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SEPHADEX LH-20 COLUMN SEPARATION OF THYROIDAL IODOAMINO ACIDS

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SUMMARY

A solvent system consisting of low-boiling organic solvents and ammonium hydroxide effectively separated iodoamino acids of the thyroid on Sephadex LH-20 gel filtration columns. The iodoamino acids were eluted from the column in a sequence unrelated to their molecular weights suggesting that adsorptive properties of the dextran gel rather than gel exclusion effects governed the elution volume of the compounds.

The eluted iodoamino acids were recovered in a pure form by low-temperature evaporation of the solvents at reduced pressure. Good recovery of iodoamino acids from the column was obtained and reproducibility of column performance was satisfactory.

INTRODUCTION

Various methods have been described for the separation of mixtures of thyroidal iodoamino acids by filter paper and thin-layer chromatography[1–5], ion exchange column chromatography[6,7], gas-liquid chromatography[8–10] and by Sephadex gel filtration columns[11–14].

Each of these techniques has certain advantages as well as disadvantages in terms of sample size needed, requirements for the preparation of the samples or derivatives for separation, speed of separation, complexity of equipment involved and recovery of the compounds of interest.

Gel filtration column procedures with an automatic fraction collector have the advantage of requiring little attention during the separation procedure and are reproducible with adequate recovery of the compounds from the columns. While proteins generally separate on gel filtration columns according to their molecular weights, crosslinked dextran gels often show strong adsorptive properties for aromatic substances in some cases favoring their separation[15,16]. Compounds such as thyroidal iodoamino acids also have been shown to be reversibly adsorbed to Sephadex gel filtration column beds[11,17,18].

Procedures for the separation of iodoamino acids on Sephadex G-25 gel filtration

columns using buffered salt solutions, dilute sodium hydroxide or higher boiling organic solvents have been described[12–14]. With such eluting mixtures, recovery of the separated compounds for further analysis or use was complicated by the presence of salt or alkaline residues when the pooled peaks from the column containing the compounds of interest were taken to dryness.

Sephadex LH-20 has been reported to be stable in organic solvents[17] and has been used as a desalting step in a purification procedure for the analysis of serum iodoamino acids[19]. We have developed a solvent system composed of ethyl acetate, methanol and aqueous ammonium hydroxide which effectively separated thyroidal iodoamino acids on Sephadex LH-20 gel filtration columns.

EXPERIMENTAL

Reagents

All reagents used were analytical grade. The eluting system for the Sephadex LH-20 column was a mixture of ethyl acetate–methanol–2 *N* aqueous ammonium hydroxide (400:100:40).

Solutions of thyroxine (T_4), triiodothyronine (T_3), diiodotyrosine (DIT) and monoiodotyrosine (MIT) (Calbiochem, Los Angeles, Calif.) were used at a concentration of 5 mg/ml in 1% methanolic NH_4OH (methanol–concentrated ammonium hydroxide (99:1)) to standardize the Sephadex LH-20 column.

Iodine assay

The iodine content of the samples was determined by a chloric acid wet ashing procedure previously described[20]. It is important that iodine assays on samples dissolved in the ethyl acetate–methanol–NH_4OH eluting mixture or in the 1% methanolic NH_4OH solvent be done on aliquots which have been dried completely prior to digestion with the chloric acid reagent: *methanol reacts violently with hot chloric acid.*

Thin-layer chromatography

The thin-layer chromatographic procedure was described previously by us[4].

Preparation of gel filtration columns

Sephadex LH-20, particle size 25–100 μ (Pharmacia, Uppsala, Sweden) was suspended in the ethyl acetate–methanol–NH_4OH elution mixture. It was allowed to settle briefly and, after discarding the fines by decantation, was equilibrated in the solvent for 4 h. An 0.8 cm diameter glass column fitted with a sintered glass disc and a stopcock to control the flow rate was attached to a reservoir filled with the eluting solvent mixture. Glass ball and socket joint column connectors were used since the solvent was found to extract ultraviolet-absorbing materials from rubber or plastic connectors.

The equilibrated Sephadex was poured into the reservoir and with stirring was allowed to flow into the column in which it was packed by gravity flow to a column height of 60 cm. The solvent was permitted to flow through the gel bed in the column for 16 h before use. Between separation runs the solvent mixture was passed through the packed column continuously to maintain equilibrium conditions in the gel bed.

A fraction collector (LKB-Produkter AB, Sweden) was used to collect 4 ml

fractions of the column effluent at an elution rate of 0.5 ml/min during separation procedures. A complete separation of iodoamino acids required the collection of 100–110 × 4 ml fractions from the column.

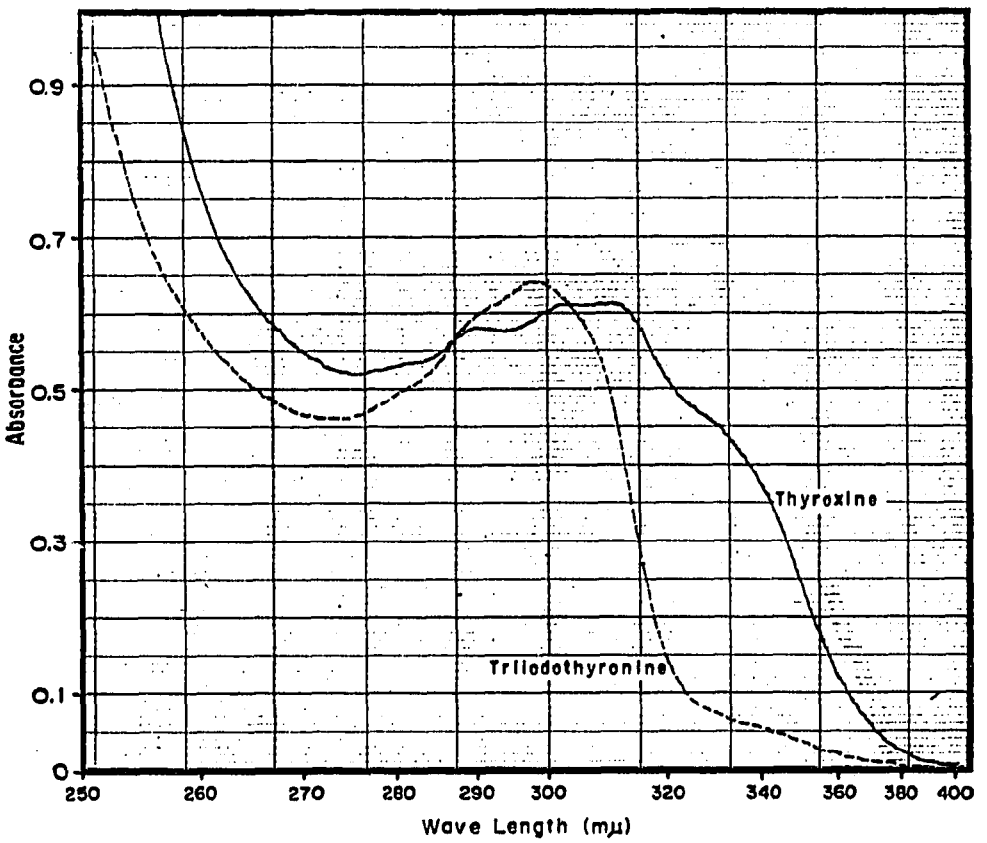

Fig. 1. Absorbance spectra of pure thyroxine and triiodothyronine in ethyl acetate–methanol–NH_4OH. 1.5×10^{-4} *M* solutions.

Spectrophotometric characteristics of iodoamino acids

A Beckman Model DK-2 recording spectrophotometer was used to obtain spectral absorbance curves of the pure iodoamino acids. The sequence of elution of the pure compounds from the column was monitored at 297 and 310 mμ using a Beckman Model DU spectrophotometer. Absorbance measurements were done on each 4 ml fraction collected from the column.

The spectrophotometric absorbance characteristics of the iodoamino acids at a concentration of 1.5×10^{-4} *M* in the eluting solvent system are shown in Fig. 1 for T_4 and T_3 and for MIT and DIT in Fig. 2. The molar extinction coefficient and the ratio of absorbance at 297 and 310 mμ of each iodoamino acid in the ethyl acetate–methanol–NH_4OH solvent is listed in Table I.

Sephadex LH-20 column separation of a pure iodoamino acid mixture

In preliminary studies, 0.5 ml of each pure iodoamino acid standard solution (5 mg/ml in 1% methanolic NH_4OH) was chromatographed individually on a Sephadex LH-20 gel filtration column. The compound was eluted from the column with

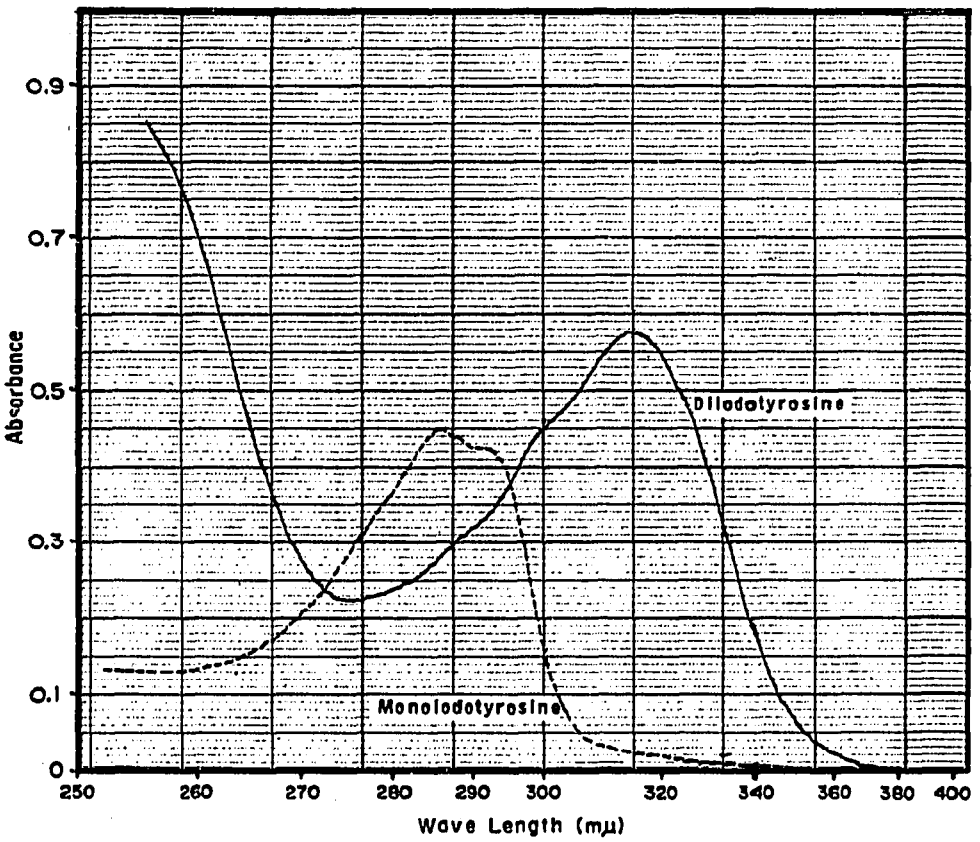

Fig. 2. Absorbance spectra of pure diiodotyrosine and monoiodotyrosine in ethyl acetate–methanol–NH_4OH. 1.5 × 10^{-4} *M* solutions.

TABLE I

SPECTROPHOTOMETRIC CHARACTERISTICS OF PURE IODOAMINO ACIDS

Molar extinction coefficient (1 cm light path) at 297 mμ in the ethyl acetate–methanol–NH_4OH eluting solvent.

Compound	*Extinction*	*Ratio 297/310 mμ*
Thyroxine	3900	0.956
Triiodothyronine	4253	1.276
Diiodotyrosine	2720	0.744
Monoiodotyrosine	2053	9.626

the ethyl acetate–methanol–NH_4OH eluting mixture to determine the effluent volume at which it emerged from the column. The column effluent was monitored spectrophotometrically.

After defining the elution pattern for each individual iodoamino acid, a mixture of the four iodoamino acids was prepared (5 mg of each compound per ml of 1% methanolic NH_4OH). An 0.5 ml aliquot of the standard mixture was applied to the column bed, allowed to penetrate the upper surface of the gel and the iodoamino acids were eluted from the Sephadex LH-20 column with the ethyl acetate–methanol–

NH_4OH eluting mixture. After spectrophotometry, iodine analyses were done on small aliquots of each 4 ml fraction. The elution pattern of the standard iodoamino acid mixture from the Sephadex LH-20 column is shown in Fig. 3. The line connecting the solid circles represents the 297 mμ absorbance while the dotted line connecting the triangles is the iodine content (μg/ml) of each fraction. Fractions composing the areas under each peak were pooled and taken to dryness *in vacuo* at less than 40° and the dried samples were redissolved in a small volume of 1% methanolic NH_4OH. In most cases, spectrophotometric studies revealed no appreciable differences between the reconstituted pooled peaks and the original pure samples. One lot number of Sephadex LH-20 (different from the batch used for the major portion of this study) appeared to produce an unexplained spectral shift when DIT was eluted from the gel bed.

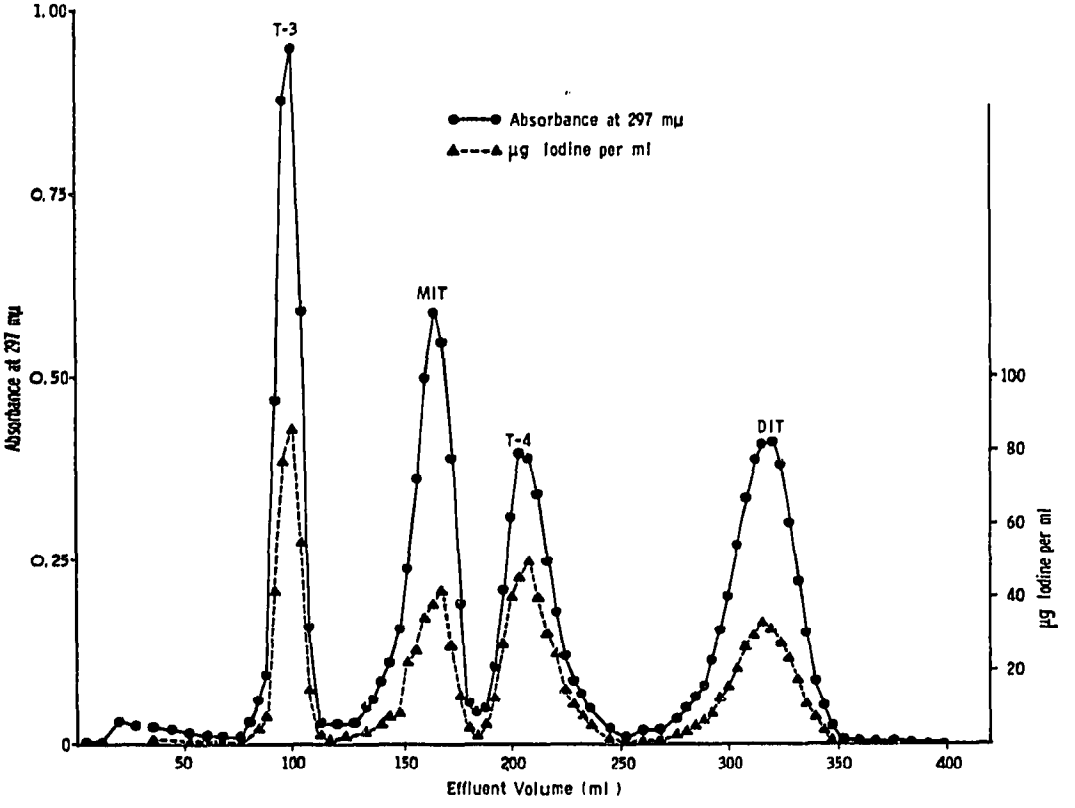

Fig. 3. Elution pattern from a Sephadex LH-20 gel filtration column of a mixture of pure iodoamino acids. Solid line = absorbance at 297 mμ. Dotted line = μg iodine per ml.

Thin-layer chromatographic analysis of the reconstituted pooled peaks revealed single spots traveling to positions occupied by known pure iodoamino acid standards with no evidence of cross contamination of a pooled peak by adjacent peaks.

Sephadex LH-20 column separation of ^{131}I-labeled rat thyroid iodoamino acids

The thyroid gland was removed from a normal rat that had been injected with ^{131}I 24 h prior to sacrifice. The gland, dissected free from connective tissue, was hydrolyzed enzymatically with Pronase[21] and subjected to a preliminary purification

on a Dowex-50 cation exchange resin column[4]. This procedure was used to remove inorganic iodide and lipids from the enzymatic digest.

A small volume (0.5 ml, equivalent to about 4 mg of thyroid) of the purified Pronase digest was placed on the Sephadex LH-20 column for separation of the ^{131}I-labeled iodoamino acids. Radioactivity in each 4 ml fraction eluted from the column was measured in a well crystal gamma counter. The results of this experiment are given in Fig. 4. The radioactivity in each peak eluted from the column was calculated as a percentage of the total radioactivity from the column. The values were: T_3 = 2 %, T_4 = 10 %, MIT = 32 %, and DIT = 55 %.

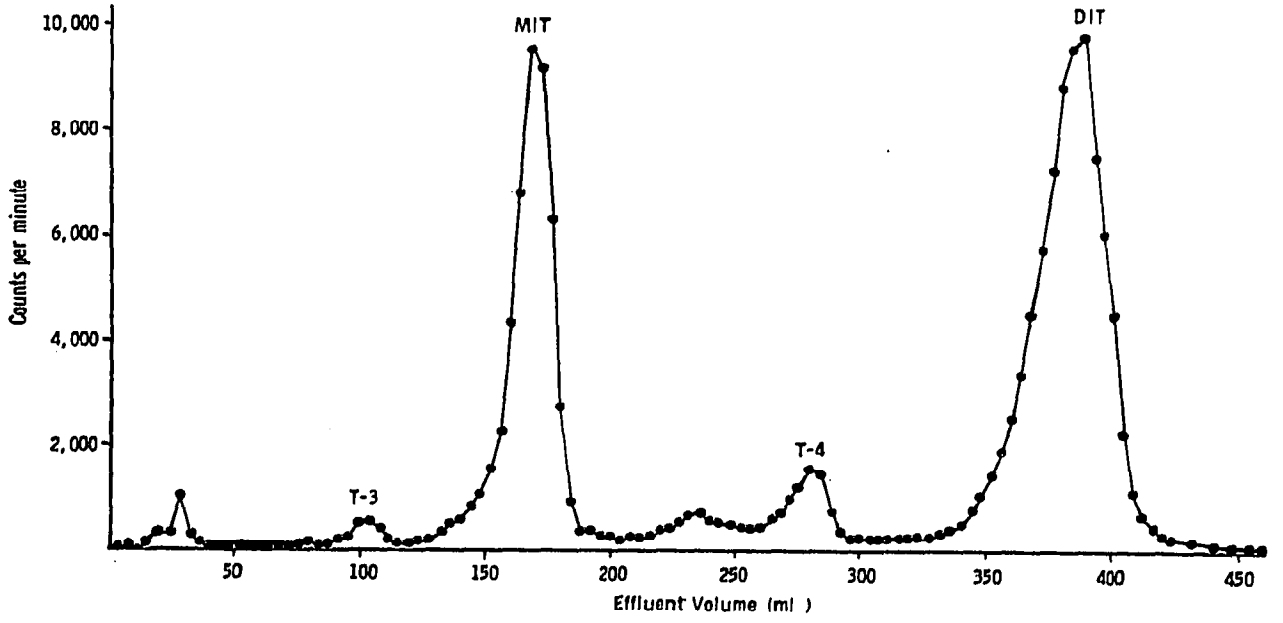

Fig. 4. Elution pattern from a Sephadex LH-20 gel filtration column of ^{131}I-labeled iodoamino acids from a rat thyroid hydrolysate.

Recovery of iodoamino acids from Sephadex LH-20 columns

To evaluate the recovery of iodoamino acids from Sephadex LH-20 columns, the standard solution of each iodoamino acid was assayed for total iodine content. In a series of five replicate column runs for each compound, 0.5 ml of the standard solution was placed on the column bed. Following elution of the sample from the column, fractions composing the peak area were pooled, taken to dryness *in vacuo* and reconstituted in a small volume of 1% methanolic NH_4OH. Iodine analyses of the reconstituted pooled peaks from the columns were done and the results are given in Table II. These values, expressed as percentage of iodine in the sample applied to the column, show recovery of from 86.9 to 98.7%.

Reproducibility of Sephadex LH-20 columns

In using the LH-20 Sephadex columns for the separation of iodoamino acids, the performance of the column was checked frequently by placing 0.5 ml of the standard iodoamino acid mixture on the column bed and eluting the compounds from the column with the ethyl acetate–methanol–NH_4OH mixture. The absorbance at 297 mμ for each fraction collected was measured. The sum of the absorbance in the fractions

TABLE II

RECOVERY OF IODOAMINO ACIDS FROM SEPHADEX LH-20 COLUMNS

Compound	*Total iodine applied to column (mg)*	*Total iodine from column (mg)*[a]	*Recovery (%)*[a]
Triiodothyronine	1.206	1.054 ± 0.0188	87.4 ± 1.56
Thyroxine	1.240	1.224 ± 0.0562	98.7 ± 4.53
Monoiodotyrosine	0.916	0.873 ± 0.0340	95.3 ± 3.71
Diiodotyrosine	1.241	1.079 ± 0.0198	86.9 ± 1.60

[a] Mean ± standard deviation; $n = 5$.

TABLE III

SEPHADEX LH-20 COLUMN REPRODUCIBILITY

Compound	*Absorbance of compound as % of total 297 mμ absorbance in mixture applied to column*[a]	*Absorbance in pooled peak as % of total 297 mμ absorbance eluted from column*[b]
Triiodothyronine	26.1	25.3 ± 2.05
Thyroxine	20.2	20.9 ± 1.91
Monoiodotyrosine	27.6	25.6 ± 1.18
Diiodotyrosine	26.0	28.1 ± 1.49

[a] Calculated from theoretical values for each compound.
[b] Mean ± standard deviation; $n = 24$.

composing the peak for each compound was expressed as a percentage of the total absorbance eluted from the column. The mean percentage distribution of absorbance for 24 different column separations of the standard iodoamino acid mixture is given in Table III. For comparative purposes, the absorbance of each compound expressed as percentage of the total 297 mμ absorbance in the mixture applied to the column also has been listed in Table III. These are theoretical calculations based on the known spectrophotometric absorbance values for each iodoamino acid. The standard deviations for the mean values as well as the agreement with the theoretical distribution values indicate good reproducibility of column performance.

RESULTS AND DISCUSSION

The spectral absorbance curves of Figs. 1 and 2 and the results in Table I illustrate that evaluation of possibly useful solvent systems for Sephadex LH-20 column separation of iodoamino acids may be done conveniently by monitoring the column effluent spectrophotometrically. It was found that the type of solvent used noticeably affects the absorbance characteristics of iodoamino acids. An increased ethyl acetate concentration in the solvent system decreased T_4 absorbance and shifted the peak absorbance to a lower wavelength. Ethyl acetate had little effect on the T_3 absorbance characteristics. High concentrations of ethyl acetate in the solvent

system depressed DIT absorbance and caused a small shift in the absorbance peak to a lower wavelength. The 310 mμ absorbance of MIT is sharply reduced by increasing the ethyl acetate content of the solvent mixture.

Observations of the spectral absorbance characteristics in the ethyl acetate–methanol–NH_4OH eluting mixture led to the selection of 297 mμ as the optimal wavelength for the most sensitive monitoring of iodoamino acid elution from Sephadex LH-20 columns. Calculation of the ratio of absorbance at 297 and 310 mμ was helpful in identifying each iodoamino acid as it emerged from the gel bed (Table I). These considerations were useful in preliminary work in which a number of different solvents and combinations were evaluated.

The eluting solvent mixture finally selected is a useful one which permits complete separation of the iodoamino acids on Sephadex LH-20 columns although more effective solvent systems undoubtedly exist. The ethyl acetate–methanol–NH_4OH eluting mixture can be evaporated easily at low temperature *in vacuo*. This property offers the advantage of facilitating recovery of the iodoamino acids in the column effluent free from the eluting solvent mixture.

It was found that increasing the water content of the eluting mixture resulted in a more rapid elution of DIT and T_4 from the column bed. When excessive water was included in the eluting solvent mixture, the DIT and T_4 peaks were eluted with the MIT peak.

Fig. 3, an elution pattern of a pure iodoamino acid mixture, illustrates the type of separation obtainable. Sharp peaks appear with good separation of each compound from the other components of the iodoamino acid mixture. Each of the iodoamino acids can be recovered uncontaminated by adjacent peaks, as shown by thin-layer chromatographic studies on the pooled fractions representing each elution peak from the column.

The elution sequence of the iodoamino acids from the Sephadex LH-20 column is not directly related to the molecular weight of the compounds although in conventional gel filtration, compounds are usually eluted in decreasing order of molecular size. Retardation of phenolic and other types of aromatic molecules on Sephadex gel filtration columns has been described previously[11,17,18] and it is likely that the adsorptive properties of the dextran gel for iodoamino acids may create a favorable condition for their separation on Sephadex LH-20 columns.

The iodine content of the molecule also appears to be a factor which affects the elution sequence of iodoamino acids. In the case of the thyronine iodoamino acids, the compound with the higher iodine content is retarded on the column. The same effect is seen with the tyrosine derivatives in which MIT is eluted from the column more rapidly than DIT. It is also possible that the elution sequence of the iodoamino acids is a function of the solubility of the compounds in the eluting solvent mixture.

Fig. 3 also indicates that the progress of the Sephadex LH-20 column separation of iodoamino acids can be monitored by chemical analysis of the iodine content in each fraction of the column effluent. Good correspondence exists between the 297 mμ absorbance and the iodine content of the fraction. Spectrophotometric absorbance at 297 mμ is a better procedure for monitoring the column effluent since the procedure is non-destructive and the entire fraction can be recovered for further use. However, effluent concentrations of the iodoamino acids must be in the range of $1.0–1.5 \times 10^{-4}$ *M* or greater in order to obtain meaningful 297 mμ absorbance readings. Iodine

analysis of the column effluent fractions can be done at much lower iodoamino acid concentrations but this results in a loss of some of the sample as a consequence of the analytical procedure.

Radioactivity measurements can also be used to follow the elution of iodo amino acids from the Sephadex LH-20 gel filtration columns if radioactive compounds are available. Separation of biologically labeled iodoamino acids from a ^{131}I-labeled rat thyroid tissue hydrolysate is shown in Fig. 4 which illustrates the separation of radioactive peaks. The nature of the small radioactive peak between MIT and T_4 is unknown but has been observed in several hydrolysates of ^{131}I-labeled rat thyroids. The percentage distribution of the radioactivity in the iodoamino acid peaks is in good agreement with other reports[4,5].

Good recovery of pure iodoamino acids from Sephadex LH-20 gel filtration columns was obtained (Table II). These recovery values were based on a calculation of the iodine content in the eluted, pooled fractions composing the compound expressed as a percentage of iodine in the pure sample applied to the column bed. Although somewhat lower recovery of DIT and T_3 iodine compared to T_4 and MIT recovery suggests the possibility of some deiodination of DIT and T_3 on the gel bed, no appreciable amount of inorganic iodide has been located in the column effluent during fractionation of these iodoamino acid solutions.

Good reproducibility of Sephadex LH-20 column performance is indicated by the results in Table III. In these studies, the sum of the 297 mμ absorbance in the fractions composing the peak for each iodoamino acid eluted from the column was expressed as a percentage of the total 297 mμ absorbance eluted from the column in the complete separation run. The low standard deviation values for the mean percentage distribution of absorbance for each compound from the column and the agreement with the theoretical values in the standard mixture applied to the column indicates that a reproducible separation of iodoamino acids on Sephadex LH-20 columns is obtainable.

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