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Note

^{125}I-Somatostatin analogues: high-performance liquid chromatography profiles and antibody binding properties following three methods of radioiodination

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Since the discovery of somatostatin (SRIF)[1] in 1973, interest in this cyclic tetradecapeptide has grown due to its ability to inhibit the actions of a number of metabolic hormones including growth hormone, thyroid stimulating hormone and insulin[2]. The development of radioimmunoassays for somatostatin has been achieved by using an iodinated tyrosine-containing analogue[3] and a variety of methodologies affording a reliable ^{125}I-tracer have been reported. Several of these investigations[4,5] have used the chloramine-T-based Hunter-Greenwood technique[6] or variations thereof, whereas Conlon *et al.*[7] employed the Iodogen method and Arimura *et al.*[3] chose an enzymatic form of iodination, all of which afforded satisfactory radioligands.

R_1-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-R_2-Thr-Ser-Cys

	R_1	R_2
Tyr^0-Somatostatin	Tyr	Phe
Tyr^{11}-Somatostatin	H	Tyr

Fig. 1. Tyrosine-containing analogues of somatostatin.

We report here a comparison of our chloramine T radiolabelling technique[8] with that of standard Iodogen and Enzymobead methodologies in the preparation of ^{125}I[Tyr^0]-somatostatin (Fig. 1). Each label was initially purified by Sep-Pak filtration and its radiochemical purity assessed from high-performance liquid chromatography (HPLC) radioactivity profiles. In the case of the chloramine T procedure, the Sep-Pak purified but unfractionated ligands and the HPLC-fractionated products of radioiodinated [Tyr^0]- and [Tyr^{11}]-somatostatin have been individually assessed for their ability to estimate an antibody titre in several plasma samples. The HPLC profiles of aged ^{125}I[Tyr^0]- and ^{125}I[Tyr^{11}]-somatostatin labels are also discussed.

EXPERIMENTAL

Instrumentation

Chromatography was conducted using Waters Assoc. equipment consisting of

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Model 510A and Model M45 pumps controlled by a Model 660 gradient programmer and monitored using a Model 481 Lambda-Max variable wavelength detector set at 273 nm. Samples were delivered through a Waters Assoc. U6K injector onto a reversed-phase Nova Pak C_{18} analytical column.

Separation was performed using a gradient elution of 0–40% solvent B over 50 min with a flow-rate of 1 ml/min. Solvent A comprised 0.1% trifluoroacetic acid (TFA) in acetonitrile–water (20:80) and solvent B was 0.1% TFA in 100% acetonitrile. HPLC fractions were collected using a Pharmacia Frac-100 set at 0.5 min collection interval and monitored with a Packard Auto Gamma scintillation spectrometer Model 5260. Radioactive counts of Sep-Pak eluates were performed on an Ortec Model 776 gamma counter.

Chemicals and reagents

Somatostatin, [Tyr^{11}]- and [Tyr^{0}]-somatostatin were obtained from Peninsula Labs. Carrier-free $Na^{125}I$ was purchased from Amersham, Enzymobeads from Bio-Rad Labs. and Iodogen from Pierce. HPLC-grade acetonitrile and Sep-Pak cartridges were purchased from Waters Assoc. UV-grade TFA was obtained from Merck and redistilled from glass prior to use.

Antisera

A somatostatin–HSA conjugate was prepared by the method of Atassi *et al.*[9]. To obtain antisera for antibody binding studies, crossbred sheep were vaccinated with the conjugate (500 μg) in Freund's complete adjuvant emulsion (2 ml) on four occasions at four weekly intervals. Blood for antibody titre measurement was taken by jugular venepuncture one week after the final booster vaccination and was collected in heparinized tubes. Cells were removed by centrifugation and the antisera stored at $-20°C$.

Radioiodination methods

Chloramine-T. A variation of the method of Hunter and Greenwood[6] was used. The peptide (10 μg in 10 μl of 0.05 *M* phosphate buffer, pH 6.8), 100 μl of the phosphate buffer and 5 μl (18.5 MBq) carrier-free $Na^{125}I$ were added to a small stoppered fusion tube (2.0 cm × 0.5 cm). The reaction was initiated by the addition of 5 μl of chloramine-T solution (1 mg/ml of the phosphate buffer) and allowed to proceed at room temperature with occasional agitation (0.5 min, Fig. 2 or 1.25 min, Fig. 3). The reaction was "terminated" by the addition of distilled water (500 μl) and the reaction mixture directly applied to an activated Sep-Pak cartridge[10].

Iodogen. Iodogen (2 μg) was coated to the inner bottom surface of a small fusion tube (2.0 cm × 0.5 cm) as described[11]. The peptide (10 μg in 10 μl of 0.05 *M* phosphate buffer, pH 6.8) was added followed by the addition of carrier-free $Na^{125}I$ (5 μl; 18.5 MBq). The reaction mixture was allowed to react at room temperature (15 min), diluted with distilled water (500 μl) and then directly applied to an activated Sep-Pak cartridge.

Enzymobead. The peptide (10 μg in 10 μl of 0.05 *M* phosphate buffer, pH 7.2), phosphate buffer (50 μl, 0.2 *M*, pH 7.2) and an aliquot of the rehydrated Enzymobead reagent (50 μl) were added to a small stoppered fusion tube (2.0 cm × 0.5 cm). The reaction was initiated by the addition of $Na^{125}I$ (5 μl, 18.5 MBq) and allowed to

continue at room temperature for 20 min. The reaction mixture was then diluted with distilled water (500 μl) filtered and the filtrate applied to an activated Sep-Pak cartridge.

Reversed-phase Sep-Pak cartridges were prepared for use by elution with methanol (5 ml) followed by distilled water (10 ml). The radioiodination mixtures applied to these cartridges were then washed with distilled water (5 ml), to remove any unreacted iodine, followed by a wash with 0.1% TFA in acetonitrile–water (10:90) (5 ml) and finally eluted with 0.1% TFA in acetonitrile–water (60:40) (5 ml). These eluents were applied manually with a 10-ml disposable syringe at a flow-rate of *ca.* 10 ml/min and the eluent radioactivity monitored using an Ortec Model 776 gamma counter. All radioligands were stored in acetonitrile–water (60:40) at 0°C.

To fractionate the radiochemical products, a 10-μl sample (*ca.* 100 000 cpm) of the radioligand eluted from the Sep-Pak was purified using the above mentioned HPLC conditions (see *Instrumentation*). Fractions were collected every 0.5 min and radioactivity monitored on a Packard Model 5260 gamma counter. The elution of radioactivity in relation to unlabelled peptide is shown in Figs. 2 and 3.

Antibody titre assays were carried out with the ^{125}I radioligands using the method of Abraham[12].

RESULTS AND DISCUSSION

When the three labelling techniques described above were applied to [Tyr^0]-somatostatin, radiolabels with only marginally differing HPLC elution profiles were produced. The HPLC traces (Fig. 2) indicate a single major radiochemical product in each case but varying proportions of two minor products, depending on the method. These minor products are quite prominent in the profiles of both the Iodogen and Enzymobead labels but are less pronounced with the chloramine-T label.

An unanticipated problem associated with both the Iodogen and Enzymobead methodologies centred on the remarkable tendency of the somatostatin analogues to adsorb to hydrophobic surfaces. With Iodogen, transfer of the radiolabelled peptide from the silanized reaction tube to the activated Sep-Pak cartridge often resulted in the retention by the tube of almost 30% of the radioactivity. With Enzymobeads, approximately 40% of the radioactivity was retained by the beads. In either case, at

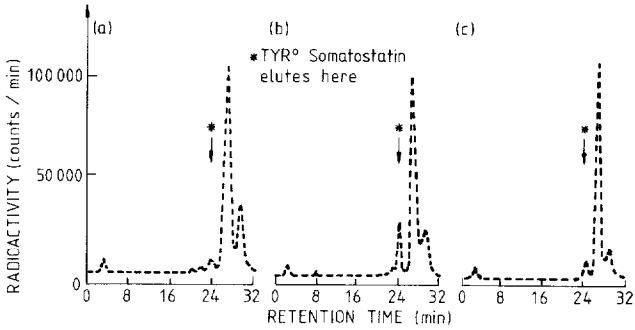

Fig. 2. HPLC radiochemical elution profiles of ^{125}I[Tyr^0]-somatostatin. (a) Enzymobeads; (b) Iodogen; (c) chloramine T.

TABLE I

COMPARISON OF THREE UNFRACTIONATED ^{125}I[TYR^0]-SOMATOSTATIN RADIOLIGANDS FOR THE MEASUREMENT OF ANTISOMATOSTATIN ANTIBODY TITRE IN SHEEP SERA

Values for sheep 1306 are means of 4 replicates. All other values are means of duplicates.

Ligand preparation (see Fig. 2)	*Reciprocal antisomatostatin antibody titre in sheep sera No.*			
	1306	*4416*	*1319*	*6187*
Chloramine T	28 363 ± 4176	6675 ± 754	4064 ± 43	1245 ± 16
Iodogen	25 457 ± 4010	4245 ± 99	3932 ± 303	631 ± 26
Enzymobead	18 887 ± 2952	2617 ± 202	1455 ± 47	544 ± 26

the risk of introducing contaminants, the retained radioactivity could be eluted by flushing the Iodogen reaction vessel or the Enzymobeads with 0.1% TFA in acetonitrile–water (60:40).

In antibody-binding studies (Table I) the antibody titre obtained with unfractionated, chloramine-T-derived ^{125}I[Tyr^0]-somatostatin tended to be greater with each of four antisera than that found for the alternative radioligands. Because this ligand had greatest radiochemical purity the observation suggests that the minor products of radioiodination may have important competitive effects on the antibody binding properties of the unfractionated ligand. The effect was examined further with HPLC-purified fractions of the one major and the two minor radiochemical products formed during prolonged chloramine-T labelling of both [Tyr^0]- and [Tyr^{11}]-somatostatin. With both somatostatin analogues peak B (Fig. 3), believed to be the respective monoiodo derivative, has superior antibody-binding properties when judged by the higher antibody titre determined for a standard antiserum with this fraction (Table II). Surprisingly, the antibody titres measured with unfractionated preparations of ^{125}I[Tyr^0]- and ^{125}I[Tyr^{11}]-somatostatin (Table II) were more comparable with those obtained with the fractionated minor products (peaks A and C) than with the fractionated major product (peak B), an effect that reinforces the qualitative nature of such measurements.

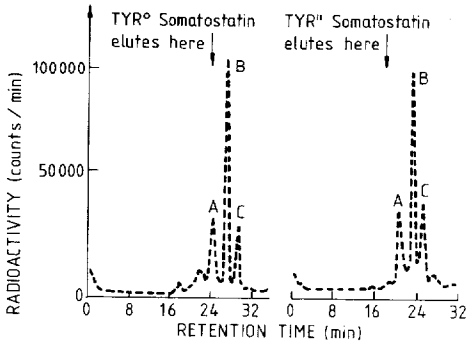

Fig. 3. HPLC radiochemical elution profiles of ^{125}I[Tyr^0]- and ^{125}I[Tyr^{11}]-somatostatin prepared with chloramine T and extended reaction conditions (1.25 min).

TABLE II

COMPARISON OF FRACTIONATED AND UNFRACTIONATED ^{125}I[TYR^0]- AND ^{125}I[TYR^{11}]-SOMATOSTATIN RADIOLIGANDS FOR THE MEASUREMENT OF ANTISOMATOSTATION ANTIBODY TITRE IN A STANDARD ANTISERUM

Values are means of duplicate measurements.

Ligand preparation (see Fig. 3)	*Reciprocal antisomatostatin antibody titre in a standard antiserum using*	
	^{125}I[Tyr^0]-somatostatin	*^{125}I[Tyr^{11}]-somatostatin*
Unfractionated Sep-Pak eluate	7618 ± 147	10 371 ± 129
Fractionated peak A	10 759 ± 521	823 ± 60
Fractionated peak B	100 000 ± 5000	67 230 ± 6260
Fractionated peak C	10 202 ± 862	2629 ± 425

The HPLC profiles shown in Fig. 4 are those of "aged" chloramine-T prepared, Sep-Pak purified ^{125}I[Tyr^0]- and ^{125}I[Tyro^{11}]-somatostatin. Comparison of these profiles with those of Fig. 2 indicates minor differences only in the amount of radioactivity eluted in the first 4 min and this is believed to be due to inorganic iodide. Thus the chemical integrity of the ligands was very largely maintained for 4 months even though a decrease in specific activity would be expected.

In conclusion, our results suggest that the chloramine-T procedure is the method of choice for the radioiodination of tyrosine-containing analogues of somatostatin. This methodology is fast, reliable and produces a more homogeneous radioligand with better antibody binding properties.

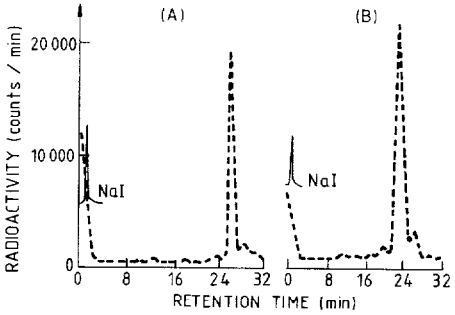

Fig. 4. HPLC radiochemical elution profiles of aged (4 months) chloramine-T-derived ^{125}I[Tyr^0]-somatostatin (A) and ^{125}I[Tyr^{11}]-somatostatin (B).

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