A METHOD FOR CHROMATOGRAPHIC SEPARATION OF IODO COMPOUNDS WITH A COMPUTER PROGRAM FOR DATA ANALYSIS

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There are many methods using chromatographic separation whereby iodinated organic compounds are partitioned from a complex mixture (*1*). To identify and quantitate these compounds by chemical means as they exist in biological solutions, relatively large amounts of solution must be used and usually a medium other than paper is required. While there are a number of methods for paper chromatographic partitioning and identification of the small amounts of stable monoidotyrosine (MIT), diiodotryrosine (DIT), 3, 5, 3′-triiodothyronine (T_3) and tetraiodothyronine (T_4) in blood, the chemical identification used in most of these methods lacks specificity (*1,2*). Overspraying of the ceric sulfate-arsenious acid-treated chromatogram with methylene blue and ammonia does not increase specificity (*3*). Therefore, the use of radioactivity for both identification of and quantitation of the small amounts of iodinated compounds found in biologic fluids has been widespread (*4–6*). A method has been devised using radioactivity as the means of identification and quantitation in the absence of iodide contamination. Particular concern has been directed in the present study to the problems of peak identification, separation and quantitation, and a technique for handling has been devised so that these chromatographic strips could be analyzed without prior bias as to what compounds would be present. Unfortunately, strip-scanning techniques using gamma-sensitive 2π detectors sacrifice sensitivity. Reasonable, statistically significant data cannot be obtained with tracer doses of much less than 500 μCi with such methods. Even 4π geometry is probably not as sensitive as the method described here, although specific counting data were not available for ready comparison. The combination of a miniature ion-exchange column (*7*) to concentrate the labeled organic iodinated compounds in the absence of inorganic iodide, a Packard Liquid Scintillation Counter Model 666 for detection and a computer program of data analysis has made it possible to identify and measure reliably with some estimate of error the small amounts of iodinated compounds appearing in plasma following a tracer dose of only 100 μCi of ^{131}I.

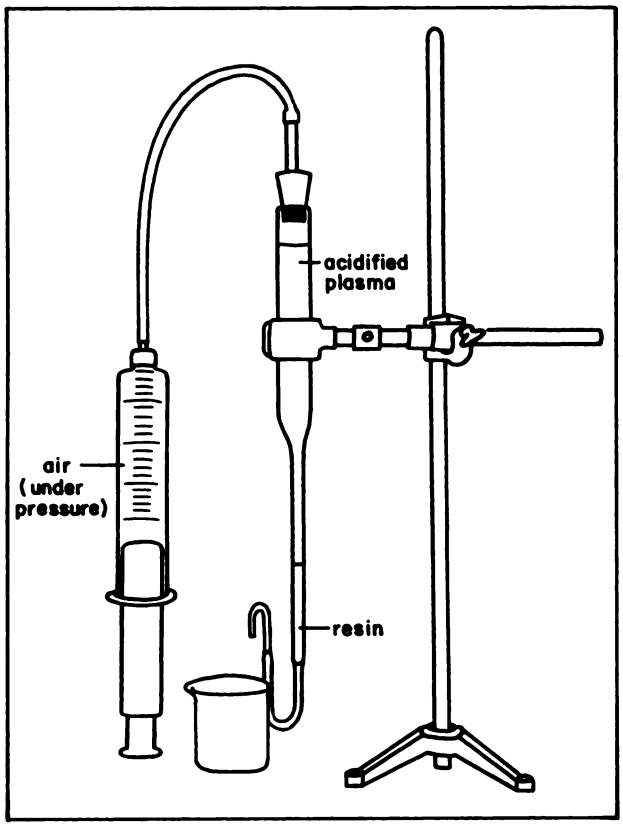

FIG. 1. Apparatus for column separation preparatory to chromatography.

METHOD

Preparation of resin and packing of column. The resin, analytical grade Dowex [50-W-x8(200-400 mesh)], is prepared by washing, first in 1 *N* sodium hydroxide followed by 2 *N* hydrochloric acid and then distilled water, until chloride free. The resin is stored on the shelf in an airtight container.

For use, 600 mg of resin is suspended in 10 cc distilled water. A miniature glass column 1 cm in diameter is plugged with glass wool and stoppered: 2 cc water are added. The slurry is poured into the column and allowed to settle for 20–30 min. The

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column is set up with a small U-shaped piece of glass tubing and allowed to drain (Fig. 1) The column is then washed several times with 2 cc aliquots of water, taking care that the level of water remains slightly above the upper level of the resin bed.

Preparation of blood specimen. Two aliquots of 5 cc plasma are processed for each specimen. To each 5-cc aliquot of plasma are added 25 mg of thiourea and approximately 7 cc 8 *N* acetic acid to achieve a pH of 2. The acidified plasma is poured into the column and allowed to drain. A flow rate of at least 1 cc in 5 min is maintained by the use of gentle pressure from a hypodermic syringe fitted with a rubber tube inserted through the rubber stopper which seals the top of the column. The column is washed with 3 cc of 5 *N* acetic acid and then with 2 cc water.

Elution of column. The resin retains the MIT, DIT, T_3, T_4 and other free amino acids. Proteins, peptides, sugars and electrolytes including iodide pass through the column. The iodinated tyrosine derivatives and free-plasma amino acids are eluted from the resin by the addition of 4 cc of 2 *N* triethylamine (TEA) prepared just prior to use from 7 cc reagent grade TEA and 25 cc 20% acetone in water (vol./vol.). Each drop of the eluate is tested with pH paper and discarded as long as the pH remains below 7. When the pH of the eluate becomes basic, the eluate is collected in a small evaporating dish. The eluate, containing iodinated organic compounds and amino acids, is evaporated to dryness. The resin can be used repeatedly provided care is taken to prevent channeling and drying. The resin is reconstituted by passing a solution of 2 *N* hydrochloric acid through until the eluate is acid, followed by washing with distilled water until the wash is chloride-free.

Chromatography. The residue is taken up in 150 μl of 1/1 mixture of methanol and 1 *N* ammonium hydroxide and spotted on Whatman No. 4 filter paper for standard partition chromatography. The container is rinsed with two additional 100-μl aliquots of methanol-ammonia which are applied on top of the original spot, taking care to maintain a spot size of no more than 10 mm. Solutions of stable MIT, DIT, T_3, T_4 and iodide (20 mg/100 ml dissolved in methanol-ammonium hydroxide solution) are prepared. Five microliters (1 μg) of each standard are applied to a separate spot on the same chromatogram with the residue from the blood specimen.

Duplicate chromatograms are prepared from the two aliquots of the same plasma and run in separate solvents. The systems used consisted of 120 cc n-butanol, 30 cc glacial acetic acid and 30 cc water (BuAc), and of 160 cc tertiary butanol, 60 cc chloroform, 15 cc ethanol, 7 cc concentrated ammonium hydroxide and 30 cc water (TBu). The chromatograms are placed in the solvent systems in ascending fashion and allowed to remain overnight (16 hr). They are then removed from the solvent and dried in air at room temperature. With every run of unknowns, one identical chromatogram is made of plasma to which is added enough stable MIT, DIT, T_3 and T_4 to allow for chemical identification with ceric sulfate-arsenious acid reagent. The Rf value of each "standard" is thereby established for that run. The chromatographic strips containing the unknown plasma samples are prepared and counted according to the method of Horwitz *et al* (*8*). This technique involves cutting the chromatograms transversely into strips 1 cm in width; each strip is placed into a separate polyethylene counting bottle. These bottles are then prepared in the usual manner for liquid scintillation counting.

Presentation of chromatography counting data. The data obtained from the counting system, 31 points of discontinuous type information for most chromatograms, were written onto magnetic tape. For clear interpretation of chromatographic data in the form given by the measuring process, some means of data smoothing was necessary; to this end the method of Fourier series was used. The basic problem was to find a continuous function to represent the data. The general form of this function is

$$f(x) = \sum_{m=-\infty}^{m=\infty} C_m \exp\left\{\frac{im\pi x}{L}\right\} \qquad (1)$$

where C_m, the so-called expansion coefficients, are given by the integrals

$$C_m = \frac{1}{2L}\int_d^{d+2L} f(x) \exp\left\{\frac{im\pi x}{L}\right\} dx. \qquad (2)$$

In the present application the Fourier expansion was treated in a more familiar form, i.e.

$$f(x) = \frac{A_o}{2} + \sum_{m=1}^{\infty} A_m \cos\frac{m\pi x}{L} + \sum_{m=1}^{\infty} B_m \sin\frac{m\pi x}{L} \qquad (3)$$

$$A_m = \frac{1}{L}\int_d^{d+2L} f(x) \cos\frac{m\pi x}{L}\, dp;$$

$$B_m = \frac{1}{L}\int_d^{d+2L} f(x) \sin\frac{m\pi x}{L} dx. \qquad (4)$$

Although there are mathematical restrictions to the use of the above analysis, they do not affect the treatment of the problem of interest.

For analysis of experimental data, f(x), the func-

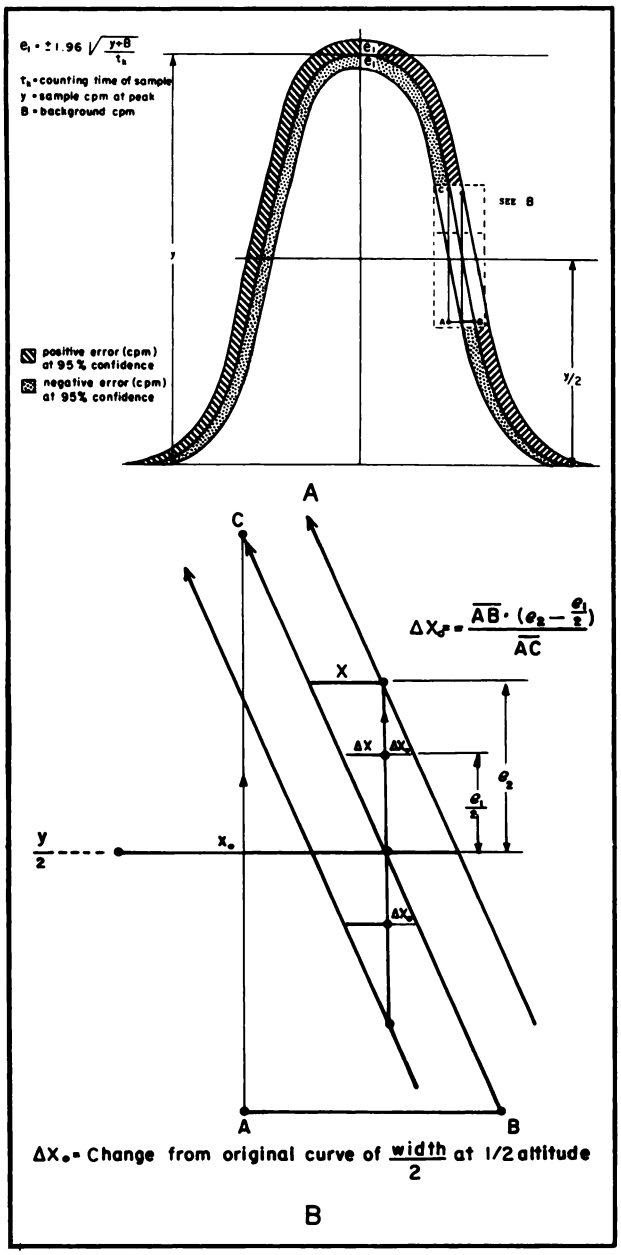

FIG. 2. Representative peak and associated mathematical treatment.

tion sought, requires that one use means other than the integrals in Eq. 2 and Eq. 4 to represent the function (Eq. 3). Several numerical methods are available for this and involve a vast amount of computational labor for required accuracy; however, with the availability of a digital computer, these analyses can be carried out efficiently on a large number of chromatograms in a short period of time. A standard Fourier coefficient routine was employed to compute the A_m's and B_m's. These coefficients were used to evaluate the function at 100 uniformly spaced intervals.

The Fourier fit was then used to estimate the percent of total activity associated with each chromatographic peak. This was accomplished by fitting to each peak a normal distribution and calculating the area under the distribution in fractional units of the total area under the function (see Fig. 2A). Some cases arose in which the peak was not symmetric with respect to the maximum; in this event an appropriate "half-normal" curve was fitted to the ascending and descending portion of the peak, the area under which was taken to be the sum of the respective half-areas. The location of the peaks was determined from the first derivative of the fitted function (Eq. 1). This procedure was continued until 95% of the area under the curve was accounted for. Since the original data points followed a Poisson distribution, the values of the function fit possess the same random error plus an "incompleteness" error, discussion of which appears below. With the distribution governing the error in the original data points known, one can assign this error to the Fourier fit and compute at any desired level of confidence the associated "area error" estimate for each peak (Fig. 2B).

One problem remains: that of spurious peaks that can occur due to the fact that only a finite number of terms can be used in representing the experimental data. In the practical case the function

$$f(x) \cong \frac{A_o}{2} + \sum_{m=1}^{k} A_m \cos\frac{m\pi x}{L} + \sum_{m=1}^{k} B_m \sin\frac{m\pi x}{L} \qquad (5)$$

must be used since it is impossible to enumerate an infinite number of A_m's and B_m's. This "incompleteness" leads to spurious higher order "harmonics;" consequently it is necessary to distinguish these "peaks" from those which bear chemical significance. One means of detecting these bogus peaks is to perform multiple analysis of the data using successively larger values of k in Eq. 5. The false peak will tend to change its abscissa value as well as its amplitude with changing k, while the genuine data peaks will tend to remain fixed in abscissa as well as amplitude. Experience indicates that spurious peaks have an amplitude less than the average value of the Fourier fit; this affords an upper limit in amplitude as to which peaks are to be studied during the variation of k. The changes in amplitude and abscissa versus k which will constitute rejection of one of these smaller peaks as spurious is still under investigation. A typical printout for the peak analysis is shown in Fig. 3.

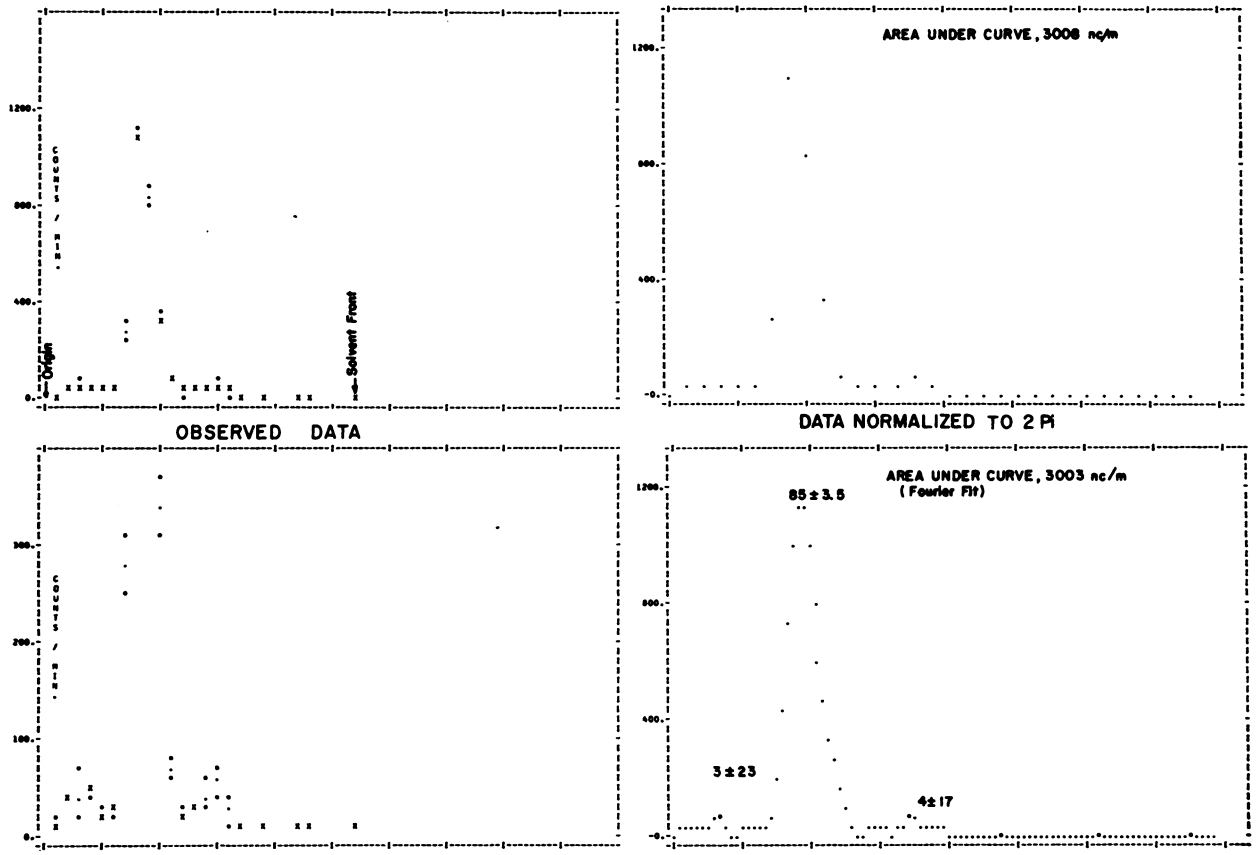

FIG. 3. Typical printout. Upper left corner: observed data; * = ±2 s.d.; x = corresponding observed data and confidence limits coincide. Lower left corner: same data with expanded ordinate. Upper right corner: observed data normalized to zero to 2, 31 points. Lower right corner: Fourier fit of observed data, 100 points normalized to zero to 2. Numbers ± a number represent percent of activity under entire curve present under that peak with error in units of percent of that percent.

RESULTS

Column recovery. The column step was first tested for recovery of gross radioactivity. Plasma obtained from each of seven subjects given 100 μCi of ^{131}I 72–120 hr earlier was counted in a gamma-sensitive well counter and then poured over the Dowex column. The various fractions were collected and later recounted in a gamma-sensitive well counter. A mean recovery value of 101% of added activity was obtained when the sum of activity found in the TEA eluate, pooled washes and the column eluate was compared to that present originally.

Plasma, to which had been added solutions of various commercially prepared iodoamino acids labeled with ^{131}I, were also poured over columns, and the individual recoveries were estimated. All standards were purified by preparatory chromatography the day of study. The activity was measured in each of three fractions: (1) the TEA eluate (containing amino acids and amines), (2) the column eluate plus wash solutions (containing proteins and inorganic ions including iodide) and (3) an aqueous suspension of the washed resin. When MIT was the standard (mean of six studies), 80% of the activity was recovered in Fraction 1, 1% in Fraction 2 and 17% in Fraction 3. When DIT was the standard (mean of 12 studies) 73, 2 and 26% of the activity was found in Fractions 1, 2 and 3, respectively. When T_3 was the standard (mean of 21 studies), the values for each fraction were 36, 39 and 22%. When T_4 was the standard (mean of 15 studies) there was 35, 43 and 24% of the activity in the various fractions.

It is not apparent why all of the original activity was recovered from the column eluate plus wash plus TEA eluate with endogenously labeled material while some 20% of the exogenous label stayed on the column regardless of the standard used. One possibility is that binding of the amino acid to plasma proteins is different under these two circumstances. This might allow for retention of amino acid-bound label by the column when exogenous standards are used. Deiodination obviously occurs as a result of column passage, and the amount seems to be proportional to the starting amino acid iodine content (T_4>T_3>DIT>MIT). Other investigators using analytic methods for thyroxine determinations have described similar results (9). However, slightly bet-

ter recovery from resins for these compounds has been reported (*10*).

When the TEA eluate obtained in a specific study with T_4 was evaporated to dryness, redissolved in acid solution and reapplied to a Dowex column immediately, an average 42% of the newly applied radioactivity was found in the TEA eluate (compared to 35% originally). When a pooled solution containing column eluate and wash radioactivity was extracted with acid butanol, 47% of the activity in that fraction, Fraction 2, passed into the butanol phase but did not migrate from the origin during the chromatography with TBu, demonstrating the absence of T_4 in Fraction 2. When the washed and eluted resin residue was extracted with acid butanol, 25% of the activity that had remained on the column, Fraction 3, went into the butanol phase and, on subsequent chromatography, appeared with an Rf value of 0.32. This suggests that some T_4 may be retained by the column after elution with TEA. When ^{14}C-labeled phenylalanine and ^{14}C-labeled tyrosine were passed over a similar column, more than 80% of the activity was recovered in the TEA eluate.

Because relatively large fractions of added standard T_4 activity appeared in the column eluate and wash solutions, recovery studies were repeated using ioresin (anion exchange resin supplied by Abbott). The ioresin, after appropriate washing, was found to bind 38% of the added radioactivity while only 44% was recovered in the acid butanol extract of the pooled supernate solutions. This suggests that over 50% of the T_4 activity was lost using this particular resin. When the pooled supernate solutions were evaporated to dryness, redissolved in saline and mixed again with ioresin, only 28% of the activity originally present in the supernate could be recovered in the new supernate: 72% stayed with the resin. These data are similar to those found using the Dowex resin and suggest that deiodination again occurred. Acid butanol did not extract activity from the washed ioresin in any instance. Continued extraction and column elution would eventually lead to total deiodination. The addition of stable T_4 did not alter the results.

TABLE 1. IDENTIFICATION OF STANDARDS

Labeled standard applied (number of separate runs)	Area associated with specific peaks* Percent area under total fitted curve† (Rf)	Percent added ^{131}I† (Rf)
Iodide‡ (2)	0	0
MIT‡ (4)	94 (0.30)	88 (0.30)
DIT‡ (4)	82 (0.58)	83 (0.58)
	6 (0.30)	6 (0.30)
T_3‖ (6)	62 (0.52)	39 (0.52)
	22 (0.32)	13 (0.32)
	6 (0.40)	3 (0.40)
T_4‖ (8)	7 (0.52)	2 (0.52)
	76 (0.32)	27 (0.32)
T_3, T_4, I‖ (2)	50 (0.52)	35 (0.52)
	36 (0.32)	20 (0.32)
	4 (0.40)	2 (0.40)

* Both chromatography systems were used but results were reported only for one in each instance.
† Mean values.
‡ BuAc chromatography system.
‖ TBu chromatography system.

Peak identification and recovery from entire system. The chromatography systems were next tested for reproducibility of peaks, for separation of specific peaks and for recovery of radioactivity. The Rf values for iodide, MIT, DIT, T_3 and T_4 in the TBu system were 0.37, 0.06, 0.01, 0.52 and 0.32, respectively, and, in the BuAc system, were 0.30, 0.58, 0.69, 0.87 and 0.91, respectively. Both systems were used in all studies in order to make the tentative identifications of the iodocompounds more secure: identification depended solely on Rf values. Sharp peaks for the tyrosines were obtained in BuAc, and broad peaks were obtained in the TBu. When thyronines were studied, sharp peaks were found with TBu and broad peaks with BuAc. Therefore, actual calculations for the recovery of tyrosines were carried out only with results obtained with the BuAc system, and thyronine calculations were based on results obtained with the TBu system. No iodide peak amounting to more than 5% of the activity under the fitted curve was seen when the Dowex column was used. All counting was normalized with a chloride standard to minimize error introduced by fluctuations in counting equipment sensitivity.

In Table 1 the results with individual labeled iodoamino acid standards, singly and in combination, are presented so as to evaluate the factors listed before. The difference between 100% of the area under the fitted curve and the sum of the areas under individual peaks (expressed as percent activity under total fitted curve) represents activity lost to background counts as a result of smearing. The difference between 100% of the added radioactivity and the sum of the areas under individual peaks (expressed as percent added ^{131}I) represents the estimate of net recovery of radioactivity. By and large iodothyronines are relatively insoluble in aqueous solution and recovery of pharmacologic amounts from solution is poor (*9*). Small amounts can be recovered with 75–85% efficiency (*11*).

As noted in Table 1 about 90% of the radioactivity under the total curve was located under peaks which had known statistical significance, regardless of the standards used. Only runs employing MIT as the standard demonstrated the predicted single significant peak, even though the standards were all purified the day of study by prior preparatory chromatography. While the results from DIT and T_4 runs suggested that some deiodination had occurred after use of the column, the T_3 results were not explained that easily. With T_3 there was always a peak moving with Rf of about 0.30. Sequential elution of the T_3 spot from chromatograms of a labeled T_3 standard always provided this satellite peak plus T_3 when rechromatogramed. However, when the satellite peak was eluted and rechromatogramed, migration of activity revealed only a peak of 0.32. Others have reported spurious and/or satellite peaks generated during chromatography of ^{131}I-labeled iodocompounds under conditions of varying pH (*12–14*). Therefore, chromatography of T_3 standards was carried out according to the method of Tata (*12*) under the various conditions of pH noted in Table 2. A sizable spurious peak moving with an Rf of 0.32 was always observed when chromatography was carried out in the pH range from 8.6 to 11.5. The error introduced in the 0.32 peak is only a concern when the T_3 peak is appreciable and the real T_4 peak relatively insignificant. It is probable that chromatography of unknown samples in TBu, performed according to the current method, is carried out at a pH between that of methanolammonia and the TBu system (between 10.8 and 11.7). Chromatography of standards is probably carried out at a pH between that of 20% propylene glycol and TBu (between 7.3 and 11.7). The spurious peak would, therefore, be greater with standards and least with unknown solutions.

Another finding was that, under the conditions of these studies, no exchange of label occurred between iodide, T_3 and T_4. When these labeled materials were all added in equimicrocurie amounts (see Table 1), only a minimal iodide peak was identified; the T_3 peak contained the anticipated fraction when expressed as percent of activity under the curve while the T_4 peak contained only three quarters of the expected fraction of activity. The failure to recover more activity as T_3 than 1/1 ratio of added activity, with T_3 and T_4 both present, is not apparent. It may be that the expected differences are of such magnitude that the error of the method obscures them. Absolute recoveries expressed as percent of added activity were similar for T_3 and T_4 when added alone or in combination.

In Table 3 are data obtained from studies of five adults and six adolescents, each of whom received a tracer dose of 100 μCi of ^{131}I. The answers are expressed as counts per minute derived from the printout, and as percent of dose administered to the patient per 100 ml serum with the error estimate. To arrive at these answers data were transformed from counts per minute to disintegration per minute to microcuries with the dose in microcuries known (i.e., 100 in all cases).

TABLE 2. A SATELLITE PEAK FOLLOWING CHROMATOGRAPHY OF ^{131}I-LABELED T_3; EFFECT OF pH

pH	Counting rate (cpm)* Rf 0.52	Counting rate (cpm)* Rf 0.32	0.32 peak as % 0.52 peak	Mixture with this pH
1.5	6,510	533	8	
4.0	7,523	413	5	
7.3	8,871	436	5	20% propylene glycol
8.0	10,656	813	8	
8.6	11,054	997	9	
9.2	13,537	2,456	18	
9.4	7,889	1,327	17	
9.6	12,791	2,087	16	100% propylene glycol
10.0	13,513	1,653	12	
10.5	8,505	1,019	12	
10.8	7,433	1,023	14	methanol-ammonia
11.0	13,979	1,064	8	
11.5	10,550	949	9	
11.7	15,527	701	5	TBu
12.2	9,155	652	7	TEA
13.0	9,711	694	7	

* The amount of added radioactivity was not kept constant.

DISCUSSION

A method has been described which uses chromatographic mobility and radioactivity for identification and quantitation of individual iodinated amino acids. The method is sensitive enough to permit studies in man with a tracer dose of 100 μCi of ^{131}I. Using this method, iodide was completely eliminated from the mixture prior to chromatography. If this is not done there will be a potentially large error resulting from the large iodide peak and its contamination of the paper. Unfortunately, the resin used deiodinated the compounds studied to some extent; this was directly proportional to the iodine content of the compound. Spontaneous deiodination during the interval while the sample dries on the paper prior to chromatography is a factor to be reckoned with, as noted by Taurog (*15*). While this should not contribute to the appearance of a peak with Rf of 0.32 in TBu, it can be minimized by the addition of a small volume of dilute solution of

TABLE 3. ANALYSIS OF CHROMATOGRAPHY RESULTS FROM RANDOM SUBJECTS

Patient (age)	^{131}I uptake, percent (hr after admin.)	Serum PBI (μg/100 ml)	Serum BEI (μg/100 ml)	Sampling time for chromatography specimen (hr after admin.)	Chromatography findings expressed according to Rf-identification in both systems: nc/m ± 2 s.d./5 ml serum			Percent administered ^{131}I/100 ml serum*
NA†	23	5.5	5.6	120	T_4	932 ± 45		5.58×10^{-6}
(7)	(24)							
RB†	23	4.4	3.6	96	T_4	47	5	0.28
(15)	(96)							
BMcC†	40	—	—	72	T_4	889	44	5.32
(48)	(24)				T_3	54	13	0.32
					MIT	34	7	0.20
JF†	36	—	—	96	T_4	182	22	1.09
(68)	(24)				T_3	14	7	0.08
					MIT	8	5	0.05
JuFr†	18	5.2	4.2	120	T_4	64	15	0.38
(13)	(72, 120)				T_3	14	8	0.08
					MIT	10	7	0.06
					Rf 0.91 (TBu):	25	15	0.15
PS†	32	5.0	4.8	96	T_4	14	0	0.08
(13)	(96)			144	T_4	222	23	1.33
JH†	21	4.8	4.4	96	T_4	84	6	0.50
(13)	(24)				T_3	20	0	0.12
				144	T_4	37	4	0.22
					MIT	8	0	0.05
GB‡	25	—	—	72	T_4	35	10	0.21
(40)	(24)				T_3	9	6	0.05
					DIT	6	4	0.04
ET‡	34	—	—	72	T_4	77	14	0.46
(62)	(24)				T_3	11	6	0.07
					Rf 0.59 (TBu):	11	6	0.07
OS‡	35	6.8	4.6	96	T_4	2,107	60	12.60
(41)	(96)			144	T_4	1,293	41	7.70
HR‡	15	13.6	10.2	72	T_4	141	4	0.84
(13)	(24)							
HMcE‡	10	2.4	1.8	144	T_4	120	25	0.72
	(24)				T_3	26	12	0.16
					MIT	25	15	0.16

* A counting efficiency of 75 was employed to convert net counts per minute to microcuries.
† These subjects were relatively normal in terms of thyroid function from a clinical standpoint.
‡ These subjects were definitely abnormal clinically.

serum albumin to the spot. In addition, the synthetic iodoamino acids were only 75% removed from the column by elution and washing. Substitution of an anion exchange resin commonly employed for this separation step (i.e., iodoamino acids from iodide) did not alter the results significantly.

An artefactual peak with an Rf value similar to that of T_4 was seen with the T_3 standard alone. This artefact was consistently recognized only with relatively large amounts of T_3 radioactivity (0.01 μCi) in the reaction mixture and probably was related to the pH at which certain steps in the procedure took place. The excellent recovery figures for MIT and DIT, including elution from resin, chromatography of the eluate and radioactivity counting, may help explain some of the discrepancies in the literature concerning the presence or absence of iodotyrosines in the blood stream (*16*); whatever activity enters into the tyrosine pools is available for detection, while some of that in the thyronine pools is lost in the processing of the serum by deiodination and poor recovery from the resin when it is used. This may make the tyrosine fractions appear inordinately large, as it does in the present study.

The degree of recovery for the iodothyronines is a limitation of the methodology which must be appreciated. It should be noted that no exchange of radioactivity occurred between the labeled standards and that the standard-related activity was recovered almost as completely in a mixture as when alone. These findings allow for comparative quantitation. However, sensitivity is far from maximal. The absence of iodoamino acid radioactivity after a tracer dose of ^{131}I does not mean that no activity is entering iodoamino acid pools; it only means that the amount of incorporation, if any, is below the sensitivity of the method. A recent report by Favino *et al* using thin layer chromatography, a strip scan-

ner with 4π geometry and Dowex resin contains results similar to these under discussion (*17*). Those authors noted approximately 93% extraction rates from plasma; from 6 to 14% of the activity present appeared unassociated with the appropriate stable standards. No recovery studies completely comparable to those herein reported were recorded. They noted in one euthyroid subject given 100 μCi ^{131}I serum T_3 components equal to 5% of the serum T_4 fraction.

The data in Table 3 demonstrate almost a 100-fold range for T_4 incorporation values. Whereas these subjects were all relatively normal from the standpoint of thyroid function, no effort was made to obtain completely healthy subjects; age and activity were uncontrolled and the blood sampling times varied. All these factors will add scatter to the data. In seven of the 12 subjects, T_3 activity was identified in amounts up to 20% of the T_4 activity. In five subjects MIT and in one subject DIT activity was found in amounts usually smaller than that for T_3. Rates of ^{131}I incorporation cannot be calculated unless multiple sampling times are employed. The use of multiple times obviously is indicated: in those few subjects where this was done, the results were noted to change quantitatively as well as qualitatively. These results are not inconsistent with current thinking about the peripheral existence, relative protein bondage and fate of thyroid secretory products and suggest that with proper methodology and multiple sampling time, evidence for both T_3 and MIT in the blood of relatively normal subjects can be accumulated as recently discussed by Rhodes (*18*). In all probability the ratios of iodotyrosine to iodothyronine incorporation values are erroneously high since the recovery values for the iodotyrosine standards were approximately three times that for T_4 and twice that for T_3. Relatively tight protein binding of MIT and DIT may account for this. Similarly the ratio of T_3 activity to T_4 activity is spuriously high: The recovery of the appropriate standards was almost 50% greater for T_3. Adjustments based upon these considerations reduce the T_3 moiety to a maximum of 18% of the simultaneous T_4 fraction and MIT no more than 7% of T_4. Estimation of relative turnover of these various pools must await further study. Certainly the higher turnover rate for T_3 compared to T_4 accounts in part for the relatively high ratio of T_3-to-T_4-associated radioactivity.

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REFERENCES

1. SMITH, I.: *Chromatographic and Electrophoretic Techniques.* William Heinemann Medical Books, Ltd., London Interscience Publishers, Inc., New York, 1960, p. 166.

2. TSUYOSHI, K., VAN MIDDLESWORTH, L. AND ASTWOOD, E. B.: Chemical identification of iodine-containing compounds in human serum. *Endocrinology* **66**:845, 1960.

3. BLOCK, R. J., DURRUM, E. L. AND ZWEIG, G.: *A Manual of Paper Chromatography and Paper Electrophoresis.* Academic Press, Inc., New York, 1958, p. 138.

4. BENUA, R. AND DOBYNS, B. M.: Iodinated compounds in the serum, disappearance of radioactive iodine from the thyroid, and clinical response in patients treated with radioactive iodine. *J. Clin. Endocrinol.* **15**:118, 1955.

5. STANBURY, J. B., KASSENAAR, A. A. H., MEIJER, J. W. A. AND TERPATRA, J.: The occurrence of mono- and di iodo-tyrosine in the blood of a patient with congenital goiter. *J. Clin. Endocrinol.* **15**:1,216, 1955.

6. ARON, W. L. AND HYDOVITZ, J. D.: The serum pattern of thyroid hormones in euthyroidism and hyperthyroidism. *J. Clin. Endocrinol.* **19**:549, 1959.

7. HARRIS, C. K., TIGANE, E. AND HANES, C. S.: Quantitative chromatographic methods: isolation of amino acids from serum and other fluids. *Can. J. Physiol.* **39**:439, 1961.

8. HORWITZ, H., KEREIAKES, J. G., GOLDSMITH, R. E. AND POLLACK, S. V.: The study and analysis of radiochromatograms following I^{131} administrations to humans. *Intern. J. Appl. Radiation Isotopes* **16**:721, 1965.

9. WEST, C. D., CHAVRE, V. J. AND WOLFE, M.: A serum thyroxine method: application in thyroid disease and iodine-treated patients. *J. Clin. Endocrinol.* **25**:1,189, 1965.

10. REILLY, W. A., SEARLE, G. L. AND SCOTT, K. G.: Resin column chromatography by dowex 50-x-4 of I^{131} labeled iodoamino acids in human serum. *Metabolism* **10**: 869, 1961.

11. MURPHY, B. P.: The determination of thyroxine by competitive protein-binding analysis employing an anion exchange resin and radiothyroxine. *J. Lab. Clin. Med.* **66**: 161, 1966.

12. TATA, J. R.: An unusual property of thyroxine and other iodophenols. *Biochem. J.* **72**:215, 1959.

13. TATA, J. R.: A new aspect of the interaction between thyroxine and proteins. *Biochem. J.* **72**:222, 1959.

14. ANGHILERI, L. J.: A chromatographic study of the stability of iodine-131 labeled sodium o-iodohippurate. *Intern. J. Appl. Radiation Isotopes* **14**:328, 1963.

15. TAUROG, A.: Spontaneous deiodination of I^{131}-labeled thyroxine and related iodophenols on filter paper. *Endocrinology* **73**:45, 1963.

16. MANDL, R. H. AND BLOCK, R. J.: Method for the qualitative, semiquantitative and quantitative determination of iodoamino acids and of inorganic iodine in iodoprotein digests and in human serum. *Arch. Biochem.* **81**:25, 1959.

17. FAVINO, A., EMRICH, D. AND VON JUR MUHLEN, A.: Separation and quantitative determination of ^{131}I-triiodothyronine and ^{131}I-thyroxine in human plasma by thin layer chromatography. *Acta Endocrinol.* **54**:362, 1967.

18. RHODES, B. A.: The circulating iodotyrosines. *Acta Endocrinol.* Suppl. 127, Vol. 57, 1968, p. 6.