p \leq 0.00$) and ELFA (mean 19.87mIU, slope 0.68, correlation coefficients 0.985, $p \leq 0.00$), with the assigned TSH values (22.54 mIU/l +/-0.96).of the six prepared pool samples. **Conclusion:** Considerable significant precision and accuracy was manifested by both ECL and ELFA methods in estimation of TSH levels, but ECL is more precise than ELFA especially in the lower TSH concentration.

Key words: Immunofloriscence, electrochemiluminescence Measurements/methods, Thyroid Stimulating hormones, Immunoassay, Accuracy, Precision

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INTRODUCTION

The hypothalamic-pituitary-thyroid axis is of particular importance for the adaptation of mammals to their environment.[1] Thyrotropin, or thyroid-stimulating hormone (TSH), is a glycoprotein produced in the anterior pituitary gland. Thyrotropin-releasing hormone (TRH), a neuropeptide produced in the paraventricular nucleus of hypothalamus, controls the secretion of TSH. Thyroid-stimulating hormone acts on receptors of the thyroid gland to promote the synthesis and release of thyroid hormones

(thyroxine [T4], tri-iodothyronine [T3]). Furthermore, hormones participate in the control of TSH secretion by a negative feedback on the pituitary gland and hypothalamus.[2] Low thyroid hormones levels due to iodine deficiency or altered utilization of iodine can increase the secretion of TSH, serving as a basis for the diagnosis of hypothyroidism in different species.[3,4,5]

Thyroid disease is one of the most common endocrine disorders.[6] Hormonal assays are now a mandatory requirement, to evaluate, diagnose and treatment of patients suffering, from thyroid disorders. The laboratory diagnosis and monitoring of thyroid diseases such as hypo and hyper thyroidism are based on serum TSH measurement along with serum T4 and T3 (both free and total).[7] The National Academy of Clinical Biochemistry (NACB) has recommended that the functional sensitivity of TSH assay be less or equal to 0.02 mIU/L. This enable to differentiate patients with nonthyroid illness from those with primary hyperthyroidism. This is particularly important in patients hospitalized with nonthyroid illness where TSH concentration as low as 0.02 mIU/L may be encountered.[8]

The analytical accuracy and precision of TSH assay and its ability to reliably distinguish between euthyroid and hyperthyroid patients especially in subclinical stages, where T4 and T3 levels are in normal range makes it a very sensitive marker of primary thyroid function abnormalities.[9] Over the past five decades, improvements in the sensitivity and specificity of thyroid test methodologies have dramatically impacted the clinical strategies for detecting and treating thyroid disorders.[10]

Traditional competitive RIA methods for thyrotropin (thyroid-stimulating hormone, TSH) determination have been increasingly replaced by the so-called ultrasensitive or supersensitive immunometric assays (IMAs) based on noncompetitive “two-site” technology.[11] Radioimmunoassay was considered as the first generation method , IRMA was the second generation method, from the 1990s to date,and the third generation method was electrochemiluminescence assay that had been introduced with improved functional sensitivity.[12]

Electrochemiluminescence or electro generated chemiluminescence (ECL) is a kind of luminescence produced during electrochemical reactions in solution. In electro generated chemiluminescence, electrochemically generated intermediates undergo a highly exergonic reaction to produce an electronically excited state that emits light.[13] ECL excitation is caused by energetic electron transfer (redox) reactions of electro generated species. Such luminescence excitation is a form of chemiluminescence where one/all reactants are produced electrochemically on the electrodes.[14] ECL is usually observed during the application of potential (several volts) to electrodes of electrochemical cell that contains solution of luminescent species (polycyclic aromatic hydrocarbons, metal complexes) in aprotic organic solvent (ECL composition). ECL proved to be very useful in analytical applications as a highly sensitive and selective method. It combines the analytical advantages of chemiluminescent analysis with ease of reaction control by applying electrode potential. Enhanced selectivity of ECL analysis is reached by variation of electrode potential thus controlling species that are oxidized/reduced at the electrode and take part in ECL reaction.[14,15]

The chemiluminescent reactions that lead to the emission of light from the ruthenium complex are initiated electrically, rather than chemically, this is achieved by applying a voltage to the immunological complexes (including the ruthenium complex) that are attached to streptavidin-coated microparticles. ECL is heavily used commercially for many clinical laboratories applications.[15,16,17]

Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation. It also occurs when molecules are excited to a higher electronic states by energetic electron bombardment.

Fluorescence labeling is a process of covalently

attaching a fluorophore to another molecules, such as a protein or nucleic acid. This are generally accomplished using a reactive derivative of the fluorophore that selectively binds to a functional group contained in the target molecules. The most commonly labeled molecules are antibodies, proteins, amino acid and peptides which are then used as specific probes for detection of aparticular target.[18,19.20]

Following a fluorescent labeling reaction, it is often necessary to remove any non reacted fluorophore from labeled target molecules. This is often accomplished by size exclusion chromatography, taking advantage of the size difference between fluorophore and labeled protein, nucleic acid, etc.., fluorophore may interact with the separation matrix and reduce the efficiency of separation. For this reason, specialized dye removal columns that account for the hydrophobic properties of fluorescent dyes are sometimes used.

Common fluorescence dyes are cyanine, fluorescein, rhodamine, alexa fluors, dylight fluors, ATTO dyes, BODIPY dyes, SETA dyes.[21,22]

With respect to the increasing competition among laboratories in order to define the best method with good reliability and practicability, for the measurement of TSH level, we performed an analytical evaluation of the new electrochemiluminescence assay (ECL) and immunofloriscence assay (ELFA) for serum TSH, and compared the results of this method with those of Elisa for TSH determination.

MATERIALS AND METHODS

The study was conducted between February 2014 to November 2014. College of postgraduate studies, University of Al-Neelain, Khartoum, Sudan. In central laboratory of military hospital and Altiqanas hospital laboratory, in Khartoum state. To evaluate the accuracy and precision of immunofloriscence assay (ELFA) and electrochemiluminescence assay (ECL) for determination of TSH. The imprecision studies for the two method was assessed by measurement of three commercial quality control materials covering low, normal and high levels of TSH. For within day imprecision of the TSH, each level of the three control sera was measured in duplicate twenty times in one day, by ECL and ELFA methods. For between day imprecision of the TSH each level of the three control sera was measured in duplicate for twenty days consecutively (from 10.8.2014 to 30.8.2014), by ECL and ELFA methods. For every run, a bottle of each level of control material was retrieved from −20°C storage, thawed, and tested in duplicate by all methods.

In addition to 120 patient samples with TSH concentrations that spanned the analytic range of each assay, 20% low, normal (50%), and 30% high TSH levels, assayed by immunofloriscence assay (ELFA) and electrochemiluminescence assay (ECL), and used for the assessment of the methods accuracy .Six prepared pool assigned serum sample with TSH concentration spanned the analytical range of the methods, were enrolled for the evaluation of the methods linearity.

The patients sample, assigned pool sample, and the control sera all were kept at −20°C prior to analysis.

INSTRUMENTS USED

For ECL method, elecsys e411 made by ROCHE company, and reagent made by ROCHE company used in military hospital central laboratory-Khartoum.

For ELFA method, AIA 600II made by TOSOH company, and reagent made by TOSOH company LOT no EUrev.TSH -010413, used in Altiqana hospital laboratory – Khartoum.

According to the manufacturers' information, all assay calibrators are traceable to the WHO Second International Reference Preparation 80/558.

QUALITY CONTROL

Three levels (low, normal, and high) of control sera of TSH values were used to verify the performance of measurement procedure, results of +/- 2SD of target values of the control sera were accepted.

STATISTICAL ANALYSIS

Data was analyzed by computer soft ware, using SPSS program manual master sheet. The mean and standard deviation of TSH hormone concentration were obtained, and t test was used for the comparison, ($p \leq 0.05$) considered as significant .Regression analysis used for the correlation between methods, and considered significant at $p \leq 0.05$.

RESULTS

Three levels of the commercial control sera spanned low, normal, and high of TSH levels, were used for the assessment of ECL and ELFA imprecision. In, addition to 120 patients sample that covered hypo, normal, and hyper TSH levels used for the methods comparison, and six assigned prepared sample for linearity check of ECL and ELFA.

IMPRECISION PROFILE

Inter and intra-assay imprecision profiles are shown in table-I & II, is low and in the acceptable range. Intraassay CV% for ECL was 2.9%, 2.74%, and 2.55% for low, normal, and high respectively TSH levels of the control sera. Intraassay CV% for ELFA was **3.95**%, **3.75**%, and 5.73% for low, normal, and high respectively TSH levels of the control sera.

Interassay CV% for ECL was 3.0%, 2.75%, and **2.81**% for low, normal, and high respectively TSH levels of the control sera. Intraassay CV% for ELFA was **4.26**%, **4.0**%, and 5.75% for low, normal, and high respectively TSH levels of the control sera, shown in table-I & II.

Methods	Low level				Normal level				High level			
	N	mean mIU/L	SD	CV%	N	mean mIU/L	SD	CV%	N	mean mIU/L	SD	CV%
ECL	20	0.611	0.018	2.9	20	6.679	0.183	2.74	20	35.049	.894	2.55
ELFA	20	0.506	0.02	3.95	20	6.540	0.245	3.75	20	31.035	1.779	5.73
Control sera	20	.488	.078		20	6.016	0.96		20	33.65	5.39	

Table-I. Within day imprecision of the three levels of TSH control sera estimated by ECL and ELFA methods

Methods	Low level				Normal level				High level			
	N	mean mIU/L	SD	CV%	N	mean mIU/L	SD	CV%	N	mean mIU/L	SD	CV%
ECL	20	0.601	0.018	3.0	20	6.678	0.184	2.75	20	35.512	.998	2.81
ELFA	20	0.587	0.025	4.26	20	6.836	0.272	4.0	20	31.954	1.836	5.75

Table-II. Between days imprecision of the three levels of TSH control sera estimated by ECL and ELFA methods

METHOD AGREEMENT

Table-III,IV,V show the mean TSH levels of the control sera measured by ECL & ELFA method : For ECL method there is significant difference when compared with assigned TSH values (low 0.488+/-0.078,normal 6.016+/- 0.952,high 33.651+/-**5.39**) of the control sera , low 0.611 +/- 0.01809 . $p \leq 0.001$, normal 6.6785 +/- 0.18259. $p \leq 0.00$, high 35.0485 +/- 0.8941. $p \leq$ **0.02**) .

For ELFA method there is significant difference when compared with assigned TSH values of the control sera, low 0.50545 +/- 0.02006. . $p \leq 0.00$, normal 6.5395 +/- 0.244357. $p \leq 0.00$, high 31.0350 +/- 1.779496. $p \leq 0.001$.In contrast, the mean values measured by ECL and ELFA of the three control sera level is within the target values of the mean.

Methods	Low level			Normal level			High level		
	N	mean mIU/L	Sign	N	mean mIU/L	Sign	N	mean mIU/L	Sign
ECL	20	0.611	0.001	20	6.679	0.00	20	35.049	0.02
ELFA	20	0.506	0.000	20	6.540	0.00	20	31.035	0.001
Assigned value	20	0.488	-	20	6.016		20	33.651	-

Table-III. Comparison of ECL and ELFA methods in estimation of TSH levels of the three levels of the control sera

Methods	N	Mean mIU/L	SD	Significance
ECL	120	15.74	1.88	0.00
ELFA	120	13.76	1.59	0.00
A signed patients sample	120	14.56	1.65	

Table-IV. Comparison of ECL and ELFA methods in estimation of TSH levels in patients samples

As illustrated in table-IV the mean TSH levels of the 120 patients samples measured by ECL & ELFA method: For ECL method there is significant difference (15.74+/- 1.181. $p \leq 0.00$) when compared with the mean of the assigned TSH value (14.56 +/- 1.65) of the patients samples. For ELFA method there is significant difference (13.76 +/- 1.59, $p \leq 0.00$) when compared with mean of the assigned TSH values (14.56 +/- 1.65) of the control sera, but within the mean range.

Table-V, and figures 1& 2 showed strong relation between the two TSH levels measured by ECL(mean 15.74 mIU/L, slope 0.67 , correlation coefficients 0.991, $p \leq 0.00$) and ELFA (mean 13.76 mIU/L, slope 0.54, correlation coefficients 0.995, $p \leq 0.00$) with the assigned values (14.56)of 120 patients sample.

Methods	N	Mean mIU/L	slope	Correlation Coefficient	Significance
ECL	120	15.74	0.76	0.991	0.00
ELFA	120	13.73	0.54	0.995	0.00

Table-V. Relationship of TSH levels in patients sample estimated by ECL and ELFA with the assigned values

LINEARITY ASSESSMENT

As shown in table-VI, there is no significant difference of TSH mean level in six prepared pool samples measured by ECL(**22.63** mIU/l +/- **1.12** , $p \leq 0.1$) and ELFA(**19.87**mIU/l +/- **1.15** , $p \leq 0.11$) when compared with the TSH assigned values**(22.54** mIU/l +/-0.96).

Methods	N	Mean mIU/L	SD	Significance
ECL	6	22.63	1.12	0.1
ELFA	6	19.87	1.15	0.11
Pool samples	6	22.54	0.96	-

Table-VI. Comparison of ECL and ELFA methods in estimation of TSH levels in pool samples

Table-VII, and figures 3& 4 showed strong relation between the two TSH levels measured by ECL(**22.63** mIU/l, slope **0.79**, correlation coefficients **0.999,** $p \leq 0.00$) and ELFA (mean **19.87**mIU, slope **0.68**, correlation coefficients **0.985,** $p \leq 0.00$) with the assigned TSH values **(22.54** mIU/l +/-0.96).of the six prepared pool samples.

Methods	N	Mean mIU/L	slope	Correlation Coefficient	Significance
ECL	6	22.63	0.79	0.999	0.00
ELFA	6	19.87	0.68	0.985	0.00
Assigned pool samples	6	22.54	-	-	-

Table-VII. Relationship of TSH levels in pool samples estimated by ECL and ELFA with the assigned values

DISCUSSION

The last years have seen development and refinement of many new immunoassay measurement principle and system.[23,24] The major trend has been away from liquid phase assay with radio isotopic label, and toward fast solid- phase assay based on monoclonal antibodies.[25]

In the present study which compared the accuracy and precision of electrochemiluminescence assay (ECL) and immunofloriscence assay (ELFA), with Elisa for estimation of TSH levels. The intra-assay coefficient of variation (2.9%, 2.74% , and 2.55% for low, normal , and high respectively of TSH

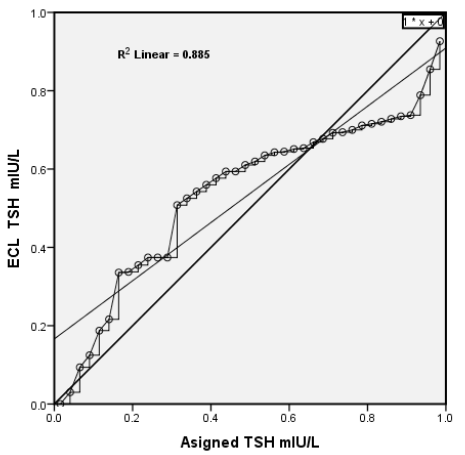

Figure-1. Regression illustrating the relationship between TSH levels measured by ECL with its assigned values

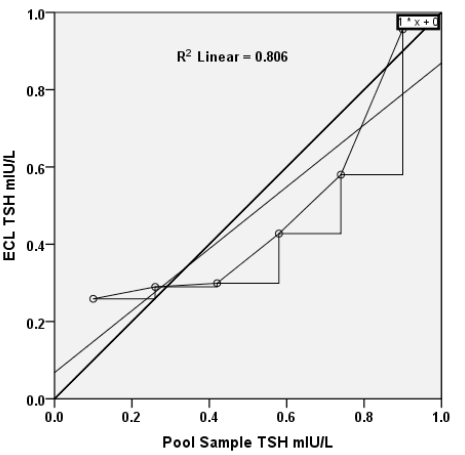

Figure-3. Regression illustrating the relationship between TSH levels measured by ECL with its assigned values in pool patients samples

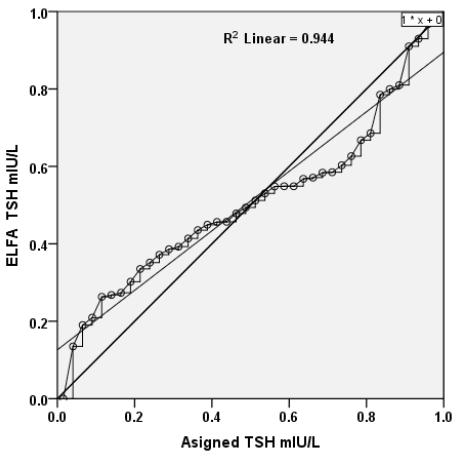

Figure 2. Regression illustrating the relationship between TSH levels measured by ELFA with its assigned values

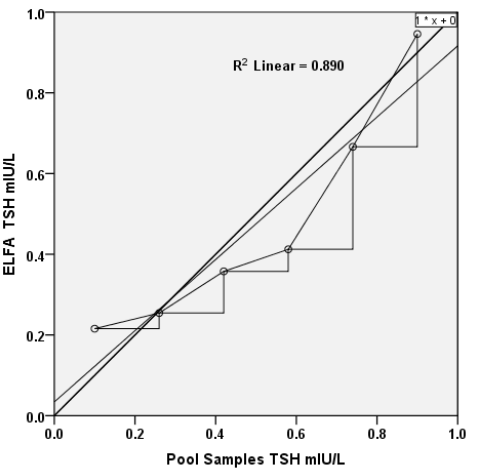

Figure 4. Regression illustrating the relationship Between TSH levels measured by ELFA with its assigned values in pool patients samples

levels of the control sera for ECL) , and(**3.95**%, **3.75**% , and 5.73% for low, normal , and high respectively of TSH levels of the control sera) for ELFA.

Interassay CV% for ECL was 3.0%, 2.75%, and **2.81**% for low, normal, and high respectively of TSH levels of the control sera. Intraassay CV% for ELFA was **4.26**%, **4.0**%, and 5.75% for low, normal, and high respectively of TSH levels of the control sera.The Inter- and intra-assay coefficient of variation for ECL and ELFA is in the acceptable variation range of the international system, and less than the coefficient of variation required by the manufacture of both ECL and ELFA .In contrast, ECL have lowest CV% than ELFA in both intra and inter-assay imprecision.[26]

Although the current study showed statistical difference in the mean TSH levels of the three levels of the control sera measured by ECL & ELFA methods, but the values is within the range of the mean of the assigned means values.

For assessment of ECL and ELFA accuracy our study deduced significant correlation in mean TSH

of the 120 patients samples measured by ECL (r =**0.991,** p ≤ 0.00) & ELFA (r = **0.995,** p ≤ 0.00) methods with its assigned value, although there is mean difference but within the mean range of TSH assigned values. Based on the results, ECL is more accurate than ELFA especially at lower and higher TSH concentration, ie it provided values closer to the assigned values, especially at lower and higher TSH concentration. The enhanced accuracy of the third generation assay permitted quantification for patients with TSH to a lower reportable value. This additional information was clinically useful in many newly diagnosed and treated hyperthyroid patients and in all patients with subnormal TSH receiving exogenous thyroid hormone for hypothyroidism, for suppression of goiter or nodular thyroid disease, or for thyroid cancer.(27) In addition, our data suggest that the information provided by more accurate TSH assays is useful when assessing thyroid function in nonthyroidally ill patients, patients with sub acute thyroiditis, and patients with hypopituitarism.[27]

In evaluation of ECL and ELFA linearity , the present study observed significant correlation of TSH values in six prepared pool samples measured by ECL(r = 0.999, p ≤ 0.00) and ELFA (r = 0.985, p ≤ 0.00) with assigned values. In addition to no significant difference of TSH mean level in six prepared pool samples measured by ECL (p ≤ 0.1) and ELFA (p ≤ 0.11) when compared with the TSH mean assigned values in the six prepared pool samples. Based on the results obtained. ECL illustrated more satisfactory linearity than ELFA especially at lower and higher TSH concentration. Our results show that ECL method has enhanced signal/noise ratio, broader linear range on logarithmic scales and better linearity within the same range. In addition, ECL also has short incubation times and fast result turnaround, hence ECL shows better lower and higher end linearity of its standard curve.[28,29]

CONCLUSION

Although ECL and ELFA methods performed equally well in measurement of TSH levels. However, better precision and accuracy were obtained with ECL method, which we consider to be the method of choice.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Bashir Elfatih Abdalla	Prepared by