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AGREEMENT BETWEEN ELECTROCHEMILUMINESCENCE AND RADIOIMMUNOASSAY METHODS FOR DETERMINATION OF PLASMA THYROID HORMONE CONCENTRATIONS IN SHEEP

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Summary

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Various methods are used to determine plasma thyroid hormones concentrations in medical diagnostic laboratories; but, some of them influence the results for thyroid hormone levels in domestic animals. The aim of this study was to compare the levels of two predominant plasma thyroid hormones using electrochemiluminescence immunoassay (ECLIA) and radioimmunoassay (RIA) methods in sheep. Blood samples were collected from the jugular vein of 30 clinically healthy and non-pregnant adult ewes. The separated plasma was analyzed to determine thyroxine (T_4) and triiodothyronine (T_3) concentrations. Results indicated significant differences in the T_4 ($P<0.05$) and T_3 ($P<0.001$) concentrations between the two methods, and only the level of T_4 was higher when using the RIA method. The linear regression analysis of these hormones showed that the RIA and ECLIA results were significantly correlated: $P=0.001$, $r=0.759$ for T_4; $P=0.008$, $r=0.638$ for T_3. The ECLIA method exhibited a good precision for determining plasma T_4 and T_3 concentrations in sheep. It was concluded that ECLIA can be used as an alternative to RIA for assaying sheep T_4 and T_3 in clinical laboratories, but it has some limitations for application in veterinary diagnostic laboratories.

Key words: electrochemiluminescence, plasma, radioimmunoassay, sheep, thyroid hormones

INTRODUCTION

Various methods were used to determine thyroid hormones concentrations until now: latex immunoassay (Mareschal *et al.*, 1990), mass spectrometry (Lee *et al.*, 2008), RIA (Kapoor *et al.*, 2001; Anderson *et al.*, 2002; De Blasio *et al.*, 2006; Šamanc *et al.*, 2010), bioluminescent immunoassay (Frank *et al.*, 2004), fluoroimmunoassay (Nargessi *et al.*, 1980; Wu *et al.*, 2003), chromatography (Gordon *et al.*, 1982), equilibrium dialysis (Sapin & d'Herbomez, 2003) and ultrafiltration (Bartkowski *et al.*, 2002).

The RIA method is widely used to determine plasma thyroid hormones concentrations in most researches in livestock (Solter & Farner, 2000). It is a reference method for thyroid hormones analysis. Its advantages compared to the other thyroid hormones assays are reliability and simple performance. However, the RIA method has major disadvantages inclu-

ding: radioactive label and waste, special storage facility and handling, need to generate a midpoint standard curve each time when the test is performed, harm to the operators and expensive instrumentation (Solter & Farner, 2000; Eshratkhah *et al*., 2010c).

With progress in technology, new methods were introduced for measurement of these hormones. Two non-radioactive methods such as chemiluminescence immunoassay and electro-chemiluminescence immunoassay (ECLIA) are used routinely in medical diagnostic laboratories.

ECLIA measures chemiluminescence produced as a result of electrochemical reactions. Numerous elements such as ruthenium, osmium, rhenium are involved in the processes. In this method, highly reactive species are generated from stable precursors at the surface of electrode and react one with another producing light. In recent years, the ruthenium chelate is commonly used in the chemiluminescence reactions as a complex for the development of light that leads to emission of light from the ruthenium complex by applying a voltage to the immunological complexes (Mathew *et al.,* 2005). This method has some advantages over the other methods such as liquid reagent convenience, short incubation time, high quality, fast result turnaround, low handling time and repeat casts, and provides a solid platform for menu expansion (Sanchez–Carbayo *et al.,* 1999; Mathew *et al.,* 2005). Generally, electrochemiluminiscence technology is based on three principles: competitive principle for analytes with extremely small and low molecular weight e.g. free T_4, free T_3, cortisol, testosterone, estradiol; sandwich principle for larger molecules such as thyroid stimulating hormone, follicle stimulating hormone, luteinizing hormone; and bridging principle to detect antibodies, not antigens (e.g. IgG, IgM and IgA) (Mathew *et al.,* 2005). The ECLIA has been validated for analysis of T_4 and cortisol in dogs, cats and horses (Singh *et al*., 1997; Solter & Farner, 2000; Wenger-Riggenbach *et al.*, 2010). It has been used for the determination of T_4, T_3, free T_4, free T_3 in sheep (Eshratkhah *et al.*, 2010b); troponin T, lactate dehydrogenase (LDH) and creatinine kinase-muscle brain (CK-MB) in rabbits (Zayerzadeh *et al.*, 2010).

This study was conducted to compare the results from the determination of blood T_4 and T_3 concentrations in sheep using the RIA and ECLIA methods.

MATERIALS AND METHODS

Animals

Blood samples were collected directly into vacutainer tubes containing heparin as an anticoagulant (Becton Dickinson, NJ, USA) from 30 ewes. The animals were reared in one group at the animal house with slatted floor at the Islamic Azad University, Shabestar branch, East Azarbaijan, Iran. Sheep had *ad libitum* access to water, and were fed a relatively low protein (95 g crude protein/kg) grass meal. The age of the ewes ranged from 1 to 3 years. All animals were clinically healthy, non-pregnant, and free from internal and external parasites. The experiment was performed in the spring (ambient temperature 27 ^{o}C).

Blood samples, analyses and assay performance

Blood samples were taken from the jugular vein and the plasma was separated by centrifugation at 750×*g* for 15 min and then frozen at –20 $^{\circ}$C until used. The levels of plasma T_4 and T_3 were measured in duplicate by RIA kits (BioSource

Europe SA 8, Belgium) with the Kontron analyzer (Kontron Co., Sweden), and Cobas ECLIA kits (Roche Boeringer-Mannheim, USA) with the Elecsys 2010 analyzer according to the kit manufacturer recommendations. The data were analyzed using SPSS v. 17 software. The significance of differences between RIA and ECLIA values was determined with independent sample *t*-test at the $P<0.05$ level. The linear regression analysis was performed for determining the percentage coefficient of variation (CV %), coefficient of determination (r^2), correlation coefficient (r), 95% confidence interval (CI) and the slope of the curve. As the hypothyroidism is the commonest type of thyroid disorders in the small ruminants (Gupta *et al.*, 2010), intra-assay precision of the ECLIA and RIA methods was determined by evaluating 10 pooled plasma samples with low T_4 and T_3 levels, three times within the same run of assay by both methods, then the percentage coefficient of variations (CV %) were calculated. For inter-assay precision, 3 pooled plasma samples with low level of T_4 and T_3 were analyzed every day for 10 days. The assay linearity was determined using two pooled plasma samples with low and moderate T_4 and T_3 concentrations by ECLIA method which were diluted 1⁄2, 1⁄5 and 1⁄10 with saline. Then, each dilution was measured in duplicate. The evaluations were made by the percentage of difference between the expected and observed values. For recovery studies, a pooled sample with low T_4 and T_3 concentrations was selected. The levels of T_4 and T_3 were determined 5 times a day by ECLIA. Different amounts of this plasma sample were added to the two plasma samples at different concentrations of T_4 and T_3. The recovery percentages were calculated by the differences between the expected and observed values. Validation of the RIA method for determination of the plasma thyroid hormones in sheep has been performed by Mostaghni *et al.* (2005); Nazifi *et al.* (2007; 2008).

RESULTS

The values of plasma T_4 and T_3 in ewes and their intra- and inter- assay percentage coefficients of variations results when using the RIA and ECLIA methods are presented in Tables 1 and 2, respectively. The T_4 concentrations in samples obtained from ewes, showed a significant difference between ECLIA and RIA methods ($P<0.05$) and its values were higher when using the RIA method. Also, we found a significant difference between two methods concerning the T_3 concentrations ($P<0.01$), but its value was higher when using the ECLIA method. Additionally, according to the 95% CI values of each hormone that were calculated in the both used methods; there was no overlap concerning the 95% CI values of T_4 and T_3 concentrations.

Table 1. Plasma concentrations of T_4 and T_3 in ewes (n=30) analyzed by radioimmunoassay (RIA) and electrochemiluminescence immunoassay (ECLIA) methods. Data are presented as mean ± SD (95% confidence interval).

	Mean±SD	95% CI	P value
T_4 (nmol/L)			
RIA	96.8±14.8	91.3–102.3	<0.05
ECLIA	84.9±16.8	78.6–91.2	
T_3 (nmol/L)			
RIA	1.36±0.38	1.22–1.50	<0.01
ECLIA	1.90±0.48	1.72–2.07	

Table 2. Precision of electrochemiluminescence immunoassay (ECLIA) and radioimmunoassay (RIA) for measurement of thyroid hormones in sheep blood plasma

	T_4 (ECLIA)	T_4 (RIA)	T_3 (ECLIA)	T_3 (RIA)
Number of replicates	30	30	30	30
Mean value (nmol/L)	12.8	14.1	0.38	0.30
Standard deviation	0.53	0.87	0.04	0.02
Inter-assay % CV	4.2	6.2	10.9	8.4
Number of replicates	30	30	30	30
Mean value (nmol/L)	11.3	15.2	0.37	0.31
Standard deviation	0.47	0.63	0.02	0.02
Intra-assay % CV	4.2	4.2	5.9	7.3

The distributions of T_4 and T_3 were linear when RIA values were plotted against the ECLIA values (Fig. 1 and 2). Both the T_4 (r=0.759) and T_3 (r=0.638) values by the ECLIA method showed a significant positive correlation to their measured values using RIA.

Recovery and linearity analyses of the T_4 and T_3 ECLIA method are presented in Tables 3 and 4. The results from the Kolmogrov-Smirnov tests indicated that both RIA and ECLIA methods had a normal and relatively similar distribution.

On the basis of the 95% CI, the correlation between ECLIA and RIA methods for T_4 was not significantly different from that of T_3 (0.56–0.88 and 0.37–0.82, respectively).

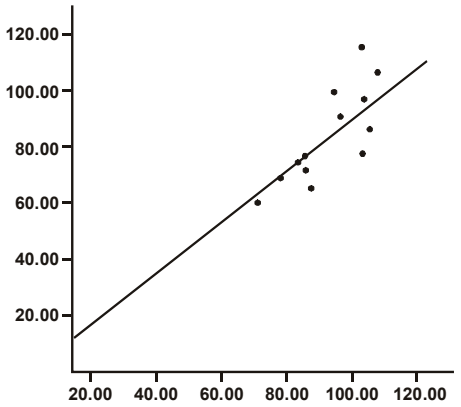

Fig. 1. Distribution of sheep blood plasma T_4 (nmol/L) concentrations in samples determined using radioimmunoassay (RIA) and electrochemiluminescence immunoassay (ECLIA). RIA values (X-axis) were compared with the ECLIA values (Y-axis) by linear regression analysis (Y = 1.561 + 0.861.X, r^2=0.576; r=0.759, P=0.001).

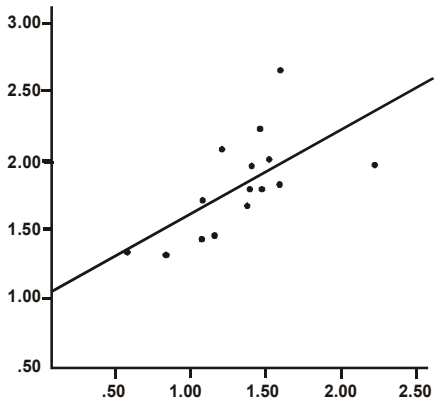

Fig. 2. Distribution of sheep blood plasma T_3 (nmol/L) concentrations in samples determined using radioimmunoassay (RIA) and electrochemiluminescence immunoassay (ECLIA). RIA values (X-axis) were compared with the ECLIA values (Y-axis) by linear regression analysis (Y = 0.796 + 0.811.X, r^2=0.407, r=0.638, *P*=0.008).

Table 3. Recovery analysis of the T_4 and T_3 assay using the ECLIA method

Theoretical values	Measured values	Recovery (%)
T_4 (nmol/L)		
12.2	13.1	107.4
31.1	30.9	99.4
T_3 (nmol/L)		
0.45	0.48	106.6
0.46	0.45	97.8

Table 4. Linearity analysis of the T_4 and T_3 assay using the electrochemiluminescence immunoassay

Theoretical values	Dilution	Measured values	Difference, %
T_4 (nmol/L)			
	1⁄2	21.5	108.7
19.8	1⁄5	22.9	116.3
	1⁄10	23.5	118.3
	1⁄2	15.7	102.6
15.3	1⁄5	14.9	98.0
	1⁄10	15.3	100.6
T_3 (nmol/L)			
	1⁄2	0.54	105.9
0.51	1⁄5	0.56	109.8
	1⁄10	0.58	113.7
	1⁄2	0.44	104.8
0.42	1⁄5	0.46	109.5
	1⁄10	0.48	114.3

DISCUSSION

The ECLIA method yielded >90% recovery of the T_4 and T_3 in sheep. The results of this study showed that the intra- and inter-assay CV of T_4 were <20 %, which indicates good precision for T_4 using previously mentioned methods. Also, on the basis of findings of this study, the T_4 level was higher than values reported in sheep and calves (Eshratkhah *et al*., 2010 a, b, c).

There was a little difference with the value reported by Nazifi *et al*., (2008) in sheep. Similarly to T_4, the intra- and inter-assay CV for T_3 were <20%, so the determination of this hormone using the ECLIA and RIA methods had also a good precision. The slope of the RIA-ECLIA curve for T_4 was higher than T_3, which suggested that ECLIA method yielded higher plasma T_3 concentration than RIA method in sheep. Unlike T_3 concentrations, the RIA method yielded higher T_4 concentration.

The coefficient of determination (r^2) of T_4 was 0.576, and that of T3 was 0.407; therefore, about 57.6% and 40.7% of the variation in the T_4 and T_3 concentration using the ECLIA method explained by the RIA method, respectively. These findings indicate that the regression equations of T_4 and T_3 appear to be relatively strongly and moderately useful, respectively, for making predictions about their concentrations using the aforementioned methods. The Elecsys T_4 and T_3 employ a competition principle assaying antibodies specifically directed against T_4 and T_3, respectively. Endogenous T_4 and T_3, released by the action of 8-aniline-1-naphthalene sulfonic acid, competes with added biothinylated T_4- or T_3-derivative for the binding sites on antibodies labeled with the ruthenium complex. It seems that, the binding affinity of T_3 is higher compared to that of T_4 for sites on the labeled antibodies. Therefore, the value of T_3 was higher than the T_4 value when using the ECLIA method. Indeed, our results indicate that, T_4 and T_3 values in sheep are consistent with ones reported by the kits' manufacturers in man. It is particularly important for T_4 and T_3 assays used in sheep to yield accurate and precise T_4 and T_3 values at their relatively low concentrations. Also, the calibrators in the ECLIA kit when used for ruminants contain lower T_4 and upper T_3 concentra-

tions than those used to analyze human samples.

In conclusion, the performance of RIA and ECLIA methods suggested that they can be used for the quantitation of T_4 and T_3 in sheep. In contrast to T_3, the analysis of blood samples by ECLIA provided lower T_4 values than did RIA in the same samples although both methods exhibited approximately similar good precision for determination of the T_4 and T_3 in sheep. Therefore, veterinary researchers and clinicians must be aware of the limitation of this method when measuring T_4 and T_3 in sheep blood samples. In a clinical setting, thyroid hormone values obtained by ECLIA should be compared to reference ranges created in the respective laboratory according to the breed, age, season and physiological state of sheep in order to report reliable results.

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