Measurement Method for Radioactive Thyroxine, Triiodothyronine, Iodide, and Iodoprotein in Samples with Low Activity

Marguerite T. Hays, Robert A. McGuire, Johannes Th. Hoogeveen, and Kim N. Diezeraad

VA Medical Centers, Buffalo, New York, and Washington, DC, VA Central Office, Washington, DC, and State University of New York at Buffalo, New York

A method is described that incorporates resin extraction and thin layer chromatography to isolate and separate radioiodinated thyroxine (T_4), triiodothyronine (T_3), iodoprotein, and iodide in samples of human plasma up to 3 ml. Tracer studies using this method showed that reverse T_3 and 3′,5′ diiodothyronine (T_2), as well as T_4, were detected in the "T_4 fraction," and that 3-3′ T_2 and 3′ monoiodothyronine, as well as T_3, were detected in the "T_3 fraction." Monoiodotyrosine and diiodotyrosine (DIT) migrated more slowly than did T_4 on the chromatogram, and a large amount of DIT was in the unextracted "iodoprotein fraction."

Kinetic studies in 14 normal subjects given intravenous commercial [^{125}I]T_3 (T_3*) and [^{131}I]T_4 (T_4*), confirmed the quantitative importance of an iodoprotein in later samples after T_3* administration, and its presence after T_4*. T_4* contamination of commercial T_3* also became quantitatively important. On the other hand, despite confirmation of in vivo conversion of T_4* to T_3*, T_3* contributed little quantitatively to the total concentration of radioactivity present even late after T_4* injection, due to the more rapid turnover and greater distribution volume of T_3*.

J Nucl Med 21: 225–232, 1980

Studies with labeled thyroxine (T_4) and triiodothyronine (T_3) in human serum have been of great value in the understanding of the kinetics of these hormones. In addition to the value of such studies as tools in elucidating the normal and abnormal physiology of thyroid hormones, they have also been useful in the diagnosis of thyroid disorders. The usual studies done with labeled T_3 or T_4 follow the distribution of the tracer, with little or no attempt to identify the chemical state of the iodinated substances. Relatively simple techniques are available for separating the iodide fraction, and these are often used. In recent years, however, it has been clearly demonstrated that T_4, in its metabolism, is converted in significant amounts to T_3. It has also been shown that the metabolism of T_3 involves formation of significant amounts of an iodoprotein as well as 3-3′ diiodothyronine (T_2), 3′ monoiiodothyrosine (T_1), and iodide (I). Ideally, kinetic studies of T_4 and T_3 metabolism should include quantitative assessment of these degradation products. We have found that the usual methods for separation of these compounds are not suitable for accurate measurement of the small concentration of label found in human subjects undergoing T_4 and T_3 distribution studies with radioactive thyroid hormones. For this reason, we have developed a technique for concentrating the labeled materials from such samples, separating out some of the components of interest. This method is presented here in some detail, with brief comments about its development. We also present preliminary results, in which the method was applied to plasma samples from normal subjects undergoing T_4 and T_3 distribution studies using labeled hormones.

Received May 17, 1979; revision accepted Sept. 21, 1979.

For reprints contact: Marguerite T. Hays, M.D. (15), Research and Development, VA Central Office, 810 Vermont Ave., NW, Washington, DC 20420.

MATERIALS AND METHODS

Chromatographic method. In triplicate, 1.4 ml 1% 8-anilino-1-naphthalene sulfonic acid Mg salt (A.N.S.)

are added to 3-ml heparinized plasma samples in 13-ml well-counter tubes. The mixture is shaken for 30 min. It is then diluted to a total volume of 12 ml with distilled water (with pH adjusted to 9.2 using 1 *N* NaOH) and rotated for 1 hr at 4°C. Then 1.2 g Dowex AG 1 × 2 resin is added and the pH is adjusted to 8.1–8.4 with 1 *N* NaOH. The mixture is then rotated overnight at 4°C. The next day it is centrifuged at 3500 rpm for 10 min, and the supernatant is collected in a 50-ml polypropylene wide-mouthed tube. The precipitated resin is then washed three times with 10 ml distilled water, centrifuging to terminate each wash. The combined supernatant and washings are then concentrated by freeze-drying, followed by solution in 2 ml distilled water. This concentrate is counted as the "iodoprotein" fraction.

The Dowex AG 1 × 2 resin precipitate is eluted for 1 hr with 10 ml 9% methanol containing sufficient *N* HCl to adjust the pH to 1.4. This is followed by two similar elutions of 45 min each. During each elution the tube is rotated at room temperature. Each elution is terminated by centrifuging for 10 min at 3500 rpm at 4°C. The eluted resin is counted as the "I" fraction.

The collected eluates are concentrated by evaporation at 50°C until the amount of liquid is reduced to 0.1 ml or less. The entire concentrate is then applied to a thin-layer chromatography film precoated with PEI cellulose† that has been predried in an oven at 60–75°C for at least 30 min before the sample is applied. The site of application is 2 cm from the bottom of the film and extends 16 cm across the plate between points 2 cm from the left and 2 cm from the right of the plate margin. A line is drawn along the application site with a pencil, and the sample is applied as dots, using a Pasteur pipette.

Thin layer chromatography is performed horizontally in enclosed tanks. The solvent used contains 100 ml tertiary butanol, 28 ml 2.8 *N* ammonia, and 24 ml chloroform. At the end of the chromatography (generally after 4–5 hr) the chromatograms are air-dried and cut into 5-mm strips paralleling the line of application, starting 1 cm from the bottom and extending to the solvent front. These strips are counted individually in order to determine the location of the T_4 and T_3 peaks. Four fractions from the plate are identified:

1. "Post-T_4 fraction" (contains monoiodotyrosine (MIT) and diiodotyrosine (DIT)), generally in strips 1–6;
2. "T_4 fraction" (contains T_4, reverse T_3 (rT_3), 3′-5′T_2, and I), generally in strips 7–13;
3. "T_3 fraction" (contains T_3, 3′-5′T_2 and 3′T_1), generally in strips 14–23; and
4. "Solvent front," generally in strips 24–32.

Finally, the strips belonging to each of the four identified fractions from the chromatogram are pooled and counted together, in order to improve the counting statistics for the total count in each fraction.

Validation studies. Initial exploratory studies, performed on pooled serum or plasma obtained from the clinical laboratories, included comparisons of serum and plasma, comparisons of 3-ml samples with smaller plasma volumes, and validation studies with added radiopharmaceuticals. These comparisons were later repeated on multiple individual blood samples obtained from volunteer donors.

Radiopharmaceuticals. The commercial T_3* and T_4* used in these studies were "off the shelf" radiopharmaceuticals obtained from several commercial firms during 1974–76, and had been labeled on the alcoholic ring of the thyronine moiety. Final dilution used for the predialysis and dose preparation was 25 μCi/ml, containing less than 1 μg/ml of T_4 or T_3. Radioiodinated serum albumin (RISA*), I*, MIT*, and DIT* were also obtained commercially. The rT_3*, 3-3′T_2*, 3′-5′T_2*, and 3′T_1* used in the validation studies were kindly donated by Dr. Kenneth Burman of Walter Reed Army Hospital. RISA*, T_3*, and T_4* were predialyzed before use to minimize I* contamination. The other compounds were used as received.

Studies in human subjects. Kinetic studies of commercial T_4* and T_3* were performed on 14 normal paid volunteer subjects (nine men and five women), ages 21–29 yr, with informed written consent. Subjects were euthyroid clinically and chemically. Each received a combined intravenous dose of 25 μCi each of commercially purchased [^{125}I]T_3 (commercial T_3*) and [^{131}I]T_4 (commercial T_4*). Radiopharmaceuticals were prepared in a 1% solution of human serum albumin in saline and were predialyzed as previously described (*1*). Thyroidal uptake of radioiodide was inhibited by potassium iodide administration (10 drops saturated solution 1 hr before radiopharmaceutical administration, followed by 1 drop every 12 hr for 5 days).

A control plasma sample was enriched with an aliquot of the injected material and then processed as were the plasma samples obtained after injection of the radiopharmaceuticals. Distribution percentage of labeled materials in this preinjection enriched sample were entered into the analysis as the "zero time" values.

Subsequent to radionuclide injection, plasma samples were obtained for overall kinetics at multiple time points, usually at 2, 5, 10, 20, 30, 40, and 50 min, and at 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, 48, 72, 96, and 120 hr. Full chromatographic analyses were done on the 10-min, 4-, 24-, 48-, 72-, 96-, and 120-hr samples. For overall kinetics studies 10 ml blood (4 ml plasma) were collected. When chromatographic analysis was to be done, 30 ml blood (10 ml plasma) were collected.

Iodine-125 and I-131 were counted in a two-channel gamma spectrometer. Iodine-131 crossover into the I-125 channel, which averaged 13%, was measured separately with each group of counts from the channel ratio when a sample of I-131 without I-125 was counted. This crossover was corrected for in all I-125 counts, using

the ratio determined for the particular group of counts. Counting standards were dilutions of the injected materials.

Nomenclature. In this paper, when the *unmodified* term (T_3, T_4, I, indicating iodide, or iodoprotein) is used, reference is to the pure compound in question. The modifier "*commercial*" (e.g., commercial T_3) is used to indicate materials purchased from a commercial supplier, ordinarily containing some contaminants. The term "*fraction*" (e.g., T_3 fraction) is used to indicate the output fraction from the separation technique that contains most of the compound in question. An *asterisk* added to any of these (e.g. T_3*, commercial T_3*, T_4* fraction) indicates that the material referred to is radioactively labeled.

RESULTS

Development and validation of the method. *Extraction of compounds from test substances.* Multiple pilot studies performed with serum and plasma samples enriched with I*, RISA*, T_3* and/or T_4* demonstrated that the radioiodinated substances were extracted equally well from serum or plasma by this system, and that efficiency of resin adsorption and methanol extraction was not altered by variations in serum or plasma volume up to 3 ml.

Thin-layer chromatography technique. In searching for a clean separation of T_3 and T_4, several thin-layer systems were studied, together with various forms of the tertiary butanol/ammonium chloride/chloroform solvent system.

With regard to the solvent system, comparative studies on Eastman Chromatogram Sheets 6064 Cellulose showed that an ammonium hydroxide content of 28 ml (pH 11.52) gave a separation clearly superior to 20 ml (pH 11.37) or 10 ml (pH 11.08). Chloroform contents of 24, 32, and 40 ml gave equally good separations. Tertiary butanol content of 100 ml gave cleaner separation of T_3 and T_4 than did 110, 120, or 132 ml.

Using the final solvent system arrived at after these studies, comparative chromatograms were performed on the following thin-layer cellulose sheets:

1. Eastman Chromatogram Sheet 6064 Cellulose without fluorescent indicator; and on polygram sheets from Macherey, Nagel & Co.[‡] coated with:
2. MN cellulose 300,
3. MN cellulose 400 (microcrystalline),
4. MN acetylated cellulose 300, 10%,
5. MN carboxymethyl cellulose 300,
6. MN DEAE cellulose 300,
7. MN Ecteola cellulose 300,
8. MN PEI cellulose 300.

Cellulose 6064, MN acetylated cellulose 300, and MN cellulose 300 gave qualitatively similar motility of both T_3 and T_4, with some overlap between the two fractions. MN cellulose 400 showed less motility of both T_3 and T_4 than the three previously mentioned coatings, but spots were denser.

DEAE cellulose and Ecteola cellulose, both anion exchangers, showed little motility of T_4, most of which remained at the origin, whereas T_3 was mobile but with some tailing. Carboxymethyl cellulose 300 absorbed both T_3 and T_4 poorly, with wide streaking and merging of T_3 with the solvent front.

The best separation obtained was with PEI cellulose 300, which gave good separation of T_3 and T_4, both spaced well above the origin and below the solvent front. Concentrations of T_3 and T_4 were dense in their final locations after chromatography.

Distribution of radioiodine containing compounds. Separation of various radioiodinated substances, which had been added in tracer amounts to 3 ml normal human plasma and studied in this system, is shown in Figs. 1-3. Each substance studied was predominantly distributed in one of the fractions, with the exception of DIT*, which is distributed between the "iodoprotein fraction" and the "post-T_4 fraction." The MIT* studied simultaneously with DIT* distributed primarily in the "post-T_4 fraction." Except for this DIT* distribution, only RISA* showed major distribution in the "iodoprotein fraction," the fraction not absorbed onto resin. Similarly, of the substances tested, only I* distributed predominately in

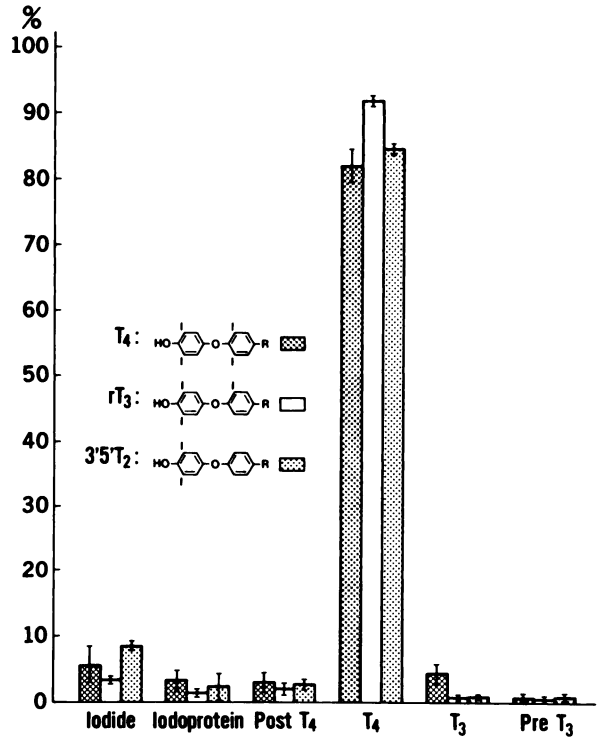

FIG. 1. Distribution of T_4, rT_3, and 3'-5'T_2, all compounds with two iodines on alcoholic ring, when plasma samples enriched with tracers are separated by method described in this paper. Labels along abscissa refer to "fractions" resulting from separation technique (mean ± s.d.).

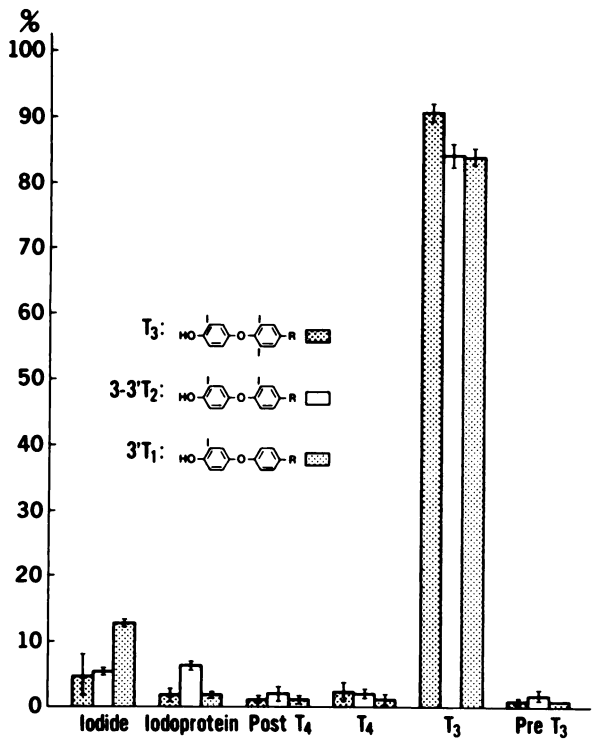

FIG. 2. Distribution patterns for T_3, 3-3'T_2, and 3'T_1, all compounds with a single iodine on alcoholic ring (mean ± s.d.). Labels on abscissa refer to separation fraction.

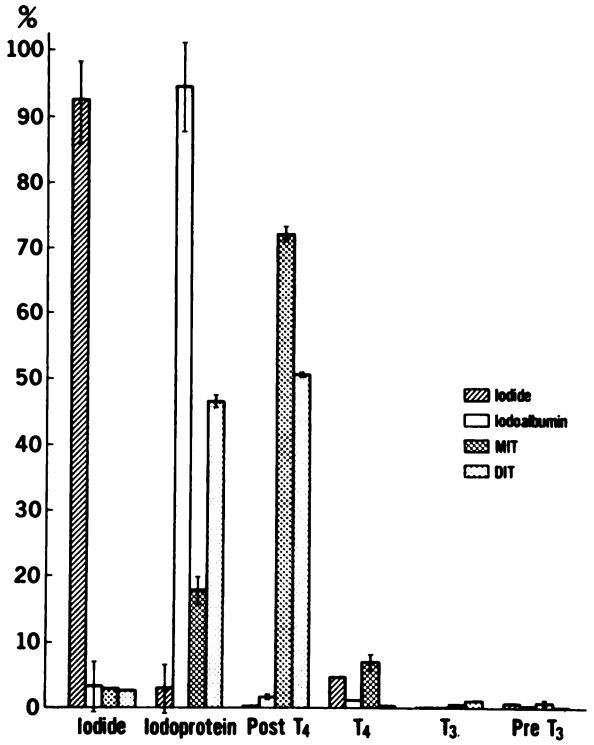

FIG. 3. Distribution patterns for iodide, iodoalbumin, MIT, and DIT (mean ± s.d.). Labels on abscissa refer to separation fraction.

the Dowex resin residual fraction (the "I fraction").

All of the iodinated thyronine substances tested were distributed predominantly in the "T_4 fraction" or the "T_3 fraction." Migration characteristics determining into which of these fractions a compound migrates appear to be associated with whether one or two iodines is present in the alcoholic (labeled) ring of the thyronine molecule (Figs. 1 and 2).

Even though the substances of interest are predominantly distributed in single fractions, cross contamination must be corrected for in order to optimize the amount of meaningful information. This was done by entering the mean proportional crossover information obtained in the validation study for each substance (Figs. 1-3) into an equation for the total radioactivity for each fraction. For example if:

a = proportion of I* measured in T_3* fraction,

b = proportion of iodoprotein* measured in T_3* fraction,

c = proportion of T_4* measured in T_3* fraction;

and

d = proportion of the I* fraction attributable to T_3*,

e = proportion of the iodoprotein* fraction attributable to T_3*,

f = proportion of the T_4* fraction attributable to T_3*;

then:

$$T_3^* = (T_3^* \text{ fraction}) - [a(I^*) + b(\text{iodoprotein}^*) + c(T_4^*)] + [d(I^* \text{fraction}) + e(\text{iodoprotein}^* \text{ fraction}) + f(T_4^* \text{ fraction})].$$

Similar equations were written to derive I*, iodoprotein*, and T_4*, each with its own proportionality constants incorporated. These four equations were then solved for the four unknowns (T_3*, T_4*, I*, and iodoprotein*) using a digital computer. In the initial solution of any subject's data set, the proportionality constants used were those observed experimentally from that subject's control ("zero time") plasma sample enriched with the injected radiopharmaceutical. The T_3*, T_4*, I* or iodoprotein* fractions used in the solution were those determined experimentally for each sample.

The proportionality constants were later adjusted for each individual subject, taking advantage of the availability of multiple samples, and using a mathematical model. Details of this model (a multicompartmental model with subsystems for T_4, T_3, iodoprotein, and I, as well as rT_3 and 3-3'T_2), will be presented elsewhere. In the present study it was used only as a technique for correcting the experimentally measured fractions for crossover.

Tables 1 and 2 present, as percentages, the observed and calculated proportionality constants for each experimental subject. The observed values are the actual

TABLE 1. FRACTIONS OTHER THAN THE "T_4 FRACTION" MEASURED AFTER [^{131}I]T_4 DOSE: CONTAMINATION AND METHODOLOGIC CROSSOVER

	T_3* Fraction (%)						I* Fraction (%)						Iodoprotein* fraction (%)		Other fractions (%)	
	Calculated						Calculated								Observed	
Subject	Initial contamination of T_4* by T_3*		Crossover		Total calculated	Observed	Initial contamination of T_4* by I*		Crossover		Total calculated	Observed	Calculated crossover	Observed	Pre-T_3	Post-T_4
MC	4.5	+	0.8	=	5.3	0.1	7.0	+	3.0	=	10.0	8.2	3.6	3.3	0.0	5.4
BO	6.0	+	1.5	=	7.5	5.3	4.0	+	5.0	=	9.0	7.5	3.0	3.4	1.0	7.3
WE	6.0	+	0.0	=	6.0	5.7	7.0	+	2.0	=	9.0	6.6	2.8	2.7	0.3	3.8
RO	5.5	+	0.4	=	5.9	5.7	3.0	+	3.5	=	6.5	5.0	4.0	5.2	0.4	2.9
TO	6.0	+	1.0	=	7.0	7.0	4.0	+	2.8	=	6.8	6.7	8.0	5.9	1.6	2.4
GA	5.0	+	0.0	=	5.0	5.2	4.0	+	1.6	=	5.6	3.6	5.0	3.2	1.3	4.6
ZO	5.0	+	0.4	=	5.4	5.8	7.0	+	6.0	=	13.0	10.6	6.0	3.2	1.2	2.1
BA	7.0	+	0.0	=	7.0	5.5	6.0	+	4.0	=	10.0	10.0	9.0	6.8	1.5	2.2
TS	4.0	+	0.0	=	4.0	4.2	5.0	+	4.0	=	9.0	7.2	3.0	1.7	0.2	2.0
BL	6.0	+	0.0	=	6.0	4.3	5.0	+	1.5	=	6.5	5.3	3.6	4.7	1.4	4.3
HO	5.0	+	0.2	=	5.2	4.8	3.0	+	7.0	=	10.0	9.8	5.0	4.4	1.0	3.3
EN	5.0	+	0.0	=	5.0	3.5	3.3	+	1.7	=	5.0	3.2	4.0	3.8	0.5	2.8
SO	5.0	+	0.5	=	5.5	5.2	10.0	+	4.0	=	14.0	9.8	4.0	6.6	0.5	5.9
SI	6.0	+	0.0	=	6.0	4.6	9.5	+	3.6	=	13.1	8.6	6.0	3.5	0.3	3.7
Mean	5.4		0.3		5.7	4.8	5.6		3.6		9.1	7.3	4.8	4.2	1.4	3.8
s.d.	0.8		0.5		0.9	1.6	2.3		1.7		2.9	2.4	1.9	1.5	2.2	1.6
P that observed differs from calculated (t-test for paired samples)						0.95						0.999		NS		

TABLE 2. FRACTIONS OTHER THAN THE "T_3 FRACTION" MEASURED AFTER [^{125}I]T_3 DOSE: CONTAMINATION AND METHODOLOGIC CROSSOVER

	T_4* Fraction (%)						I* Fraction (%)						Iodoprotein* fraction (%)		Other fractions (%)	
	Calculated						Calculated								Observed	
Subject	Initial contamination of T_3* by T_4*		Crossover		Total calculated	Observed	Initial contamination of T_3* by I*		Crossover		Total calculated	Observed	Calculated crossover	Observed	Pre-T_3	Post-T_4
MC	0.0	+	0.5	=	0.5	1.7	1.0	+	4.0	=	5.0	4.4	1.2	1.3	0.0	0.0
BO	0.2	+	1.5	=	1.7	1.9	2.0	+	7.0	=	9.0	8.2	1.5	1.6	1.0	1.6
WE	0.1	+	1.4	=	1.5	2.5	0.5	+	1.8	=	2.3	2.3	1.1	1.3	0.2	0.6
RO	0.5	+	1.0	=	1.5	1.2	2.5	+	0.8	=	3.3	3.5	1.5	1.8	0.6	0.9
TO	0.4	+	1.0	=	1.4	1.4	1.0	+	3.4	=	4.4	3.9	1.9	2.0	1.2	0.5
GA	0.2	+	1.2	=	1.4	1.8	0.5	+	2.0	=	2.5	2.4	1.4	1.2	1.5	1.5
ZO	0.5	+	0.2	=	0.7	1.1	2.0	+	8.0	=	10.0	8.6	1.0	1.0	0.6	0.8
BA	0.2	+	1.2	=	1.4	1.6	3.0	+	2.0	=	5.0	6.7	1.0	1.3	0.7	0.6
TS	0.2	+	1.0	=	1.2	1.2	4.0	+	4.0	=	8.0	6.8	1.0	1.0	0.4	0.7
BL	0.2	+	1.5	=	1.7	†	3.0	+	0.7	=	3.7	†	1.0	†	†	†
HO	1.0	+	1.8	=	2.8	3.0	4.0	+	7.0	=	11.0	7.8	1.7	1.7	0.9	0.8
EN	0.0	+	1.5	=	1.5	1.7	0.8	+	2.0	=	2.8	2.5	1.6	1.6	0.6	0.7
SO	0.7	+	1.0	=	1.7	2.6	6.0	+	4.0	=	10.0	10.0	0.9	1.7	0.9	1.9
SI	0.3	+	1.5	=	1.8	1.8	3.5	+	6.0	=	9.5	8.8	1.5	2.0	0.5	1.2
Mean	0.3		1.2		1.5	1.8	2.4		3.8		6.2	5.8	1.3	1.5	0.7	0.9
s.d.	0.3		0.4		0.5	0.6	1.6		2.4		3.2	2.8	0.3	0.3	0.4	0.5
P that observed differs from calculated (t-test for paired samples)						0.98						NS		0.96		

† *T_3 control measurements were omitted on Subject BL.

percentage of the label (in commercial T_3* or commercial T_4*) measured in the other fractions in chromatography of the subject's "zero time" plasma sample, enriched with the injected radiopharmaceuticals. These observed values are attributable to methodologic crossover and also to contamination—that is, to the actual presence of the other compound in the radiopharmaceutical (as when commercial T_3* contains some T_4*). Because of the differing in vivo kinetics of the different compounds, it was possible to use the model to separate out this initial chemical contamination from methodologic crossover. After correction for methodologic crossover, commercial T_4* was found to have T_3* and I* contamination but none from iodoprotein* or the other fractions. Commercial T_3* had T_4* and I* contamination. These separate determinations of contamination and crossover are presented as the "calculated" values in the first two columns of each data set. Their sum, in theory, should equal the observed proportionality constant derived from the "zero time" study. Occasional discrepancies can be attributed to the fact that the model calculation draws on the entire series of chromatographic analyses for a subject, whereas the observed data derive from a single sample. However, some systematic variants

were noted. In particular, the amount of T_4* seen as I* is underestimated in the enriched control sample. It could well be that, in the chromatography of the control sample, some of the I* is measured in the "post-T_4" chromatographic fraction, as I travels behind T_4 on the PEI sheets, though generally within the T_4 segment. T_4* seen as T_3* is also underestimated in the control samples, while direct measurement overestimates the combined crossover and contamination of commercial T_3* by T_4* and by iodoprotein*. While statistically significant, these differences are quantitatively small.

In order to present the observed data for T_4*, T_3*, iodoprotein*, and iodide*, corrected for methodologic crossover, the final crossover factors for each subject were used as the proportionality constants in the series of equations presented above. That is, each observed data point from a given fraction was corrected by subtracting the crossover into that fraction from measured amounts of other compounds, and by adding its contribution to other fractions. The resulting values for each substance still contain the initial contaminations in the original injected commercial compound as well as appearance of new compounds as a result of metabolism of the radiopharmaceutical. In particular, at the later times after T_3* injection, T_4* accounts for an increasing percentage of the radioactivity. The model shows this to be due to T_4* in the injected material, which disappears more slowly than does T_3*. On the other hand, iodoalbumin*, which also becomes increasingly prominent with time after commercial T_3* administration, is the result of T_3* metabolism.

Figures 4, 5, and 6 present the T_4*, T_3*, iodide*, and iodoprotein* data (corrected for crossover from and into other compounds) after injection of commercial T_4* and of commercial T_3*, in three normal studies representative of the patterns seen in the 14 subjects studied to date.

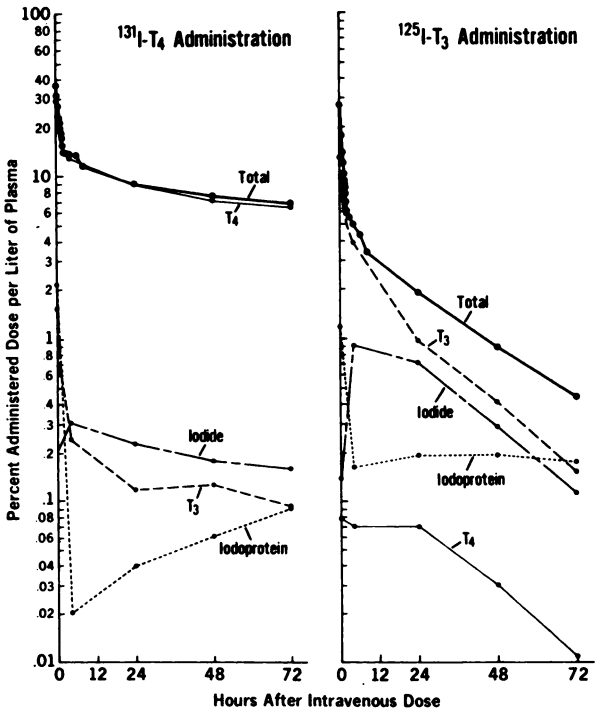

FIG. 4. Disappearance curves for control subject EN, using values corrected for methodologic crossover (A) After injection of [^{131}I]T_4, (B) After injection of [^{125}I]T_3.

DISCUSSION

Tracer studies continue to play an important role in understanding hormone disposition and metabolism. With the maturation of radioimmunoassay and related techniques, stable hormone assay is now feasible for most compounds, and studies of unlabeled hormones have supplanted tracer studies in some cases. Such studies, however, require correction for endogenous levels if pharmacologic doses are used, and these doses, if metabolically active, will alter the steady-state condition. Tracer studies remain often the most practical approach to study of a physiologic problem.

Tracer studies in human subjects are limited by considerations of radiation safety. While no absolute "safe" level of radioiodide has been established, we have limited doses in single experimental situations to 25 μCi each of [^{125}I]T_3 and [^{131}I]T_4 and have limited the number of sessions for any subject to two. When thyroidal recirculation of radioiodide is blocked, the estimated whole-body radiation to a normal human subject from a session with these doses is about 0.06 rads (*2*). In the absence of block to recirculation, the thyroidal dose is almost 15 rads (*3*). Because of the need to keep tracer dosage to a practical minimum, fairly large volumes of plasma must be studied if count rates for the fractions are to retain an acceptable level a number of days after tracer administration. The method presented here was developed in response to that need.

In early studies we used a column method, absorbing iodide and the iodotyrosines and iodothyronines onto Dowex AG 1 × 2 resin, following the method of Pileggi, et al. (*4*). This method required more technician time than the batch method presented here and it proved to be no more efficient in extraction of iodothyronines, especially when the serum or plasma is pretreated with ANS. The batch method used here is also less volume-sensitive than is the column method. We also studied column methods for selective elution of the iodotyrosine and iodothyronine fractions. However, the method presented here, using methanol extraction and the thin-layer chromatography, proved more reliable and was eventually adopted for the definitive studies.

In our developmental studies, we studied nonlabeled T_3 and T_4 and compared results with those using radioiodinated hormones. Chromatographic characteristics on PEI cellulose were identical.

This system for separation of radioiodinated com-

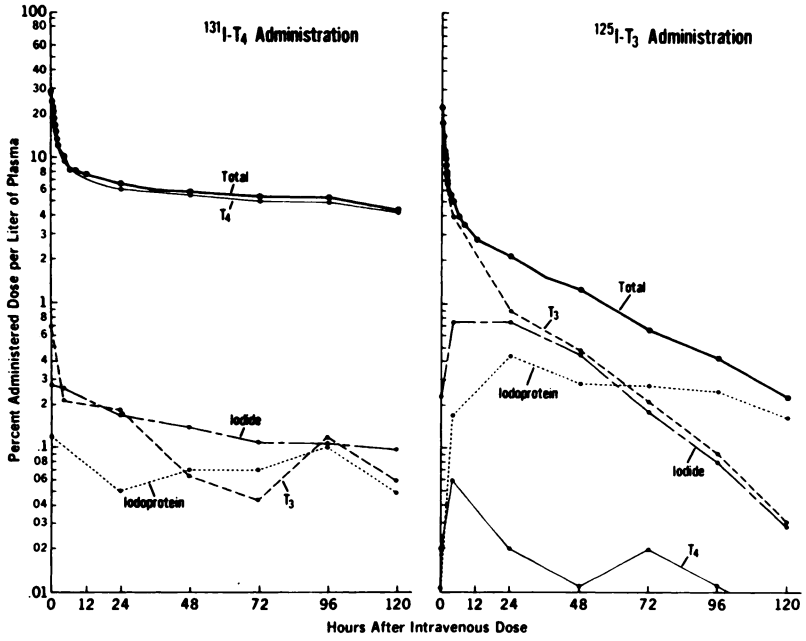

FIG. 5. Disappearance curves for control subject BL.

pounds has a number of disadvantages, which future developments may address. Because of the occasional appearance of unexplained aberrant results, we found it necessary to do triplicate studies. This means that at least 9 ml of plasma are needed for each data point. To assure adequate plasma volumes, blood samples of 30 ml were usually obtained. Further improvement in the ratio of counting efficiency to background count would permit reduction of either plasma volume or radiopharmaceutical dose.

Other methods for separation of radioiodinated compounds in human subjects have been reported recently, and a number of practical approaches to removal of iodide and of iodoprotein (without distinguishing T_3* from T_4* in the residuum) are now available (*5,6*). In addition, Inada and coworkers (*7*) have reported studies in which T_3* and T_4* of extracted serum were also separated, using paper chromatography. In their paper, these authors do not mention the dose of tracer necessary. In our hands, the paper-chromatography techniques were insufficiently precise for quantitative kinetic studies after the tracer doses we used. Rudolf and coworkers (*8*) recently presented a Sephadex technique that separates T_3* from T_4* and also identifies a fraction (apparently pooled 3-3′T_2*, 3′-5′T_2*, and 3′T_1*) separate from T_3* or rT_3*. This technique however, requires

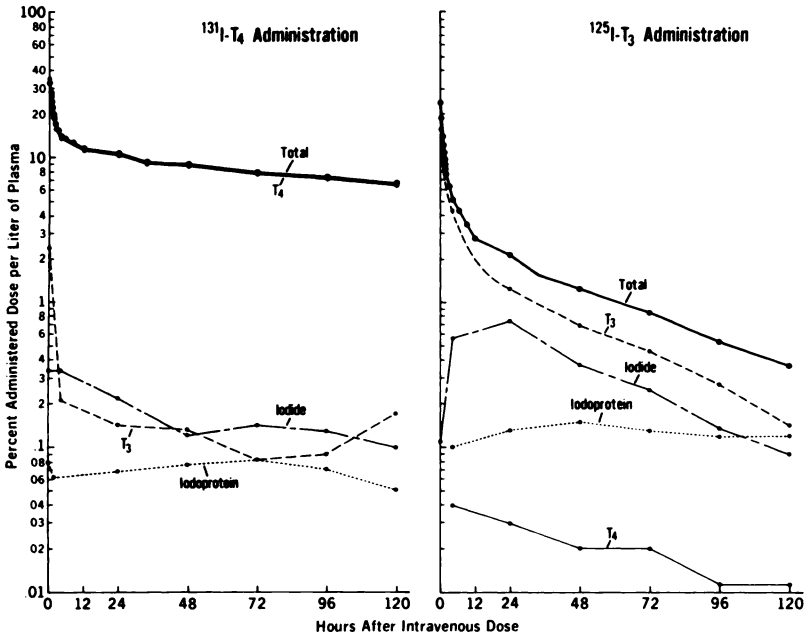

FIG. 6. Disappearance curves for control subject WE.

administration of 100 μCi of [^{125}I]T_3 to normal subjects (four times that used in this study) and 5–10 ml of serum for each determination. While it offers more chemical information than that available in the present study, the Sephadex method appears to be very limited in applicability to repeated kinetic studies because of radiation dose as well as the laboriousness of the technique. None of these authors appears to have corrected the data for methodologic crossover.

The present method is also rather laborious, especially in the methanol extraction and concentration phase, and in the detection of the major peaks on the chromatograms. Because of the low count rates in the in vivo samples, we found that there was no practical alternative to separate counting of small strips, identifying the segments on the chromatogram from these counts, and then pooling the strips for each segment to achieve a statistically valid count rate. This process prolonged the analysis, and careful attention to timing was necessary to minimize loss of I-131 counts from physical decay. Nevertheless, the method has now been applied successfully to repeated kinetic studies in normal subjects and in patients with thyroid disease.

The degree of chemical purity of commercial [^{125}I]T_3 and [^{131}I]T_4, reported in the first column in each section of Tables 1 and 2, is comparable to that which we measured when the newly received shipments were applied directly to the thin layer chromatograph. Whereas T_4* present in [^{125}I]T_3 did not exceed 1% in any of the commercial samples, T_3* contamination of [^{131}I]T_4 ranged from 4 to 7% of the initial radioactivity. The degree of I* contamination remaining in these injected substances is somewhat surprising, as the radiopharmaceuticals had been predialyzed to remove iodide. In the model solution, all iodoprotein* measured in fractions other than the "iodoprotein fraction" (Fig. 3) was due to methodologic crossover, so that apparently no significant contaminating iodoprotein* was present in the radiopharmaceuticals. In pilot studies of endogenously labeled serum from patients given I* for scanning, we found that, even after extensive dialysis, some I* remained. The possibility that I* is generated in vitro must be considered. In these studies, the endogenously labeled iodoprotein* fraction was reduced by predialysis, but chromatographic patterns of the iodothyronines were unchanged.

The data presented from normal subjects is preliminary in nature and will be expanded upon in future presentations of the mathematical model which, by utilizing information from all data points simultaneously, will minimize the impact of "noise" in the data resulting from low count rates. However, simple inspection of the data curves in Figs. 4–6 confirms the major impact of iodoprotein on the late phases of the T_3 curve, and the smaller, more variable impact of small amounts of T_4* contamination on administered T_3*. On the other hand, because T_3 is metabolised faster than is T_4, and because it distributes in a larger volume, T_3* present after T_4* administration has minimal effect on the total radioactivity curve after T_4* administration. The T_3* initially contaminating the T_4* dose disappears early and is supplanted by T_3* derived from T_4*. Important as this conversion of T_4* to T_3* is physiologically, it represents a quantitatively small portion of the distribution of I-131-labeled tracer after [^{131}I]T_4.

FOOTNOTES

† Cel 300 precoated TLC plates from Brinkman Instruments, Inc., Westbury, NY.

‡ Brinkman Instruments, Inc.

ACKNOWLEDGMENTS

Dr. Kenneth Burman kindly provided the samples of I-125-labeled rT_3, 3-3′T_2, 3′-5′T_2, and 5′T_1 used in the validation studies.

Mr. Dino Graziano provided excellent technical assistance.

This study was supported by VA Medical Research funds.

REFERENCES

1. McCall MS, Camp MF: A simple technique for prolonging the storage life of I^{131}-labeled proteins and polymers. *J Lab Clin Med* 58:772–775, 1961
2. Dillman LT: Radionuclide decay schemes and nuclear parameters for use in radiation-dose estimation MIRD Pamphlet No. 4, *J Nucl Med* 10: Suppl. No. 2, 1969
3. Berman M, Braverman LB, Burke J, et al: Summary of current radiation dose estimates to humans from ^{123}I, ^{124}I, ^{125}I, ^{126}I, ^{130}I, ^{131}I, and ^{132}I as sodium iodide. MIRD Dose Estimate Report No. 5, *J Nucl Med* 16: 857–860, 1975
4. Pileggi VJ, Lee ND, Golub OJ, et al: Determination of iodine compounds in serum I. Serum thyroxine in the presence of some iodine contaminants. *J Clin Endocrinol* 21: 1272–1279, 1961
5. Nicoloff JT, Low JC, Dussault JH, et al: Simultaneous measurement of thyroxine and triiodothyronine peripheral turnover kinetics in man. *J Clin Invest* 51: 473–483, 1972
6. Surks MI, Schadlow AR, Stock JM, et al: Determination of iodothyronine absorption and conversion of L-thyroxine (T_4) to L-triiodothyronine (T_3) using turnover rate techniques. *J Clin Invest* 52: 805–811, 1973
7. Inada M, Kasagi K, Kurata S, et al: Estimation of thyroxine and triiodothyronine distribution and of the conversion rate of thyroxine to triiodothyronine in man. *J Clin Invest* 55: 1337–1348, 1975
8. Rudolph M, Sakurada T, Fang SL, et al: Appearance of labeled metabolites in the serum of man after the administration of labeled thyroxine, triiodothyronine (T_3), and reverse triiodothyronine (rT_3). *J Clin Endocrinol* 46: 923–928, 1978