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PURIFICATION OF ^{125}I-LABELLED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE DETECTION

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SUMMARY

^{125}I-labelled compounds, including [^{125}I]triiodothyronine, [^{125}I]thyroxine and [^{125}I]estriol, have been prepared and purified by high-performance liquid chromatography (HPLC). Using a reversed-phase column, compounds of highest purity and uniformity were obtained; stereoisomers, *e.g.*, [2-^{125}I]E_3 and [4-^{125}I]E_3, were separated. A sodium iodide crystal served as an on-line γ-detector. Thus it was possible to measure the specific activity of the radiolabelled compounds prepared. The recovery of radioactivity was virtually quantitative. There is no substantial accumulation of activity on the column used. Compared with conventional techniques, *e.g.* thin-layer chromatography or gel filtration, HPLC is to be preferred with regard to specificity, handling, safety and operating time.

INTRODUCTION

The development of the radioimmunoassay (RIA) technique[1] prompted a search for convenient methods for preparing radiolabelled compounds. The sensitivity and specificity of RIA depend to a large extent on the purity and specific activity of the radiolabelled tracers. Comparatively high specific activities can be obtained by using γ-emitters, *e.g.*, iodine-131 and more recently iodine-125, instead of β-emitters, *e.g.*, tritium and carbon-14. About 20 years ago Hunter and Greenwood[2] devised a method for labelling proteins with iodine-131. Smaller molecules, including thyroid hormones and steroid derivatives, have been labelled and applied as tracers in RIA since then.

These tracers have generally been purified by gel filtration[3–5], thin-layer chromatography on different support materials[3,4,6–8], ion-exchange chromatography[4,8,9], paper chromatography[5,10] or polyacrylamide gel electrophoresis[4]. Most of these methods are slow and some are not able to separate labelled from unlabelled material or to separate isomers. Except for column chromatography, the purified tracer has to be eluted from the support material, thus requiring an additional step in the preparation of radiolabelled compounds. Moreover, it is virtually impossible to measure rather than to estimate specific activities of the isolated material, the knowledge of which is very important for the development of RIA.

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The aim of this study was therefore to develop a fast and convenient purification method that is specific and allows the measurement of specific activities. Owing to its high selectivity and speed, we chose reversed-phase high-performance liquid chromatography (HPLC) with an on-line γ-detector.

EXPERIMENTAL

Reagents

Acetonitrile, acetic acid and estriol (analytical-reagent grade) were obtained from E. Merck (Darmstadt, G.F.R.). Diiodothyronine (T_2) was purchased from Koch-Light (Colnbrook, Great Britain) and triiodothyronine (T_3) from Henning (Berlin, G.F.R.). Carrier-free $Na^{125}I$ (IMS 300), from Amersham-Buchler (Braunschweig, G.F.R.) was used for the synthesis of the iodo compounds.

Apparatus and column

The chromatographic equipment[11] consisted of a Waters Model 6000 A pump, a Waters Model U6K universal injector, a Waters μBondapak C_{18} column (10 μm; 300 × 3.9 mm I.D.; stainless-steel), a Waters Model 440 fixed-wavelength UV detector, a γ-detector and a Metrawatt Servogor two-channel recorder.

γ-Detector

The γ-detector[11] was a PTFE capillary (20 × 0.5 mm I.D.) passing through a drilled 2 × 2 in. sodium iodide crystal, type 8 SAF 8 / 2 GX (Laboratorium Prof. Berthold, Wildbad, G.F.R.). The volume of the detector cell was 3.9 μl. The photomultiplier of the crystal was connected to a rate meter (Laboratorium Prof. Berthold) and the Metrawatt Servogor recorder. The response time of the rate meter was set to 2 sec.

Preparation of compounds

All compounds were prepared according to Hunter and Greenwood[2]. Thus T_2 was labelled with $^{125}I_2$ yielding [^{125}I]T_3 and a small amount of thyroxine (T_4), T_3 yielded [^{125}I]T_3 and [^{125}I]T_4 and estriol (E_3) yielded [2-^{125}I]E_3 and [4-^{125}I]E_3.

The crude reaction mixtures were injected directly and chromatographed. For compounds of lower specific activity we added calculated amounts of $Na^{127}I$ to the $Na^{125}I$ solution.

Other compounds containing aromatic groups, including testosterone and theophylline derivatives, were labelled and purified in similar manner.

Determination of specific activity

The radioactivity of the purified ^{125}I-labelled compounds collected at the Waters 6000 A pump was measured with a Capintec Model CRC-10R Curiemeter. From the amount of a particular compound, derived from UV data, we determined the specific activity in mCi/mg or TBq/nmol. Work is currently in progress to calibrate the γ-detector and calculate the specific activity on-line with a two-channel Spectra Physics SP 4200 integrator system.

Recovery of radioactivity

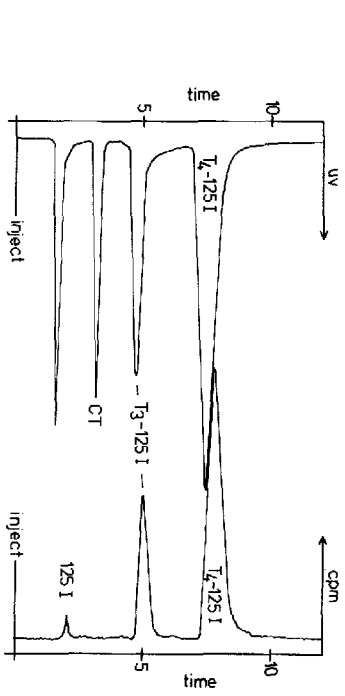
The radioactivity of the complete eluate from different labelling mixtures was

measured with a Capintec Model CRC-10 R Curiemeter. The syringe was flushed four times with 25 μl each of methanol and water. The radioactivity of the washings and the reaction vial was subtracted from the radioactivity of the labelling mixture, thus yielding the radioactivity applied to the column.

RESULTS

Purification of compounds

Reaction mixtures for labelling procedures[2] usually contain labelled products, radioactive iodide, unreacted starting material, reduced chloramine-T (CT), decomposition products and salts. As the performance and specificity of RIA depend to a large extent on the purity of the applied tracer, we selected HPLC for the isolation of the ^{125}I-labelled compounds. Fig. 1 is a typical chromatogram obtained in the preparation of [^{125}I]T_4. The UV trace shows clearly that the salts, reduced CT and the starting material T_3 are well separated from the desired product, [^{125}I]T_4. The radiochromatogram contains the peaks of Na^{125}I, [^{125}I]T_4 and additionally [^{125}I]T_3. [^{125}I]T_3 originates from a side reaction, *i.e.*, exchange reaction, in the labelling process. Using increasing amounts of starting material we were able to reduce the amount of [^{125}I]T_3. Virtually all [^{125}I]T_4 is eluted within 1.5 ml.



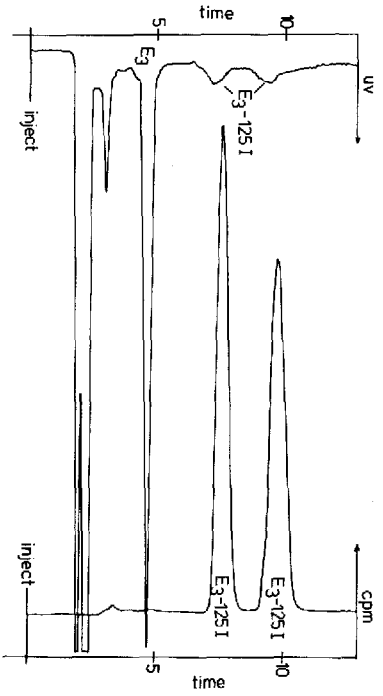


Fig. 1. HPLC of [^{125}I]T_4 prepared from T_3. Column, μBondapak C_{18} (10 μm, 300 × 3.9 mm I.D.); mobile phase, water–acetonitrile–acetic acid (60:38:2); flow-rate, 1.5 ml/min; detection, UV (254 nm), 0.005 a.u.f.s. and γ-detector.

Fig. 2. HPLC of [^{125}I]E_3 prepared from E_3. Column, μBondapak C_{18} (10 μm, 300 × 3.9 mm I.D.); mobile phase, water–methanol–acetic acid (50:50:2); flow-rate, 1.5 ml/min; detection, UV (254 nm), 0.005 a.u.f.s. and γ-detector.

With a modification of the qualitative and quantitative composition of the mobile phase, *e.g.*, replacement of acetonitrile with methanol, we obtained similar results in the purification of [^{125}I]T_3 and ^{125}I-labelled testosterone, estriol and theophylline derivatives.

Separation of isomers

With the HPLC technique we were able to separate stereoisomers, as shown in Fig. 2. The aromatic ring of estriol was directly labelled with $^{125}I_2$, yielding [2-^{125}I]E_3 and [4-^{125}I]E_3. On the basis of chromatographic data with 2-nitro-E_3 and 4-nitro-E_3 (ref. 12) and immunological binding studies we assign the peak eluted first tentatively to [4-^{125}I]E_3 and the peak eluted second to [2-^{125}I]E_3.

Recovery of radioactivity

We evaluated the recovery of radioactivity by comparing the amount of radioactivity in labelling mixtures with the amount of radioactivity in the corresponding complete eluates. Table I gives the results. The recovery is virtually quantitative; on average $\bar{x} = 101.4 \pm 2.4\%$ ($n = 6$). The values for the expected and found amounts of radioactivity correlate very well ($r = 0.9998$, $n = 6$). The linear regression analysis yields the equation

$$y = 1.003\,x + 5.06$$

where y is the amount of radioactivity recovered and x the amount of radioactivity applied to the column. The slightly elevated values in recovered radioactivity are probably due to geometric factors, *i.e.*, a larger volume of eluate in comparison with the labelling mixture, in the Curiemeter.

The radioactivity of Na^{125}I solutions was recovered quantitatively. We did not chromatograph solutions containing $^{125}I_2$ because, owing to the sulphite added to the labelling mixtures, there is only iodide present.

So far the columns have been used for up to 6 months without a significant decrease in performance. We have no indication of μBondapak being radioiodinated or releasing radioiodide in subsequent chromatograms. Flushing the column with

TABLE I

RECOVERY OF RADIOACTIVITY

Labelling mixture	*Radioactivity applied (μCi)*	*Radioactivity recovered (μCi)*	*Recovery (%)*
T_3*	323	341	105.6
T_3*	932.3	951	102
T_4*	1958.1	1973	100.7
T_4*	928.2	919	99
E_3**	434.7	442	101.7
E_3**	405.9	404	99.5

* Conditions as in Fig. 1.
** Conditions as in Fig. 2.

organic solvents, *e.g.*, acetonitrile or methanol, and water yielded only neglectible amounts of radioactivity. The baseline of the γ-detector remained virtually constant over long periods of time. From time to time, however, we had to backflush the injector system because of accidental contamination.

Determination of specific activity

The theoretical specific activity of the compounds was calculated using the specific activity of the $Na^{125}I$ solution stated by the supplier or the specific activity of the $Na^{125}I$ solution containing additional $Na^{127}I$ and the molecular weight of the respective compounds. For the experimental values the amount of labelled product was determined from UV data. The radioactivity of the collected peak was measured with a Curiemeter. As Fig. 3 shows for [^{125}I]T_3, the experimental values correlate very well ($r = 0.996$; $n = 8$) with the theoretical values. The linear regression analysis yields the equations

$$y = 1.05\,x + 17.99$$

and

$$y = 1.05\,x + 0.44$$

when the specific activity is expressed in mCi/mg and TBq/nmol, respectively, where y is the theoretical activity and x the experimental specific activity. Similar results were obtained with [^{125}I]T_4 and other labelled compounds.

DISCUSSION

Selection of method

A method for the purification of ^{125}I-labelled compounds should be specific, fast, convenient to handle and safe. As the volumes of labelling mixtures generally

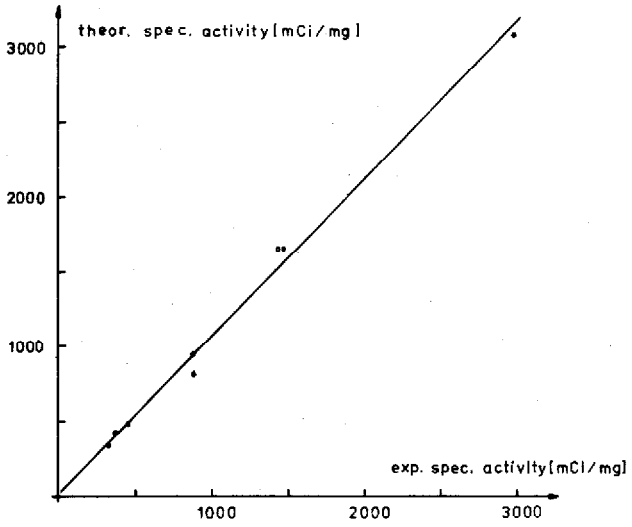


Fig. 3. Experimentally determined specific activity *versus* theoretically calculated specific activity of [^{125}I]T_3 prepared from T_2. HPLC conditions as in Fig. 1; 415.8 mCi/mg ≡ 10 TBq/nmol.

range from 25 to 500 μl the selected method should be capable of handling such volumes. For convenience and safety it is preferable to have a continuous process. Therefore, column chromatographic techniques, *e.g.*, gel filtration with Sephadex or ion-exchange chromatography, have been used more widely recently. For speed and ease of handling, however, the generally better separation power of flat-bed methods has to be traded. Using reversed-phase HPLC instead we were able to combine the advantages of both techniques.

Recovery

Glass generally absorbs substantial amounts of radioiodinated compounds or radioiodine. In our studies, between 5 and 10% of the radioactivity contained in the different reaction mixtures remained in the reaction vial and injection syringe. Therefore, in all experiments we used stainless-steel instead of glass-lined columns. As we recovered the radioactivity applied to the chromatograph practically quantitatively, we have no indication of a substantial accumulation of activity on the column, or of iodination of μBondapak C_{18} with subsequent release of radioiodine.

Mobile phase

For most purification problems associated with the preparation of ^{125}I-labelled compounds acetonitrile or methanol will suffice as organic modifier. In no instance had we to use ternary systems or gradients. As all of our radioiodinated compounds contained phenolic or other acidic groups, acetic or sulphuric acid was added to the mobile phase, thus avoiding possible interference from ion-pairing agents in RIA techniques. Owing to the small amounts of iodo compounds chromatographed, no solubility problems were observed.

Separation power

With gel filtration techniques the radioiodinated compound may not be separated sufficiently from the unlabelled starting material, *e.g.*, steroid derivatives[12], or other reaction products. With [^{125}I]T_3 we have some evidence that reduced chloramine-T is eluted on Sephadex G-75 together with [^{125}I]T_3 and interferes in the RIA. Flat-bed methods generally possess a much higher separation power, although it is sometimes difficult to separate stereoisomers. Reversed-phase HPLC yields ^{125}I-labelled compounds to highest purity in highly concentrated solutions and is able to separate stereoisomers. Hence the separation power of HPLC is at least as good as that of flat-bed methods and superior to that of gel filtration methods.

Specific activity

In contrast to flat-bed and column chromatographic methods, UV or fluorescence detection in HPLC measure quantitatively the microgram amounts of labelled compounds usually obtained in synthesis. Hence the specific activity of the radiolabelled compounds can be determined with an accuracy of $\pm 10\%$ over a wide range. Thus there is the possibility of preparing and analysing radiolabelled compounds of any desired specific activity. With the aid of a two-channel integrator system and the on-line γ-detector (work currently in progress), the specific activity of the purified material can be measured on-line.

Operating time

For the synthesis of a compound, preparation of the purification medium, isolation of the compound and determination of specific activity, HPLC takes a maximum of 30 min in operating time. This compares favourably with flat-bed methods, *e.g.*, TLC or paper chromatography, where between 90 and 120 min, and with gel filtration or ion-exchange techniques, where at least 75 min are needed.

CONCLUSIONS

Using HPLC, ^{125}I-labelled compounds of highest purity and uniformity (stereoisomers) are obtained that show much better performance in RIA than conventionally produced tracers.

Determination rather than estimation of the specific activity of compounds is possible. ^{125}I-labelled compounds of any desired specific activity can be prepared.

The operating time is reduced considerably compared with currently used techniques.

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