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# Determination of thyroxine in the hair of newborns by radioimmunoassay with high-performance liquid chromatographic confirmation

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**Abstract**

Hair analysis is often used in forensic toxicology to study, retrospectively, chronic exposure of individuals to drugs, and consequently newborn hair may become an ideal sample to study intrauterine exposure to xenobiotics as well as to endogenous compounds. As a tool to investigate a supposed maternal thyroxine ($T_4$) supply to the congenital hypothyroid fetus, we devised to use the analysis of $T_4$ extracted from newborn hair. In the present paper, the analytical method based on $T_4$ extraction from hair followed by a radioimmunoassay is described. To verify the nature of the $T_4$-like immunoreactive material present in newborn hair, it was further studied by HPLC fractionation with radioimmunoassay of the eluted fractions. On the basis of a clear correspondence between retention times of $T_4$ standard and $T_4$-immunoreactive compound extracted from hair, we assigned this immunoreactive material to $T_4$. Then, we determined $T_4$ hair concentrations in 19 control newborns at birth and 12 congenital hypothyroid infants at 22 days of life. Values obtained from hypothyroid infants ($31.47\pm8.8$ pg/$mg_{hair}$, mean±S.D.) were not significantly lower than those obtained from healthy newborns at birth ($36.10\pm13.2$ pg/$mg_{hair}$). Such results are in agreement with the hypothesis of a maternal supply of thyroxine to the fetus through placental crossing. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Thyroxine

## 1. Introduction

In the late 1970s, hair analysis was introduced in forensic toxicology to investigate retrospectively chronic abuse of illicit drugs. Hair, indeed, displays unique features which make it suitable for the monitoring of chronic exposure to a wide spectrum of therapeutic and illicit drugs, which are incorporated into the hair matrix [1]. The still prevailing theory of incorporation is drug embedding in the hair follicle, where the germination center, formed by cells in active proliferation, is surrounded by a capillary from which small molecules dialyze into the cells. The progressive keratinization and death of the cells, becoming the major constituents of the hair shaft, would lead to the incorporation of these

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molecules in the hair matrix where they last for a long time, protected from degradation and preserved from metabolism. Other theories propose drug incorporation through sebaceous and sweat glands which are associated with the hair follicles.

The scalp hair growth (rate $1.2\pm0.3$ cm/month in the adult) causes the chronological distribution of the embedded molecules along the hair shaft.

Notwithstanding still unresolved problems of scarce methodological standardization and susceptibility of environmental contamination, hair analysis turned out to be a powerful new method of investigation of chronic exposure to substances, with a diagnostic chronological window much wider than urine and blood testing. To this innovative approach an increasing number of scientific publications and symposia are being dedicated [2–4].

The non-invasive nature of hair analysis is particularly suitable for pediatric investigation, and hair testing in newborn has already been used to study maternal exposure to drugs during pregnancy [5].

Rather surprisingly, hair analysis has been limited to exogenous molecules, but it can be reasonably applied to study the time course of exposure to endogenous compounds. On this basis, in our institute we investigated the thyroid secretion through hair analysis. A preliminary study allowed us to demonstrate the presence of the thyroid hormones in hair at concentrations easily detectable by radioimmunoassay (RIA). This led us to use hair of newborns as a new tool to study the intrauterine fetal thyrometabolic status.

## 2. Experimental

### 2.1. Chemicals

All chemicals were purchased from C. Erba Analyticals (Milan, Italy) and were of HPLC grade. L-Thyroxine was obtained from Sigma (St. Louis, MO, USA).

$T_4$ determinations in the hair extracts were carried out with a commercial solid-phase RIA kit (Neonatal $T_4$ Solid Phase Component System, ICN Pharmaceutical, Costa Mesa, CA, USA), using antibody-coated tubes and $^{125}I$-$T_4$ tracer, marketed for the neonatal screening of congenital hypothyroidism.

Serum concentrations of $T_4$ were determined with the immunoassay diagnostic kit LIA-mat® $T_4$, purchased from Byk-Sangtec Diagnostica (Dietzenbach, Germany).

### 2.2. Equipment

The HPLC equipment consisted of: a high-pressure pump (PU-980, Jasco, Tokyo, Japan), fitted with a 7125 injector (Rheodyne, Cotati, CA, USA), a $250\times4.6$ mm I.D. reversed-phase column S5ODS2 (Spherisorb, Norwalk, CT, USA), a UV absorbance detector (model UV-975, Jasco), a recorder (model N1, Gilson, Villiers Le Bel, France) and a fraction collector (Microfraction Collector, Gilson, Middleton, WI, USA). The mobile phase was filtered through a 0.45-$\mu$m Durapore polyvinylidene fluoride membrane (Millipore Corporation, Bedford, MA, USA).

A vacuum centrifuge (model UniVapo 100H, UniEquip, Munchen, Germany) was used to evaporate to dryness the collected fractions.

The radioactivity was counted in a Model 1270 Rackgamma II $\gamma$-counter (LKB, Bromma, Sweden).

### 2.3. Subjects

Nineteen healthy born-at-term newborns of mothers with no thyroid-related past or present events (13 females and six males, gestational age, $40.6\pm0.5$ weeks, mean$\pm$S.D., birth weight $3520\pm320$ g) were studied after parents' informed consent. All of them resulted normal in the neonatal screening for congenital hypothyroidism. Hair was collected by cutting close to the scalp at 3 days of life.

In addition, 12 congenital hypothyroid infants born at term (nine females and three males, birth weight $3325\pm120$ g, gestational age $40.2\pm0.3$ weeks), identified by routine neonatal blood spot screening for congenital hypothyroidism, were studied at 22 days of life. Hair collection was carried out, just before the start of the replacement therapy. $^{99m}$Tc scanning showed thyroid agenesis for six of them and thyroid ectopy for the remaining six infants. Finally, hair samples from 25 young healthy adults (age $28\pm5$ years) were collected.

The present study protocol was approved by the ethical committee of the University of Verona.

### 2.4. Method

About 60 mg of hair (~30 mg in case of newborns) were cut as close as possible to the scalp from the vertex posterior of the head. Hair samples were kept in paper envelopes at room temperature until assay was performed. Hair was carefully cut into small fragments, weighed and suspended in methanol (~50 $\mu l/mg_{hair}$). After 15 min, methanol was discarded and new methanol was added for four times to wash the sample from possible external contamination. The washed hair was then incubated in fresh methanol (10 $\mu l/mg_{hair}$) at 45°C overnight in sealed glass tubes. Methanol was then evaporated to dryness in a vacuum centrifuge and an equal amount of phosphate buffer (67 m*M* $Na_2HPO_4$/67 m*M* $KH_2PO_4$, 19:1, v/v, pH 8.0) was added to the residue (10 $\mu l/mg_{hair}$). After 30 s vortex mixing, sample solutions were left in the dark at room temperature for 1 h to allow complete dissolution.

To set up the RIA calibration curve, six standard solutions were freshly prepared from a stock solution of L-thyroxine, 2.5 mg/ml in methanol–$H_2O$ (3:1, v/v), kept at −20°C and protected from light to avoid photochemical decomposition. The calibration curve was obtained from the stock solution by dilution with the phosphate buffer at concentrations ranging from 0.1 to 10 ng/ml.

The Neonatal Solid-Phase Component System radioimmunoassay was performed as follows: 100 μl of phosphate buffer standard or sample solutions were pipetted into labeled coated tubes, where 1.0 ml of $^{125}I$-$T_4$ tracer was added. After 30 min incubation at room temperature, all tubes were submitted to 30 s vortex mixing and incubated overnight (12–18 h) at room temperature. All tubes were decanted, rinsed twice with 1.5 ml of distilled water and submitted to radioactivity count for 1 min. The concentration of $T_4$ in sample solutions was determined by spline function interpolation from the standard calibration curve. The percent cross-reactivity of the antiserum was: 100% L-thyroxine, 88% D-thyroxine, 0.6% 3,5,3-triiodo-L-thyronine.

To study the effectiveness of the washing procedure, we evaporated to dryness the methanol recovered after 15 min of washing time. An equal volume of new methanol was then added to the same hair sample. The same procedure was performed 15 min later (accounting for 30 min of washing time), 30 min later (corresponding to 1 h of washing time) and so on, in order to monitor an overall time of 4 washing hours. All residues were reconstituted with phosphate buffer (10 $\mu l/mg_{hair}$) and submitted to $T_4$ RIA.

### 2.5. HPLC separation coupled to RIA detection

As mobile phase, a mixture of methanol–$H_2O$ (4:1, v/v) acidified with phosphoric acid (0.2 ml/100 ml), was used after filtration and degassing through a 0.45-μm membrane. The liquid chromatographic separation was performed on a S5ODS2 column packed with spherical reversed-phase particles. The flow-rate was 1.2 ml/min at room temperature.

A UV absorbance detector, operated at 254 nm wavelength and connected to a data recorder, was used for the initial calibration, carried out using pure standard injections (50 μl of methanol solutions of $T_4$ standard, 1 μg/ml). Since the concentration of the hormone in the extract from hair was far lower than the detection limit of the UV absorbance detector, in order to avoid any possible interference from carryover, during the experiments we calibrated the system with standard solutions at concentrations comparable to the expected levels in hair (50 μl of $T_4$ methanol solutions at concentrations ranging from 1 to 100 ng/ml). At the used concentrations, detection was only possible by RIA carried out on the fractions collected (0.5 ml each). For this purpose, the fractions were dried off in a vacuum centrifuge and reconstituted with 250 μl of phosphate buffer. After dissolution, aliquots of 100 μl from each tube were submitted to RIA as described for the hair extracts.

Between calibration runs and runs with unknown samples, blank runs with injections of mobile phase were carried out, which constantly resulted negative on RIA analysis of the fractions.

$T_4$ standard was also subjected to overnight incubation at 45°C (1 ng/ml) to check its stability and no sign of degradation was observed, by comparing the concentrations before and after incubation.

## 3. Result and discussion

### 3.1. Washing procedure

On the basis of the existing literature on the embedding in the hair matrix of drugs and xenobiotics, it can be hypothesized that also for $T_4$, the amount present in hair can originate from two sources: incorporation at the root level from blood and external contamination from the environment. In case of newborns, the external contamination with $T_4$ may originate from the amniotic liquid surrounding the fetus. For $T_4$, no environmental sources of contamination can be postulated for adults. Hair can be also 'contaminated' by sweat and sebaceous gland secretion, but this should reflect endogenous $T_4$ production.

The aim of the present work was to study $T_4$ present in the hair from endogenous incorporation and, for this reason, it was necessary to develop a suitable washing technique in order to efficiently remove $T_4$ derived from external contamination.

For this purpose, we used repeated methanol washings, as detailed above, according to procedures already reported by other authors for drugs of abuse [6,7]. In the present work, methanol was chosen also because it is known to prevent formation of $T_3$ from $T_4$ [8].

The amount of $T_4$ removed by washings was determined in 25 healthy adults and seven healthy newborns. $T_4$ removed from hair, plotted against washing time, displayed for adults a high peak of washing removable hormone in the first 2 h, while newborns' hair appears fairly 'clean' from the first washing step performed at 15 min (see Fig. 1).

Even if hair from newborns appeared substantially clean without washing, in the further experiments hair samples were submitted to four washing steps

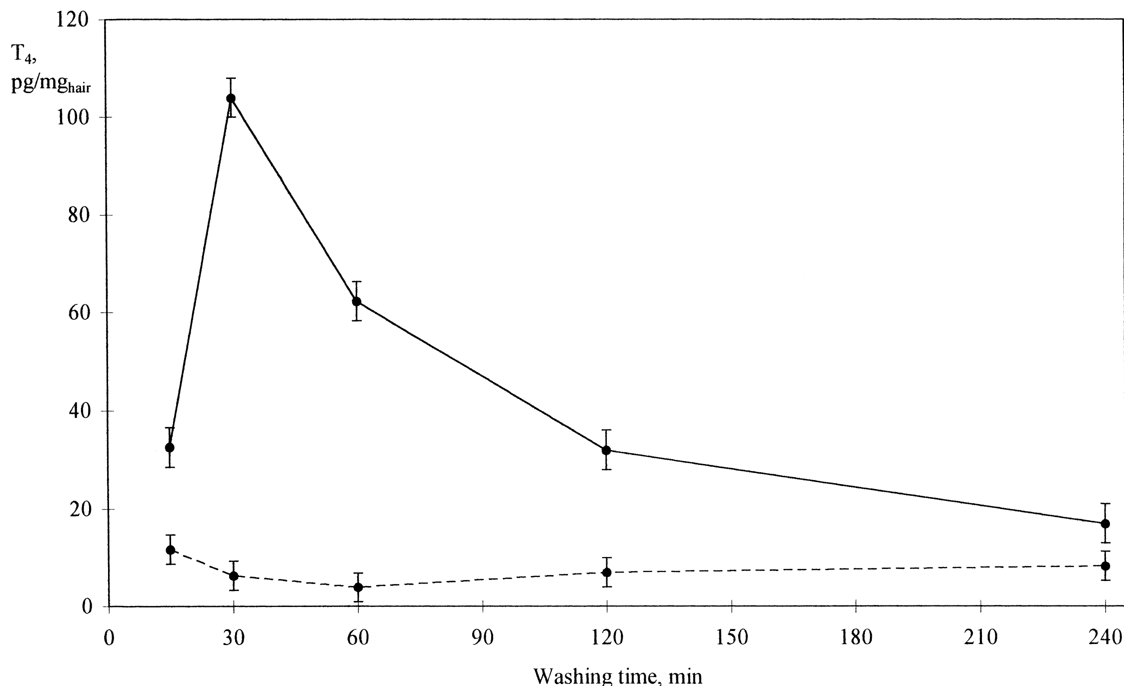


Fig. 1. Efficacy of the washing procedure in the case of hair from 25 healthy adults (solid line) and from seven control newborns (dotted line). Methanol from washings was recovered at 15 and 30 min, and at 1, 2 and 4 h, evaporated to dryness and the residue reconstituted with 10 $\mu l/mg_{hair}$ of phosphate buffer (67 m*M* $Na_2HPO_4$–67 m*M* $KH_2PO_4$, 19:1, v/v, pH 8.0). Each reconstituted residue was submitted to $T_4$ RIA.

for an overall time of 2 h, to assure beyond any reasonable doubts removal of external contamination.

### 3.2. $T_4$ in hair results

The intra-day quantitative precision, checked by five consecutive RIA determinations carried out on the same reconstituted hair extract ($0.725\pm0.05$ pg/$mg_{hair}$), resulted in a C.V. of 6.9%. The day-to-day quantitative precision, checked by seven RIA determinations carried out on reconstituted extracts prepared at different times from the same hair sample, was 20.6%.

No correlation between hair $T_4$ and any of the analytes assayed in the serum ($T_4$, $T_3$, $FT_4$ and TSH) was found, conceivably because of the different detection window of hair in comparison to serum samples. $T_4$ hair assay gives information on past thyrometabolic status, while serum assays outline present conditions.

The average $T_4$ hair content in 19 healthy newborns at birth was $36.10\pm13.2$ pg/$mg_{hair}$ (mean±S.D.).

$T_4$ average content in hair of all hypothyroid infants was $31.47\pm8.8$ pg/$mg_{hair}$ within a range between 16.60 and 48.70 pg/$mg_{hair}$. No statistical difference with Student's *t*-test was found between the hypothyroid and the normal infants ($P=0.293$). Moreover, we did not find any significant difference among infants with thyroid agenesis and those with ectopies (mean±S.D., $30.00\pm6.2$ pg/$mg_{hair}$ and $32.93\pm11.3$ pg/$mg_{hair}$, respectively, $P=0.590$). The lack of any statistical difference between controls and hypothyroid newborns, and between agenesis and ectopies, was confirmed also using the Mann–Whitney *U*-test ($P=0.320$ and $P=0.100$, respectively). On the other hand, concentrations of $T_4$ in the sera of the healthy and of the hypothyroid infants affected by agenesis or ectopies, were $129.43\pm40.9$, $13.20\pm5.6$ and $9.35\pm2.2$ μg/l, respectively.

These data look quite surprising at first glance, but are in good agreement with the hypothesis of thyroxine placental transfer from mother to fetus. This is also supported by the clinical observation of normal brain development of the hypothyroid fetus during the pregnancy. Indeed, the hypothesized transfer of maternal $T_4$ to the fetus would be particularly important to protect it from deleterious effects of hypothyroidism, especially during the myelination process. Consistently with such an hypothesis, a case reported in the literature [9] appears to be particularly significant: under conditions of both maternal and fetal TSH insufficiency, leading to the exceptional status of absolute fetal hypothyroidism, the newborn at birth showed a striking delay of development.

### 3.3. HPLC confirmation

In order to verify the chemical identity of the immunoreactive material determined in hair and tentatively identified as $T_4$, we developed an isocratic HPLC method, based on a method for purification of iodothyronines in in vivo turnover studies, reported in literature by Bianchi et al. [10], with minor adjustments.

When the HPLC separation procedure was carried out on $T_4$ standard solution (50 μl of methanolic $T_4$ solution at a concentration of 4 ng/ml), the RIA determination on the collected fractions (0.5 ml each) identified a single and symmetrical peak of the hormone, eluting in the fractions corresponding to a retention time of $20\pm2$ min ($n=5$).

The same procedure was carried out by injecting extracts from overnight incubations of hair samples from six healthy newborns and five young adults ($T_4$-like in hair, $26.56\pm8.3$ pg/$mg_{hair}$ and $9.19\pm6.5$ pg/$mg_{hair}$, respectively). Consistently with the chromatography of the $T_4$ standard, a single peak corresponding to the standard $T_4$ retention time was identified ($t=20\pm2$ min). These findings support the identity of the compound extracted from hair as L-thyroxine (Fig. 2 shows an example—taken from among all methanol hair extracts injected— compared to a $T_4$ standard injection).

The average recovery of the HPLC separation calculated by comparing the RIA analysis on the crude extracts with the sum of the collected fraction was 95%.

## 4. Conclusions

To the best of our knowledge, the present work reports for the first time the possibility of determin-

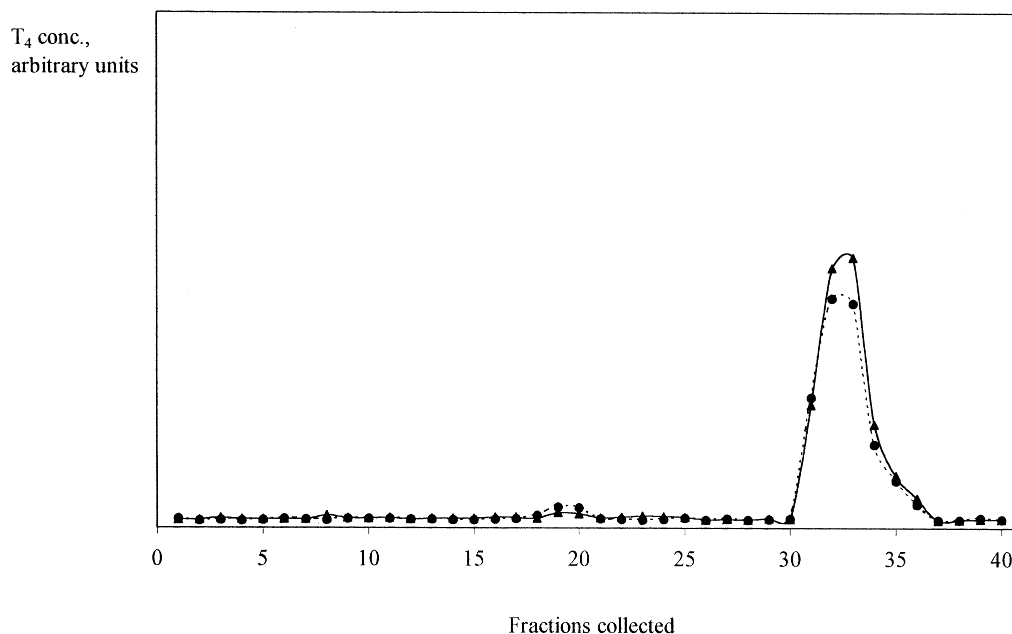


Fig. 2. $T_4$ content determined by RIA in the fractions collected in the case of $T_4$ standard injection (4 ng/ml, 50 μl injected, solid line) and in the fractions collected after 50 μl injection of methanol from overnight incubation of a newborn hair sample, taken as an example (dotted line). The first 15 fractions were discarded and not subjected to RIA.

ing $T_4$ in hair of newborns. The determination was carried out by RIA and further confirmed by HPLC.

The method has been applied to investigate possible $T_4$ placental crossing from mother to fetus showing the presence of thyroxine in hypothyroid newborn hair at concentrations lower than in controls, but not significantly. This would demonstrate a maternal supply of the hormone to the fetus through placental crossing, as already hypothesized on the basis of clinical data.

Although the mechanisms of $T_4$ incorporation in hair are still unclear, our findings point out the potential importance of studying hair as a source of precious information on the past history of the subject, in terms of chronic exposure to xenobiotics (as already accepted by the scientific community), but also to endogenous substances such as hormones and metabolites.

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