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Validated ultra high performance liquid chromatography-tandem mass spectrometry method for quantitative analysis of total and free thyroid hormones in bovine serum

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ABSTRACT

Thyroid hormones are essential hormones for regulating growth and development. Methods to accurately monitor low-levels (ppb-ppt) of these hormones in serum are needed to assess overall health, both from a clinical perspective as from environmental contaminant or drug exposures. In general, the separation of the free thyroid hormone fraction from animal sera is performed through labour intensive equilibrium dialysis, while detection of total and free thyroid hormone fractions in animals is done with commercially available radioimmunoassays (RIAs). This study reports newly developed analysis methods for both the total and free fractions of triiodothyronine (T3), reverse-triiodothyronine (rT3) and thyroxin (T4) from bovine serum, with a much higher specificity and selectivity than commercially available RIAs. The bovine serum extraction procedures of total and free T3, rT3, T4 were optimised with fractional factorial designs and consisted of, respectively, deproteinisation followed by liquid–liquid extraction, 30 kDA ultracentrifugation and solid phase extraction. Both free and total thyroid hormone UHPLC-HESI-MS/MS based analysis methods were successfully validated. The limits of quantification for T4, rT3 and T3 amounted respectively 0.04 ng mL^{-1}, 0.05 ng mL^{-1}, 0.03 ng mL^{-1} for the total fraction, and 6.6 pg mL^{-1}, 2.6 pg mL^{-1} and 2.7 pg mL^{-1} for the free fraction. Individual recoveries of total and free thyroid hormone fractions ranged between 95.6 and 106.3% and 92.1 and 106.5%. Good results for repeatability and intra-laboratory reproducibility (RSD%) were observed, i.e. respectively ≤8.0% and ≤7.3% for the total and free fractions. Excellent linearity ($R^2 \geq 0.99$) and lack-of-fit was proven for both fractions. In conclusion, these methods show excellent in-house performance and possibilities for elaboration to application in other animal sera (e.g. feline, canine, equine)

1. Introduction

The thyroid gland governs the metabolism of all species through thyroid hormone secretion. Therefore, the evaluation of its status through analysis of thyroid hormones is of great value in a veterinary clinical setting but also in other research fields where an overall view of the thyroid status is required. More specifically in reference to contaminants, like endocrine disrupting chemicals (EDC's) (e.g.: organchlorine pesticides and polybrominated diphenyl ethers)) [1–3], or to endogenous formation of certain residues affecting the thyroid function (e.g. thiouracil) [4,5] thyroid hormone analysis has proven its merits.

The thyroid gland is histologically characterised by large-follicular tissue with a monolayer of cells, which produce thyroxin (T4, 90%) and tri-iodothyronine (T3, 10%) [6]. Due to the highly vascularised gland tissue, T4 and T3 can be quickly released into the systemic circulation. Secretion of thyroid hormones is well regulated by a negative feedback mechanism involving, besides the thyroid gland, the hypothalamus and the pituitary gland [6,7]. Circulating T4 concentration is 50–60 times higher compared to T3, and both hormones are also partially bound to blood proteins, of which the thyroid-binding globulin (TGB) is the most relevant (70–80%) besides the other common plasma proteins, prealbumin and albumin, which bind the remaining fraction [6,7]. Consequently, only a very small non-bound free fraction of these hormones (0.03% of total T4 and 0.3% of total T3) remains available to directly access the target organs and this availability is known to be influenced by the physiologic status of the organism and may thus alter in case of disease [7,8]. Moreover, only T3 will exert any

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biologic activity since T4 needs an additional deiodination step in the peripheral tissues [7]. This clearly illustrates why the monitoring of free and total thyroid hormone concentrations, and the individual identification of both T3 and T4, is of major importance in a clinical setting.

A vast amount of publications on method development for the analysis of total and free thyroid hormones is available, but these mostly apply to human serum and focus solely on free or total hormone fractions [9,10], which have been reviewed elsewhere [11,12].

For human total and free thyroid hormone analysis the detection method of reference has been (radio)immunoassays (RIA/IA) [10–13]. But over the years, the evolutions in hyphenated mass spectrometry (MS) based techniques (gas chromatography (GC)-MS; liquid chromatography (LC)-MS and LC-MS/MS) have led to the increasing replacement of RIAs [14] in favour of more sensitive and specific mass spectrometric applications for human total and free thyroid hormone analysis [10,15–18]. For total thyroid hormone analysis in particular, laborious sample preparations were common [19–21] with the first GC-MS applications, but the evolution towards LC-MS methods, with eventually high performance liquid chromatography isotope dilution tandem MS (HPLC-ID-MS/MS) applications, allowed faster and more generic analysis of total thyroid hormones in humans [9,22,23]. Free thyroid hormone extraction, on the other hand, is still generally based on equilibrium dialysis (ED) combined with immunoassays, but mass spectrometric applications have also been described, e.g. ED LC-ID-MS/MS, which has been put forward as a human reference method [12,16,24]. Nevertheless, ED has the disadvantage to be a labour intensive, imprecise, technically demanding and costly procedure, which is hard to perform in a clinical laboratory [11,25]. Therefore, ultrafiltration (UF) was proposed as a valuable alternative for ED [18,24,26], which further led to its implementation in many North American laboratories for human serum analysis nowadays [11]. The separation through UF takes in average about 30 min, whereas ED requires 17 h to 24 h [11,25]. Only very few publications deal with method development for thyroid analysis in non-human species [10] and none are able to both asses free and total fractions of thyroid hormones. In animal medicine, several immunoassays are commercially available that detect total thyroid hormones, while the free fraction is generally determined by an ED immunoassay combination (e.g.: in donkeys, dogs and cats) [27–29]. Since thyroid hormones are involved in a variety of critical metabolic pathways and growth processes, it is clear that animals, veterinarians and researchers would benefit from the development of a fast, cost-effective and specific method allowing thyroid hormone monitoring. This paper therefore describes the development and validation [30] of rapid and sensitive methods combining ultra high performance liquid chromatography (UHPLC) with MS/MS detection preceded by two fast and cost-effective extraction procedures, for free and total thyroid hormone fractions in bovine serum, respectively.

2. Material and methods

2.1. Reagents and chemicals

The analytes, 3,3′,5,5′-tetraiodo-L-thyroxine (T4); 3,3′,5-triiodo-L-thyronine (T3); 3,3′,5′-triiodo-L-thyronine (reverse T3; rT3) were purchased from Sigma–Aldrich (St. Louis, USA). The internal standard, 3′,5,5′-tetraiodo-L-thyroxine-$^{13}C_6$ (T4-$^{13}C_6$) was acquired from Toronto Research Chemicals (Toronto, CA). All used solvents and reagents were obtained from VWR International (Merck, Darmstadt, Germany). Solvents were of 'pro analysis' grade when used for extraction purposes and of 'LC-MS Optima' grade when used for U-HPLC-MS/MS application. Ultrapure water (UPW) was obtained from a purified-water system (Sartorius Stedim Biotech, GmbH arium 611 UV, Germany).

Primary stock solutions were prepared in LC-MS optima grade methanol (MeOH) with 25% ammonium hydroxide (100 μL NH_4OH, 12 mL^{-1} MeOH) according to Van Houcke et al., [31] at a concentration of 200 μg mL^{-1} and stored in dark glass flasks at −20 °C. Working solutions were made up in MeOH, except for total thyroid hormone extraction where spiking solutions were made up in ethanol (EtOH) to match the deproteinisation conditions.

2.2. Sample preparation

2.2.1. Sample

Bovine blood (Belgian Blue) was collected from Belgian slaughterhouses (without anticoagulant) on the slaughter line during exsanguination. Subsequently, the coagulated blood was centrifuged for 20 min at 2,625 × *g*. The obtained bovine serum was pooled and stored at −20 °C in falcon tubes.

2.2.2. Statistical designs for the extraction procedure

Statistical model designs were used to optimise the analytical extraction procedures of both, total and free, thyroid hormone fractions in bovine serum. The dependent variables that might significantly affect the extraction efficiency were screened with a Plackett–Burman fractional factorial design (PB) for the free thyroid hormone fraction and a D-optimal experimental design for the total thyroid hormone fraction. These variables were selected based on a literature survey for both free and total thyroid fractions [9,10,15,16,22,23,26,32–36]. This allowed further optimisation with only the influential variables through response surface modelling (RSM) (i.e. central composite face-centered design (CCF)). If a factor proved to be non-critical ($p > 0.05$), the best level according to its effect, was chosen. Generally, this was the least time-consuming or most result effective level of the factor, which was then built into the protocol [37].

For free thyroid analysis, the 11 selected variables and their respective levels are shown in Table 1. Based on this statistical design (PB), only 12 experimental runs and three central value runs were required generating enough information to calculate the main effect of each variable and to eventually specify a particular combination of variable levels. Subsequently, critical variables with a significant ($p < 0.05$) influence (conditioning, wash and elution volumes) were subjected to RSM optimisation, resulting in the final protocol.

For total thyroid analysis, eight variables were selected with up to four levels per variable, as shown in Table 1. Therefore, a total of 26 experimental runs and three central value runs were needed to specify this particular combination of settings. After performing the prescribed scenario, the main effect of each variable was calculated. Subsequently, the critical variables ($p < 0.05$) (serum volume) and the most potent sample preparation procedure (liquid–liquid extraction) ($p > 0.05$) were subjected to further optimisation by response surface methodology (CCF design). First the number of liquid–liquid extraction steps was evaluated (1.5 mL–1 mL/3 mL–3 mL–2 mL/5 mL–4 mL/5 mL–4 mL–3 mL). Subsequently, the volumes in the first (1.5–3 mL) and second (1–3 mL) liquid–liquid extraction steps were optimised along with the serum volume (100–1000 μL) in a CCF setup.

The software program Modde 5.0 (Umetrics, Umea, Sweden) was used to build the experimental design matrix and carry out data analysis. The parameters were inserted into the program and depending on the purpose of the analysis (screening or RSM), the most adequate model was proposed by the program. Evaluation of the models was done through a one-way variance analysis (ANOVA)