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Note

Analysis of term human milk concentrations of 3,5,3′-triiodo-L-thyronine by high-performance liquid chromatography and radioimmunoassay: correlation with circulating serum levels in lactating women

LINDA V. OBERKOTTER

Department of Biological Sciences, Florida Institute of Technology, Melbourne, FL 32901 (U S A.)

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The presence and significance of thyroid hormones in milk have been actively investigated, as reviewed recently [1]. From the maternal view, these hormones may play an active or permissive role in initiation and/or maintenance of lactation [2], and the lactating breast may also provide an alternative site for thyroid hormone deiodination at a time when hepatic deiodinase activity is low [3]. From the suckling infant's perspective, the thyroid hormones secreted into the milk may stimulate gastrointestinal growth and absorptive capacity, and also enter the circulation, where they may feedback to the hypothalamo-pituitary thyroid axis, and exert other local tissue effects.

The analysis of hormones in milk is fraught with difficulties, due to the complex nature of the fluid and the presence of interfering substances which preclude direct quantitation by conventional techniques. In the present study, we have assessed milk triiodothyronine (T_3) levels in full-term lactating women using high-performance liquid chromatographic (HPLC) separation followed by radioimmunoassay (RIA) and then correlated these values with circulating T_3 concentrations in paired serum specimens, in order to determine whether serum T_3 levels are a good predictor of T_3 concentrations in milk and the extent to which T_3 is concentrated in milk, relative to serum levels.

EXPERIMENTAL

Samples

Term human milk and serum specimens were obtained at the same visit from twelve mothers who gave written informed consent to participate in the study.

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The women were multiparous, well nourished, and between 22 and 38 years of age. All had delivered, five to thirteen weeks earlier, full-term appropriate-for-gestational age infants who were exclusively breast-fed from birth. Blood was collected by intravenous puncture; milk was collected by manual pump, and a 10-ml aliquot was drawn from the full breast sample. Serum and milk were then frozen at −50°C until assayed. T_4 and T_3 standards were purchased from Sigma (St. Louis, MO, U.S.A.) and reverse-T_3 (rT_3) from CalBiochem (San Diego, CA, U.S.A.). The other thyromimetic amino acid metabolites, triiodothyroacetic acid (TRIAC), triiodothyropropionic acid (TRIPROP), and tetraiodothyroacetic acid (TETRAC), were obtained from Henning-Berlin (Berlin, F.R.G.). Frozen samples were thawed in an ice–water bath prior to extraction. Serum T_3 concentrations were measured directly in unextracted specimens using a T_3 RIA kit (T_3-Quanticoat, Kallestad Labs., Chaska, MN, U.S.A.). For each of the milk specimens, tracer quantities of [^{125}I]T_4 and [^{125}I]T_3 (Amersham, Arlington Heights, IL, U.S.A.) were added to each 5-ml sample in order to monitor recovery. Chloroform–methanol (2:1, v/v) extraction was followed by precipitation with 0.05% calcium chloride as described by Gordon et al. [4] with minor modifications. Following lyophilization of the lipid-free extract, an iodothyronine-enriched fraction was prepared by adding the reconstituted extract to a 1.0-ml bed volume of AG 1X2 (200–400 mesh, acetate form) anion-exchange resin (Bio-Rad Labs., Richmond, CA, U.S.A.) and eluting in step-wise manner monoiodotyrosine (MIT; 0.25 ml/l acetic acid), diiodotyrosine (DIT; 10.0 ml/l), and the iodothyronines (in the 500 ml/l wash) as described previously [5]. Metabolite recovery from the ion-exchange resin was determined empirically by addition of known quantities of standard preparations to the columns. The resulting iodothyronine region (50% acetic acid) was evaporated under nitrogen and then reconstituted in 200 μl of a 50:50 mixture of acetonitrile and 1% acetic acid. The reconstituted iodothyronine preparations were pre-filtered prior to column injection using a Millex HV 0.45-μm filter unit (Millipore, Bedford, MA, U.S.A.); 50–100 μl of this filtered material were used for injection onto the column. Standard metabolite mixtures (2 μg/ml) were prepared fresh weekly from a stock solution (1 mg/ml) using a 50:50 mixture of acetonitrile and 1% acetic acid. A standard T_3 preparation of 25–250 ng was injected onto the column each day prior to the extracted biological samples, in order to assess the reproducibility of the retention times observed.

Apparatus

The HPLC system employed included a Model 655A-11 delivery pump with tertiary gradient capabilities and a Model 655 data processor (EM Science, Cherry Hill, NJ, U.S.A.). Samples were injected onto a C_8 (250 mm×4.6 mm I.D.) reversed-phase column (DuPont HPLC Products, Wilmington, DE, U.S.A.) followed by a step-wise binary gradient of acetonitrile and 1% acetic acid ranging from 30:70 at 2 min to 50:50 at 28 min followed by isocratic elution to 70 min. The flow-rate was 2.0 ml/min. Absorbance at 300 nm was monitored using a variable-wavelength flow-through spectrophotometer (Kratos Analytical, Ram-

sey, NJ, U.S.A.). Fractions (0.5–1.0 ml) were collected with an LKB Redirac (LKB Instruments, Gaithersburg, MD, U.S.A.) collector. Fractions eluting within the retention time range established for the T_3 standard were pooled and evaporated under nitrogen at 28°C. The residue was reconstituted in 200 μl of thyronine-depleted serum [6]. This material was then further diluted, as required, with thyronine-free serum and assayed for T_3 by RIA as noted above. The relationship between serum and milk T_3 concentrations was determined by curve-fitting procedures applying the method of least squares, using a Hewlett-Packard HP-41C calculator with an advanced statistical package.

RESULTS

A representative HPLC elution profile for a mixture of the major iodothyronine metabolites is seen in Fig. 1. Both labelled and unlabelled T_3 eluted from the column at 11.10+0.46 min (mean ± S.D.; $n=20$). Average recovery of [^{125}I]T_3 tracer from the combined extraction and pre-column procedures was 62%; recovery of material applied to the HPLC column was > 95% in all samples examined. All milk T_3 values reflect corrections for recovery. It is significant to note that the discrete separation of the iodothyronines by HPLC combined with the low cross-reactivity for structurally similar metabolites such as rT_3 (0.9%) and diiodothyronine (T_2, 0.26%) in the RIA permits a high degree of specificity in the analytical method employed.

The range of serum T_3 concentrations measured in this group of lactating women

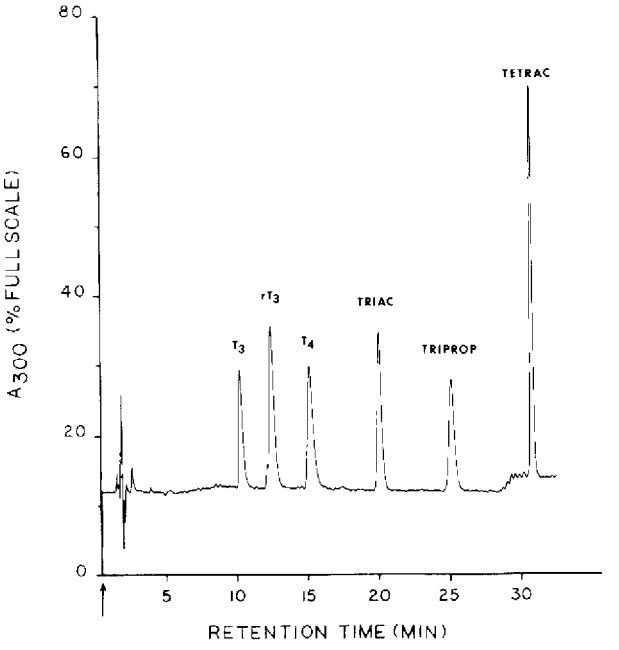

Fig. 1. Elution profile of major thyroid hormone metabolites on reversed-phase HPLC. Typical profile resulting from injection of a mixture of 100 ng per metabolite, with baseline adjusted for gradient changes See text for description of operating conditions.

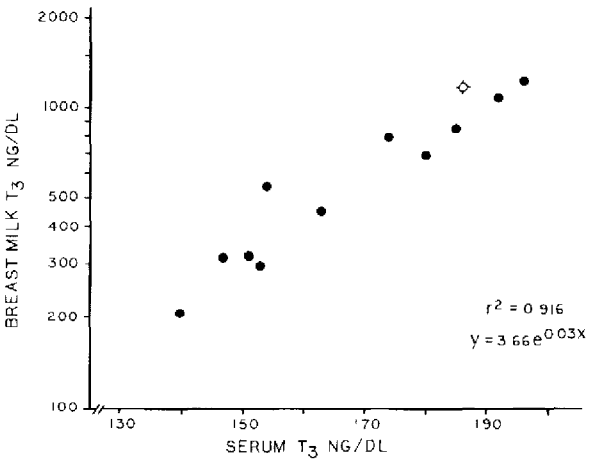

Fig. 2 Serum versus milk T_3 concentrations in full-term lactating women. Each point represents T_3 levels for paired serum and milk specimens from an individual subject. ⊹ represents a hypothyroid woman on 0.1 mg Synthroid (Levothyroxine, Boots-Flint, Deerfield, IL, U.S.A.) per day. Correlation, $p < 0.0005$.

was 139–196 ng/dl. The range and mean (168 ± 6 ng/dl, $n = 12$) observed suggest that T_3 levels in the study population were in the mid- to high-normal region of the normal distribution observed in the general population (where approximately 95% fall between 85 and 185 ng/dl). Milk T_3 levels in these same women ranged from 200 to 1220 ng/dl and averaged 669 ± 107 ng/dl. The relationship between serum (as the independent variable) and milk (as the dependent variable) T_3 is seen in Fig. 2. Using an exponential curve fit as defined by the equation $y = 3.66e^{0.03x}$, a highly significant correlation ($p < 0.0005$) between the two variables was found. We observed no statistically significant correlations between time post-partum and neither serum nor breast milk T_3 concentrations, strongly suggesting that at least in the period of one to three months of lactation, maternal circulating T_3 level, rather than duration of lactation, is the primary predictor of hormone concentration in her milk.

DISCUSSION

The mean T_3 levels observed in the present study are higher than those reported in our previous investigations [7] and those of others [1]. Several factors may account for these differences. First, milk specimens used in our initial study were random samples collected from women at 10–36 weeks of lactation, and it has been suggested that thyroid hormone concentrations in milk may decline over time with prolonged lactation [8]. In addition, the original extraction (via ethanol) and separation (a low-pressure, low-resolution LH-20 column without pre-column thyronine separation) procedures may have resulted in suboptimal estimates of hormone content. Lastly, we did not collect serum specimens from mothers enrolled in the earlier study, and the present data suggest that if circu-

lating T_3 concentrations in the original group of mothers were generally lower, significantly lower milk T_3 levels would be expected. It is likewise, for this reason difficult to compare our data with those of Varma et al. [9]; interestingly, however, they found T_3 concentrations as high as 500 ng/dl in "day 8 and onward" milk specimens. Our data suggest that the lactating woman in a hyperthyroxinemic condition (e.g. the overmedicated hypothyroid patient) is capable of transferring very high concentrations of T_3 to her offspring. The impact upon the suckling infant under such clinical circumstances is presently unknown, but would most certainly relate to the length of time over which this condition persisted.

The present data are consistent with our earlier findings in rat pups of control and T_4-treated dams [1]. T_4 concentrations of the two groups of pups were comparable (although T_4 levels of the treated dams had increased more than two-fold), but pups of the T_4-treated dams exhibited a *four-fold* increase in circulating T_3 levels (vis à vis a two-fold increase in maternal T_3 levels). Hence, it is reasonable to hypothesize that transfer of T_3 via the milk may contribute to these observed differences. Fukuda et al. [10] reported a depression of maternal T_4 and T_3 levels during lactation in the rat, which may reflect a net loss of hormone through the milk. Further, Giralt et al. [3] have observed a significant depression of the T_3/T_4 ratio in lactating rats vis à vis virgin females. This alteration reflects to some extent slightly lower T_4 levels, but it is the significantly lower serum T_3 concentrations in lactating dams which predominantly depresses this ratio. The latter authors focused upon differences in hepatic 5'-monodeiodinase activity (lower in lactating dams) in their experiments. The role of lactating mammary tissue, however, in the generation, secretion, and transfer of T_3 is poorly understood, and its function in affecting both maternal and neonatal metabolic function via thyroid and thyromimetic hormones remains to be elucidated.

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