Chromatographic Identification in Serum of Endogenously Radioiodinated Thyroid Hormones After Iodine-131 Whole-Body Scintigraphy in the Follow-up of Patients with Differentiated Thyroid Carcinoma

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Patients with differentiated thyroid cancer (DTC) are conventionally followed with serial ^{131}I whole-body scintigraphy (WBS) and serum thyroglobulin (hTg) assay. Given the 15%–20% incidence of discordant results, we developed a sensitive and specific procedure for monitoring such patients, based on the assumption that ^{131}I uptake, even if too low to be detected by ^{131}I WBS, could be assayed in serum as thyroid products (hTg, T_3 and T_4) endogenously labeled with ^{131}I. Our study included 125 patients routinely monitored for tumor recurrence or for the persistence of functioning thyroid tissue after complete primary treatment for DTC (surgery and ^{131}I ablation of remnants). A plasma sample, taken 72 hr after administering ^{131}I for WBS was fractionated on a Sephadex-G25 superfine column by first eluting all of the radioactive species except the thyroid hormones and then the radioiodothyronines. The sensitivity and specificity of chromatography in detecting functioning thyroid tissue after primary treatment for DTC were 98.4% and 100% (accuracy 99.2%), respectively, versus 90.6% and 95.1% for ^{131}I WBS (accuracy 92.8%) and 60.9% and 100% for hTg (accuracy 80%). Combining chromatography with serum hTg gave the highest gains in diagnostic performance (100% for all parameters). This chromatographic method can be used in addition to conventional procedures in the follow-up of patients with DTC and represents a highly sensitive test for assessing the results of ^{131}I ablation of postsurgical remnants.

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Differentiated thyroid cancer (DTC) derived from the follicular epithelium* displays highly variable biological/clinical patterns with frequent long-term recurrences in cases that are potentially curable by primary treatment, thyroidectomy and radiometabolic ablation of remnants with ^{131}I when indicated (*1–3*). The effectiveness of radiometabolic ablation in eliminating any tumor cells possibly harbored in residual cell clusters is under debate, in part due to the lack of an unequivocal definition of successful radiometabolic ablation in either qualitative or quantitative criteria (*4*). Tumor recurrences observed after apparently successful radiometabolic ablation clearly demonstrate that diagnostic ^{131}I uptake and/or scintigraphy and assay of serum thyroglobulin (hTg) do not fulfill absolute sensitivity criteria. Furthermore, although more sensitive in detecting recurrent/metastatic tumor than ^{131}I whole-body scanning (WBS) (*5,6*), the hTg assay performs poorly in evaluating the completeness of radiometabolic ablation (*7,8*).

The procedure described here was developed to complement the diagnostic performance of the hTg assay and ^{131}I WBS in the follow-up of DTC. This approach, based on the appearance of 3,5,3′-triiodothyronine (T_3) and thyroxine (T_4) in serum radiolabeled in vivo after administration of ^{131}I for WBS (*9*), amplifies the specific biological signal originating from cells with ongoing thyroid function. Even if this event is sometimes so weak as to result in negative hTg assays and/or ^{131}I WBS, any ^{131}I uptake appears in the serum has endogenously labeled T_3 and T_4 if the metabolic thyroidal pathways are maintained. Instead, in the absence of functioning thyroid-derived cells, no alternative pathways for producing labeled thyroid hormones exist. We evaluated the usefulness of this chromatographic procedure in monitoring patients with DTC, thus helping to clarify the controversies still surrounding this issue.

MATERIALS AND METHODS

Patients

This chromatographic procedure was included in the follow-up protocol of 160 patients with DTC: (1) total thyroidectomy (as completion surgery following lobectomy in about 15% of the cas-

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*For simplicity, general definition of "differentiated thyroid carcinoma" is used to indicate thyroid cancers derived from the follicular epithelium, including both follicular and papillary histotypes.

es); (2) radiometabolic ablation of remnants (50–100 mCi of ^{131}I, 6 wk after surgery) because all tumors were >1.5 cm in diameter; (3) serial ^{131}I WBS and hTg assays in addition to focused clinical examinations and other imaging modalities.

One hundred twenty-five patients were selected for the study based on: (1) complete follow-up for at least 3 yr (range 3–6 yr) after application of the chromatographic procedure and (2) absent circulating anti-hTg autoantibodies.

There were 84 women and 41 men (mean age: 41 ± 17 yr; range: 18–71), with 91 cases of papillary carcinoma and 34 cases of follicular carcinoma; postsurgical follow-up varied from 4–15.6 yr at the time of entering the study. For this study, "disease" was not strictly defined as histologically proven presence of neoplastic tissue, but also included the presence of functioning thyroid tissue in the thyroid bed after thyroidectomy and apparently successful radiometabolic ablation. The presence or absence of thyroid-derived tissue (tumor recurrence or unexpected remnants) was established in each patient based on a combination of at least three of the following: clinical findings, echotomography, follow-up (in all patients), standard x-ray (34 patients), ^{201}Tl scan (32 patients), positive uptake with therapeutic ^{131}I doses (25 patients) (*8*), MRI (24 patients), cytology on fine-needle aspirates (22 patients), CT (15 patients), bone scan (9 patients), WBS with ^{123}I (*10*) (8 patients), and histology (6 patients). This was the gold standard against which the results of the three tests under evaluation (^{131}I WBS, hTg, chromatography) were compared.

After assaying serum hTg while on treatment, suppressive L-thyroxine was discontinued for 4–6 wk prior to a second hTg assay and ^{131}I WBS. At this time, serum TSH was always higher than 60 μIU/ml, indicating that the possible production of thyroid hormones by residual thyroid-derived tissue was never sufficient to inhibit TSH secretion. Total T_4 concentration was in the 0.25–0.75 μg/dl range (a trace from prior exogenous administration). Total T_3 was below the useful working range for immunoassays, and free T_4 and T_3 were mostly undetectable.

Iodine-131 WBS

Patients followed a low-iodine diet for the last week before WBS. Seventy-two hours after the oral administration of ^{131}I, a Starcam 400 large field of view gamma camera equipped with a 3/8" crystal and a high-energy collimator was used to record whole-body images and spot views. In 1986–1987, a 2-mCi dose (75 MBq) was used, but was increased to 5 mCi (185 MBq) following literature trends. For the whole-body mode (routinely the anterior projection), the moving bed was set at a very low speed (13 cm/min at most, typically between 5–10 cm/min) to achieve satisfactory counting statistics and compensate for reduced counting efficiency of the gamma camera crystal for ^{131}I. A high-energy and/or a pinhole collimator was used to record specific spot views (the neck region in all patients and additional areas with dubious uptake foci), acquiring at least 100,000 counts. Scintigraphs were blindly read by three experienced specialists; discordant results (about 8% of the cases) were resolved by consensus.

Serum hTg Assay

Serum hTg was assayed using an immunoradiometric procedure (Sorin Biomedica) with an assay sensitivity of 0.35 ng/ml and a cut-off of 5 ng/ml for absence or presence of disease. The interassay coefficients of variation were 18%–25% for hTg concentrations around the cut-off level, 50%–60% in the 1.2–3 ng/ml range and about 8% in the 80–140 ng/ml range.

Chromatography

Four milliliters of serum obtained when the patients reported for WBS was saturated with 8-anilino-1-naphthalene sulfonic acid (ANS) to displace the binding of thyroid hormones to the carrier proteins. Serum was then loaded onto a Sephadex-G25 superfine column (1.5 × 21 cm) preequilibrated with 0.05 *M* phosphate buffer (pH 7.4) containing 0.15 *M* NaCl and 0.01 *M* KI.

Elution was begun with this buffer (0.7 ml/min) and 100 2-ml fractions were collected. The buffer eluted all of the radioactive components in the serum except the thyroid hormones, which are retained indefinitely at this pH and molarity. The bulk of radioiodothyronines was then eluted in one-two fractions by adding 3 ml of normal human serum to the column (followed by the same phosphate buffer) and collecting 10 additional 2-ml fractions.

Fractions were counted in an automatic gamma-counter for relatively long counting times (20–30 min) based on activity levels in the fractions of interest and taking into account an average background count rate of 26.5/min (±4.34% CV for 20 min of counting; ±3.54% CV for 30 min of counting) in the photon peak, including 80% of the ^{131}I gamma emission. Endogenously radioiodinated thyroid hormones were positively identified if radioactivity (net counts) in at least one fraction of the bulk elution of T_3-T_4 was at least twice the background activity.

The chromatographic system was tested under various conditions artificially reproduced in vitro by adding well defined radioactive species (40–500 Bq, the amount usually recovered in 4 ml serum 72 hr after a diagnostic ^{131}I dose) to cold serum. This identified the elution volumes of reference radioactive standards: radioiodine, radioiodotyrosine, radioiodinated T_3-T_4 and labeled serum albumin (a marker for "iodoproteins"). In fact, after administering radioiodine in vivo (even in athyreotic patients or during complete thyroidal pharmacologic blockade) radioactive species chromatographically indistinguishable from the main protein components appear in the serum (*11*). This is also observed when incubating radioiodine with serum in vitro (*12*), thus excluding any ongoing in vivo neogenesis of hypothetical iodoproteins, probably due to a weak-to-intermediate strength covalent bonding between plasma proteins and radioiodine.

Statistics

Paired-data Mac-Nemar's test was used to compare sensitivity and the overall diagnostic value of the three tests (^{131}I WBS, hTg assay, chromatography), with the final classification of patients achieved independently as described. Bayesian formulas were utilized to calculate the sensitivity, specificity, accuracy, positive-predictive and negative-predictive values of the three tests considered either individually, in three pair-associations (^{131}I WBS + hTg, ^{131}I WBS + chromatography, hTg + chromatography) and with all three tests combined. Each association was classified as positive when at least one of the tests in the combination was positive.

RESULTS

Chromatography

Figure 1 shows that 0.01 *M* KI in the buffer reduced the coelution of radioiodine with proteins from about 2.7% to 0.3%, while reducing its coelution with T_3-T_4 from about 0.19% to virtually 0%. Even after saturation with ANS, some fraction of T_3-T_4 (5–8%) remained bound to the carrier proteins.

In vivo labeling by [^{131}I]iodide, hypothetically due to

circulating/tissue peroxidases of T_3-T_4 traces remaining from prior exogenous L-T_4 was excluded as follows. One patient who had undergone complete primary treatment (surgery and radiometabolic ablation) was referred for ^{131}I WBS while still on suppressive L-T_4; chromatography of a serum sample obtained from this patient was completely negative (Fig. 2, upper panel). An example of a positive chromatographic profile is also shown (Fig. 2, lower panel). The overall recovery of radioactivity was 96.9% ± 4.3% (n = 10), with variability among different runs <2%.

In vitro experiments performed with radioactive standards showed that even one single becquerel of radioiodinated T_3-T_4 was detected in serum samples containing overall radioactivity in excess of 500 Bq (0.2% analytical sensitivity). Endogenously labeled T_3-T_4 recovered in a 4-ml plasma sample was as low as 10^{-8} of the ^{131}I administered dose (or 0.000135% of the dose/liter plasma, with a maximum observed value of 0.05% dose/liter plasma).

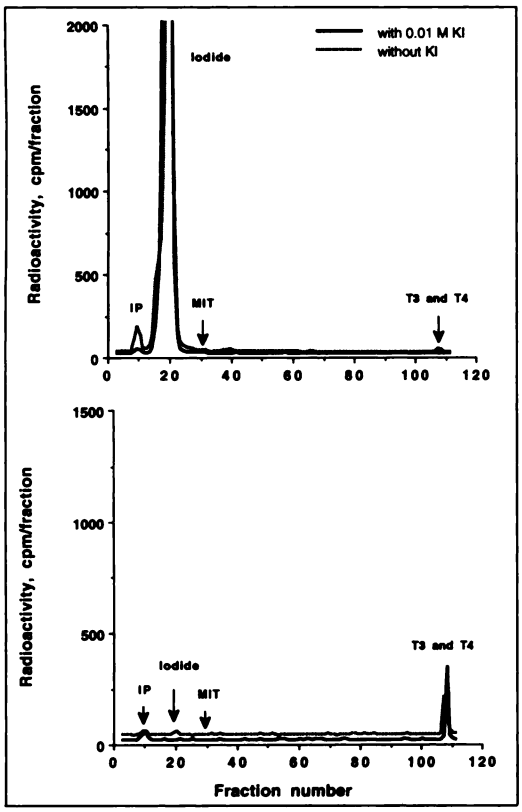

FIGURE 1. Elution profiles of radioactive standards (labeled with ^{131}I and/or ^{125}I) incubated in vitro with cold serum. Background activity has not been subtracted to show statistical significance of radioactivity counting in identifying radioiodothyronines concentrated in only two to three fractions (4–6 ml eluate). (Top) Elution profile obtained after incubating [^{131}I]iodide. Without 0.01 *M* KI, radioactivity is eluted both in specific radioiodide zone (fractions 14–24) and in the "iodoproteins" zone (IP, fractions 7–12, identified by radiolabeled human albumin), with marginal amounts eluted by the late addition of cold serum (fractions 107–109, iodothyronine zone). Adding 0.01 *M* KI greatly reduces elution of [^{131}I]iodide in the iodoprotein peak, while reducing to virtually zero [^{131}I]iodide eluted with iodothyronines. (Bottom) After incubating radiolabeled T_3 and T_4 (dotted line, shifted upwards for easier identification), 0.01 *M* KI minimizes elution of radioiodothyronines in zones other than the specific elution peak. MIT = radiomonoiodotyrosine.

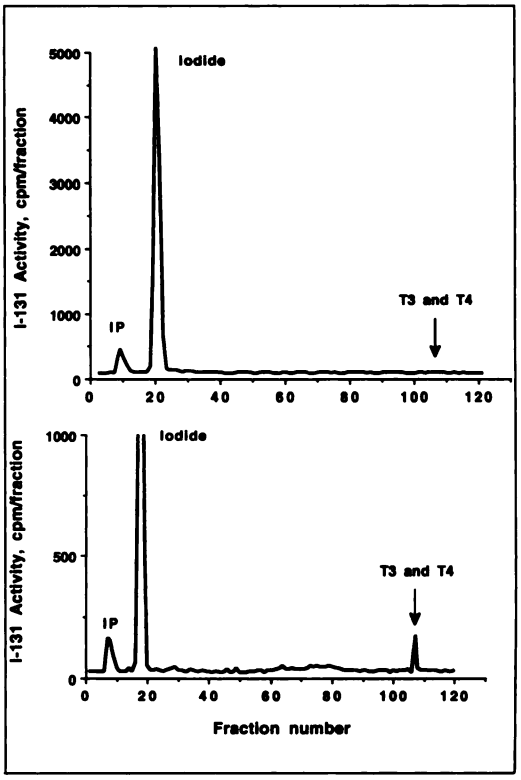

FIGURE 2. (Top) (negative for radiolabeled T_3-T_4): patient referred for ^{131}I WBS while still on suppressive L-T_4 treatment. (Bottom) Example of chromatography positive for endogenously radiolabeled T_3-T_4.

Clinical Results

Sixty-one patients (48.8%) were disease-free, whereas 64 (51.2%) were affected by "disease," including in this definition residual functioning thyroid tissue after postsurgical radiometabolic ablation of remnants (see Table 1). In most cases, this was benign thyroid tissue probably without tumor recurrence, as demonstrated by a frankly positive scan following therapeutic ^{131}I given after nondiagnostic ^{131}I WBS and negative serum hTg [handling of hTg by residual tiny thyroid remnants showing some iodofixation after postsurgical radiometabolic ablation may be normal, as also observed in thyroid remnants post-thyroidectomy (*7*)]. These patients had positive chromatography, while the likelihood of recurrent tumor was excluded based on other criteria (primarily echotomography of cervical lymph-nodes). This condition was treated with an additional ablative ^{131}I dose to eliminate possible misdiagnoses regarding locally recurring tumor and to improve the long-term prognosis (*2*). The tumor-positive patients had local recurrences or metastases primarily in regional lymph nodes, with only a few distant metastases.

Table 1 compares the results of the three tests with the patients' final classification. Iodine-131 WBS generated six false-negative and three false-positive results (false-positives: two patients with esophageal diverticulum, one pa-

TABLE 1
Comparison of the Results from the Three Procedures According to the Final Classification of Patients

No. of patients	^{131}I WBS	Serum hTg ng/ml*	Sephadex G25 chromatography	Overall follow-up
58	Negative	Negative	Negative	Negative
38	Positive	184 ± 62	Positive	Positive
20	Positive	Negative	Positive	Positive
5	Negative	Negative	Positive	Positive
1	Negative	256	Negative	Positive
3	Positive	Negative	Negative	Negative

*Negative if <5 ng/ml.

tient with ectopic salivary tissue in cervical lymph nodes). The hTg assay generated 25 false-negative and no false-positive results; chromatography produced one false-negative and no false-positive results. There were false-negative results in both ^{131}I WBS and hTg assays in five patients in whom chromatography gave true-positive results (Fig. 3). Statistical analysis showed that the overall diagnostic value of serum hTg was significantly lower than for either ^{131}I WBS or chromatography ($p < 0.0001$).

Bayesian diagnostic parameters of the three procedures are listed in Table 2. Iodine-131 WBS and chromatography exhibited high and comparable values for all diagnostic performance parameters (mean 92.8% for ^{131}I WBS, 98.4%–100% for chromatography), whereas objectively lower values for sensitivity ($p < 0.0001$ versus ^{131}I WBS and chromatography), accuracy and negative-predictive value were observed for the hTg assay. Specificity and positive-predictive values were highest (100%) for hTg and chromatography (no false-positive results).

The greatest gains in diagnostic performance were observed when associating hTg and chromatography (100% for sensitivity, specificity, accuracy, positive-predictive value, negative-predictive value) because this association identified all patients with disease without any false-positive results. Sensitivity of serum hTg was significantly lower ($p < 0.0001$) than for either of associations tested.

Overall, chromatography performed objectively better than ^{131}I WBS or serum hTg. The inclusion of chromatography results in each combination invariably caused objective improvement in all diagnostic performance parameters.

DISCUSSION

The ideal objective of cancer treatment is to eradicate all tumor cells in the body. Radiometabolic ablation of post-surgical thyroid remnants, although widely and increasingly used (*2,13*), is still controversial due to the lack of controlled prospective clinical trials and disagreements in defining ablation. Conflicting results probably arise from the different criteria employed to define successful ablation (*4*), and late tumor recurrences demonstrate that radiometabolic ablation is often only apparently successful.

Pochin first attempted to obtain quantitative information

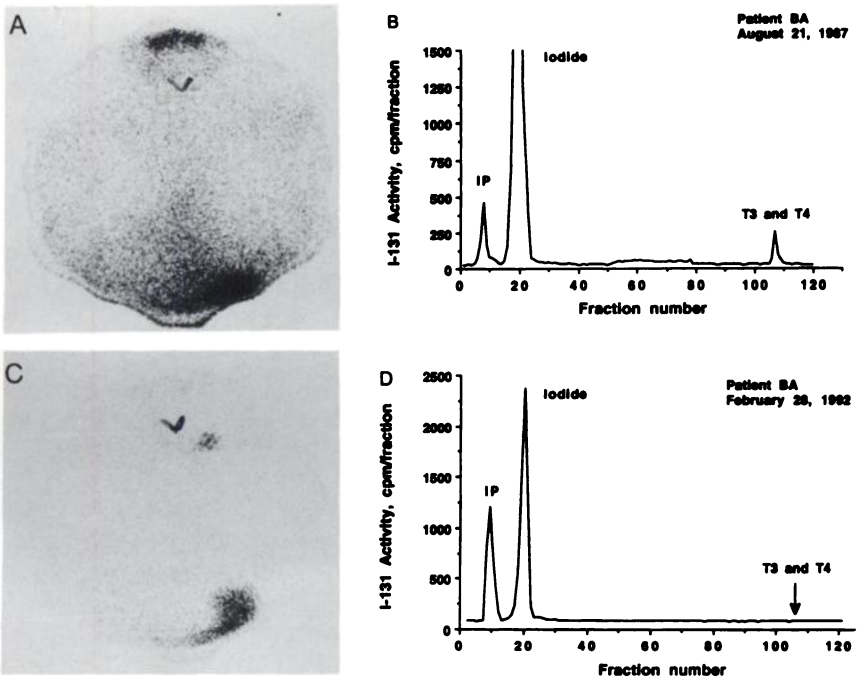

FIGURE 3. Representative patient from the five with negative ^{131}I WBS and hTg in whom chromatography correctly identified tumor recurrence or metastasis. Patient B.A. (a 32-yr-old man when entering the chromatography protocol in 1987) underwent total thyroidectomy in February 1982 (papillary cancer). Apparently successful radiometabolic ablation of remnants was carried out with 100 mCi of ^{131}I. In 1982–1984, three therapeutic doses (150 mCi each) were given because of metastatic cervical lymph nodes. Surgery became necessary to remove additional metastatic lymph nodes in 1985 (cervical) and 1986 (mediastinal). (A) Iodine-131 WBS (5 mCi) performed on August 21, 1987 was negative and serum hTg was <3 ng/ml, notwithstanding serum TSH = 240 μU/ml. (B) At this time, chromatography was positive for endogenously radiolabeled thyroid hormones (~0.0013% of dose/liter plasma), and MRI revealed an ovoidal left retroclavicular mass (1.2 cm in diameter). A therapeutic ^{131}I dose (150 mCi) was administered. (C) This scan obtained five days later was positive, with ^{131}I uptake corresponding to the mass detected by MRI. (D) Chromatography was negative both 12 mo after this last therapeutic dose and in later dates (last follow-up: February 1992, profile shown here). MRI demonstrated progressive reduction of the retroclavicular mass until complete disappearance in June 1990.

TABLE 2
Diagnostic Performance Values (Bayesian Approach) of the Three Tests Considered Either Singly or in Various Combinations (Values Given as Percentages)

	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
^{131}I WBS	90.6	95.1	92.8	95.1	90.6
Serum hTg	60.9	100.0	80.0	100.0	70.9
Chromatography	98.4	100.0	99.2	100.0	98.4
^{131}I WBS + Serum hTg	92.2	95.1	93.6	95.2	92.1
^{131}I WBS + Chromatography	98.4	95.1	96.8	95.5	98.3
Chromatography + Serum hTg	100.0	100.0	100.0	100.0	100.0
^{131}I WBS + Serum hTg + Chromatography	100.0	95.1	97.6	95.5	100.0

using an in vitro technique in addition to external counting by evaluating organically-bound radioiodide recovered in plasma (%dose/liter) after ^{131}I administration to estimate the amount of residual functioning tissue (*14*). This promising method was not further explored because of low analytical specificity. The chromatographic method proposed here completely separates in vivo labeled iodothyronines from other contaminating radioactive species (Fig. 1), thus eliminating any false-positive results. Furthermore, in the absence of functioning thyroid tissue, no synthesis of radioiodothyronines can take place (Fig. 2). These observations demonstrate the intrinsic specificity of this chromatographic system.

The high intrinsic sensitivity and accuracy of chromatography are further enhanced by direct radioactivity measurement and by coelution in a single peak of endogenously labeled T_3-T_4. Whereas the hTg assay is an indirect, immunologic-based evaluation with lower analytical accuracy, as shown by wide intra-laboratory (see "Methods") and even wider inter-laboratory variability [>90% (*15*)]. This raises problems in patient follow-up. Also, fluctuating threshold levels in the literature make intergroup comparisons difficult. Furthermore, the hTg assay is affected by intrinsic biological variables, such as anti-hTg autoantibodies and antigenic heterogeneity of hTg produced by tumors (*16*). We observed a significantly higher sensitivity of ^{131}I WBS performed with the gamma camera versus serum hTg (90.6% versus 60.9%). A similar low sensitivity for the hTg assay has recently been reported in a study where patients were frequently correctly classified based on a positive scan after a therapeutic ^{131}I dose (*8*).

This chromatographic procedure exhibits very high diagnostic sensitivity (even higher than ^{131}I WBS and serum hTg, 98.4% versus 92.2%) and the highest possible specificity (100%). These excellent diagnostic parameters are achieved after administering a conventional diagnostic dose of ^{131}I (2–5 mCi), thus eliminating the high radiation burden received after a therapeutic ^{131}I dose (*8*). Chromatography also strengthens the diagnostic performances of ^{131}I WBS and hTg. A combination of the two tests for highest specificity (serum hTg and chromatography) resulted in a 100% value for sensitivity, specificity, accuracy and positive and negative predictive values. The occasional false-negative chromatography result (one patient, in whom ^{131}I WBS was also negative out of 64 with residual disease) can be explained by the fact that in some cases tumor cells still synthesize hTg but do not trap iodide (*6*).

Chromatography correctly classified 23 of the 24 patients with discordant ^{131}I WBS and serum hTg results (Table 1), thus exhibiting a very high diagnostic value (95.8%) when the decision regarding ^{131}I treatment is most critical. Furthermore, positive chromatography correctly classified five patients with negative ^{131}I WBS and undetectable hTg (Table 1, Fig. 3). In fact, chromatography evaluates integral hormonal secretion in the serum of all functioning thyroid-derived foci however disperse in the body that are too small to be detected by ^{131}I WBS.

Given its 100% specificity and 98.4% sensitivity, the chromatographic procedure described here may be useful in the follow-up of patients with DTC, especially when the two conventional tests give discordant or dubious results (e.g., patients with negative serum hTg but positive ^{131}I WBS). This discordance emphasizes therapeutic dilemma, whether or not to administer a therapeutic dose of ^{131}I that might represent the turning point between curing the disease or seeing it progress.

Negative results for all three tests would rule out the presence of functioning thyroid tissue, whereas in those patients with discordant or inconclusive ^{131}I WBS and serum hTg, positive chromatography indicates the presence of disease. Finally, a positive chromatographic test associated with negative ^{131}I WBS and hTg would identify those patients requiring close monitoring with more frequent follow-up examinations.

In conclusion, this study demonstrates the potential clinical usefulness of a chromatographic technique optimized for identification of radioiodinated thyroid hormones neogenerated in vivo after the administration of a diagnostic dose of ^{131}I. The procedure is characterized by a 98.4% sensitivity, 100% specificity, low cost, rapidity and simplicity of operation. It could be incorporated into the clinical routine because it provides evidence for the persistence or absence of functioning thyroid tissue in patients treated for DTC. Furthermore, in vivo neogeneration of labeled thyroid hormones after diagnostic ^{131}I administration proves that residual tissue retains not only iodide-trapping capabilities, but also the metabolic pathways of iodothyronines synthesis, an important factor for predicting a favorable effect of subsequent radiometabolic treatment. Finally, the

new test could provide useful information when evaluating the impact of complete radiometabolic ablation on the long-term outcome of patients with DTC.

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