# Determination of Triiodothyronine and Thyroxine from Plasma and Milk of Lactating Cow

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**Objective:** The purpose of this study was to establish a method for the determination of T3 (triiodothyronine) and T4 (thyroxin) hormone concentrations in plasma, whole milk and after ethanol extraction, as well as to calculate the partition coefficient milk/plasma.
**Methods:** Ten Holstein Romanian friza milking cows were used to test the efficiency of the method. T3 and T4 were determined by an immunochemical ELISA competitive assay.
**Results:** Medium value of T3 in plasma was 2.78 ±1.53 ng/ml (4.27±2.35 nmol/l), in whole milk 3.72±1.44 ng/ml (5.71±2.21 nmol/l) and in extracted milk 4.97±1.67 ng/ml (7.63±2.56 nmol/l). Mean value of T4 in plasma was 50.97±7.30 ng/ml (65.60±9.39 nmol/l), in whole milk 2.12±0.87 ng/ml (2.73±1.12 nmol/l) and in extracted milk 3.60±1.15 ng/ml (4.64±1.48 nmol/l). Extraction from milk presented a good efficiency of 94.39% for T3 and 101.30% for T4.
**Conclusion:** The values obtained are in the concentration range reported by literature data for T4 and T3 from plasma and milk.

**Keywords:** triiodothyronine, thyroxin, ELISA, cow's milk, plasma

## Introduction

Variations in thyroid hormone (TH) bioactivity allow the animals to adapt their metabolic balance to different environmental conditions, changes in nutrient requirements and the homothetic changes during different physiological stages. The thyroid gland mostly secrets 3-5-3`-5`-tetraiodothyronine or thyroxin (T4) which is monodeiodinated to 3-3`-5-thiiodathyronine (T3), the active form stimulating oxygen utilization and heat production in every cell of the body [1]. Different ratios of blood/ milk TH and T3/ T4 in milk of various mammals suggest species differences in the conversions of T4 to T3 in mammary gland cells, or both [2]. Active T3 in colostrums and milk could play different physiological effects on the suckling offspring may be systematic or within the gastrointestinal tract or both: positive effects on intraluminal digestion, absorption, maturations of enzyme systems; macromolecule absorptions by the intestine; responsiveness to gastric secretions [3].

## Material and methods

milk and blood samples were obtained from 10 healthy lactating cows, 4 to 11 years of age, from the Research and Development Stations for Bovines Târgu-Mureş, Farm II Ernei, Mureş County. In order to test our method, the animal group presented a serious heterogeneity.

T3 and T4 were determined by an immunochemical ELISA competitive assay. The used kits were EIAgen total T3 and EIAgen total T4. Hormone in the sample competes with hormone conjugated with horseradish peroxidase for binding to specific antibody sites coated to the wells. At the end of the incubation, all unbound material is removed by aspiration and washing. The enzyme activity which is bound to the solid phase will be inversely proportional to the concentration of hormone in calibrators and samples and is evidenced by incubating the wells with a chromogen solution (tetramethylbenzidine, TMB) in substrate-buffer. Absorbance was measured using the photometric microplate reader, at 450 nm with a filter at 620 nm. All the samples were assayed three times. Precision of the methods was evaluated by calculating the mean intra- and inter-assay coefficients of variation (CV=SD/mean*100) of different pooled blood and milk samples [1].

### Milk and blood sample collection, post-collection handling and storage

Aliquots of whole milk samples (n=10) were used for extraction of iodothyronines with alkaline ethanol at low temperature, as described by Slebodzinski et al. (1998) [4]. Briefly, 2 ml cold (–200°C) alkaline ethanol (pH= 9.0 with NH4OH) were added to 1 ml whole cow's milk, vortexed and left overnight in the freezer (–200°C). After 24 h the milk-ethanol mixture was vortexed, left in the freezer for a further 24 hours and then centrifuged at 3500 rpm for 30 min at 0°C. Supernatant was collected and stored at –200°C.

Blood samples (n=10) were collected by jugular venipuncture in evacuated tubes containing K3-EDTA. Tubes with anticoagulant were immediately centrifuged (2500 rpm for 15 min) and the plasma aliquot. Plasma and milk samples were immediately refrigerated at –200°C.

All the reagents (ethanol, ammonium) were purchased from SC Chimopar SA, Bucharest and were of high purity.

### Milk extraction efficiency

Because T3 and T4 are normal components of milk, a blanc sample could not be obtained. Milk samples from one cow were spiked with 1.6 ng/ml T3 and 60 ng/ml T4. Samples were extracted by the same protocol.

## Results

After the samples were analyzed, two animals were excluded from the initial lot based on the elimination criteria for

Table I. T3 concentration in plasma, whole milk and extracted milk

| Sample | Plasma (ng/ml) | Whole milk (ng/ml) | Extracted milk (ng/ml) | Partition coefficient M/P |
|---|---|---|---|---|
| V1 | 4.42 | 2.21 | 3.43 | 0.78 |
| V2 | 3.19 | 3.74 | 6.25 | 1.96 |
| V3 | 1.49 | 4.72 | 5.69 | 3.82 |
| V4 | 1.70 | 3.04 | 4.45 | 2.61 |
| V5 | 1.58 | 6.21 | 7.30 | 4.62 |
| V6 | 2.34 | 1.82 | 2.08 | 0.89 |
| V7 | 5.68 | 4.64 | 5.91 | 1.04 |
| V8 | 1.85 | 3.39 | 4.63 | 2.50 |
| Average±SD | 2.78±1.53 | 3.72±1.44 | 4.97±1.67 | 2.27±1.40 |

Table II. T4 concentration in plasma, whole milk and extracted milk

| Sample | Plasma (ng/ml) | Whole milk (ng/ml) | Extracted milk (ng/ml) | Partition coefficient M/P |
|---|---|---|---|---|
| V1 | 57.42 | 1.10 | 2.62 | 0.045 |
| V2 | 57.95 | 2.11 | 3.62 | 0.062 |
| V3 | 52.59 | 2.17 | 4.97 | 0.094 |
| V4 | 48.47 | 1.70 | 3.41 | 0.070 |
| V5 | 43.74 | 3.55 | 5.19 | 0.118 |
| V6 | 60.07 | 0.94 | 1.81 | 0.030 |
| V7 | 39.60 | 2.83 | 4.18 | 0.099 |
| V8 | 47.91 | 2.58 | 3.00 | 0.062 |
| Average±SD | 50.97±7.30 | 2.12±0.87 | 3.60±1.15 | 0,072± 0,029 |

Table III. Milk extraction efficiency

| | Cp (ng/ml) | Ct (ng/ml) | Efficiency % |
|---|---|---|---|
| T3 | 4.75 (±0.67) | 5.03 | 94.39 (±13.45) |
| T4 | 3.27 (±0.69) | 3.22 | 101.30 (±21.64) |

Table IV. Plasma values for T3 and T4 compared with the literature data

| | Our values | Literature values | Reference |
|---|---|---|---|
| T3 | 2.78±1.53 ng/ml | 1.89 nmol/l (1.23 ng/ml)<br>0.90 ng/ml | [3]<br>[8] |
| T4 | 50.97±7.30 ng/ml | 67.06 nmol/l (52.09 ng/ml)<br>30 ng/ml | [3]<br>[8] |

Table V. Milk values for T3 and T4 compared with the literature data

| | Our values | Literature values | Reference |
|---|---|---|---|
| T3 | 4.97±1.67 ng/ml | 2.02 ng/ml) | [10] |
| T4 | 3.60±1.15 ng/ml | 1.90 ng/ml | [10] |

improbable statistic values from The 10th Romanian Pharmacopoeia [5].

Calibration curve: on 0.5–8.0 ng/ml for T3 and 20–300 ng/ml for T4 domain, the dependence between the inverse of absorbance and concentration was linear with the determination coefficient R2 >0.99 for both components. The medium equation (n=3) of the calibration curve was 1/A= 0.5329(±0.1255)*c+0.6764(±0.3125) for T3 and 1/A=0.0097(±0.00189)*c+0.4586(±0.1023) for T4.

T3 and T4 concentration were determined by analyzing 3 samples and calculated by the medium calibration curve (Tables I, II).

Medium values of T3 in plasma were 2.78±1.53 ng/ml (4.27±2.35 nmol/l), in whole milk 3.72±1.44 ng/ml (5.71±2.21 nmol/l) and in extracted milk 4.97±1.67 ng/ml (7.63±2.56 nmol/l).

Mean value of T4 in plasma was 50.97±7.30 ng/ml (65.60±9.39 nmol/l), in whole milk 2.12±0.87 ng/ml (2.73±1.12 nmol/l) and in extracted milk 3.60±1.15 ng/ml (4.64±1.48 nmol/l).

Milk extraction efficiency was calculated for T3 and T4 on 3 milk samples spiked with 1.6 ng/ml T3 and 60 ng/ml T4 and extracted individually.

## Discussions

Plasma values for T3 and T4 are consistent with the literature data as shown in Table IV.

Milk extraction method presented a good yield and the values obtained are with 30% better for T3 and with 70% for T4 than in unextracted milk. In consequences to determine the distribution coefficient M/P of T3 and T4 milk hormone dosage should be done after alkalinization. As milk is very perishable, the alkaline extract can be preserved longer.

The values of T3 and T4 from milk are consistent with the literature data (Table V).

Similar results were obtained also by early-phase radioimmunoassay for milk T3 and T4 values [2,7].

Plasma values of T3 and T4 do not vary with age; between the youngest animal (4 years) and the eldest (11 years) no significant differences could be observed either in plasma or milk values of T3 and T4.

The data suggest that cow's milk can provide a significant exogenous source of T4 to the infant. In the hypothyroid infant, the amount of T4 in cow milk may delay clinical recognition of the disease. Although this exogenous source of T4 may alleviate the disease, it is insufficient to prevent the detrimental effects of hypothyroidism [9,10].

## Conclusions

Our article presents a simple and precise method of T3 and T4 determination from plasma and milk, as well as an extraction method of these compounds from milk. This method was verified on a small group of cows with a large age domain. The values obtained are in the concentration range reported by literature data for T4 and T3 from plasma and milk.

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