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Preparation of Mono-^{125}I-labelled Gastrin-17 for Radioimmunoassay Measurements

J. Nemeth, M. Vecsernyes*, G. Oroszi, Zs. Balla, Zs. Helyes, B. Farkas**, Z. Szilvássy

Department of Pharmacology and Pharmacotherapy and **Department of Dermatology, University of Pecs, H-7601, P.O.Box 99, Pecs, Hungary
*First Department of Medicine, University of Szeged, H-6701, P.O.Box 469, Szeged, Hungary

SUMMARY

In the present work the efficiency of two oxidising agents (chloramine-T and iodogen) for human gastrin-17 labelling is investigated, whilst the purification of ion-exchange chromatography is compared with that of reversed-phase HPLC. In the Iodogen method the extent of radioactive iodine incorporation can be as high as 95-98 % in contrast to 60-65 % attained using the chloramine-T method. Reversed-phase HPLC purification proved to be superior to ion-exchange chromatography on the basis of a more distinct separation of mono-iodinated gastrin underlain by specific activity value (73 to 75 TBq/mmol) near identical with the theoretical one.

Keywords: Iodination, Human gastrin-17, Iodogen, Chloramine-T, HPLC, Ion-exchange chromatography

All correspondence should be addressed to: J. Nemeth, Department of Pharmacology and Pharmacotherapy, University of Pecs, H-7601, P.O.Box 99, Pecs, Hungary; Phone/Phax: +36-72-211-761; E-mail: jnemeth@apacs.pote.hu

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INTRODUCTION

Measurement of plasma gastrin, a gastrointestinal peptide hormone by means of radioimmunoassay (RIA), is of diagnostic value in various gastrointestinal diseases, particularly in establishing or excluding Zollinger-Ellison syndrome (1). Accordingly, high purity of radiolabelled gastrin as a RIA tracer is of crucial importance to carry out reliable assessments. Radiolabel ^{125}I is the most commonly used isotope for iodination of gastrin and gastrin derivatives in order to perform RIA. Due to the electron-donating effect of a neighbouring hydroxyl group, the ortho position(s) in the aromatic rings of tyrosine in peptides and proteins is (are) activated for electrophilic substitution between pH 7 and 8. Likewise, the two nitrogen atoms in the imidazole ring of histidine possess an electron-donating effect on their mutual carbon atom (C-2) at pH 9. Following conversion of $^{125}I^-$ to $^{125}I^+$ by means of distinct oxidising agents, the latter form is incorporated into tyrosyl or histidyl according to the pH conditions mentioned above (2,3). The most widely applied oxidising agents include chloramine-T (4), hydrogen peroxide (5) and iodogen (6,7). After the labelling procedure the mono-iodinated gastrin must be separated from the other components (di-iodinated peptide, unlabelled peptide, unincorporated iodine, oxidatively damaged peptides) employing various separating techniques comprising sephadex gel filtration (8), starch gel electrophoresis (9), ion-exchange chromatography (10) and reversed-phase high performance liquid chromatography (HPLC) (11, 12).

In the present study the efficacy of two oxidising agents (chloramine-T and iodogen) for gastrin labelling is compared, whilst the purification by ion-exchange chromatography is also compared with that of reversed-phase HPLC.

MATERIALS and METHODS

Reagents

Synthetic human gastrin-17 (hG-17) was purchased from Calbiochem (Lucerne, Switzerland), $Na^{125}I$ from Izinta (Budapest, Hungary), chloramine-T, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium metabisulphite,

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hydrochloric acid, sodium hydroxide, ammonium carbonate and dichloromethane from Reanal (Budapest, Hungary), bovine serum albumin (BSA) and iodogen (1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril) from Sigma (St. Louis, USA), trifluoroacetic acid (TFA) from Fluka (Buchs, Switzerland), diethyl amino ethyl (DEAE) sephadex gel from Pharmacia (Uppsala, Sweden), acetonitrile and methanol from Merck (Darmstadt, Germany).

Techniques of iodination

Iodination of hG-17 by means of chloramine-T method

The chloramine-T method described originally by Hunter and Greenwood (4) was modified and found to be effective for gastrin labelling as follows: synthetic hG-17 (1 nmol/20 μl), 18.5 MBq/5 μl $Na^{125}I$ and chloramine-T (10 μg/10 μl) were measured into a small glass reaction tube successively. Following 20s mixing at room temperature the labelling process was terminated by addition of sodium metabisulphite (20 μg/10 μl) and 5 % (w/v) BSA (50 μl). All the reagents applied were dissolved in 0.25 mol/l (pH 7.5) phosphate buffer. Eventually, the mixture was loaded onto a chromatographic column.

Iodination of hG-17 by virtue of Iodogen method

The iodogen labelling method, which was developed in the early eighties (6,7) was adjusted to meet the requirements of gastrin labelling. Iodogen (4 μg) freshly dissolved in dichloromethane (100 μl) was dispersed in the bottom of an iodination vial and evaporated to dryness under nitrogen. Subsequently, 0.25 mol/l (pH 7.5) phosphate buffer (30 μl), synthetic hG-17 (1 nmol/20 μl) was added along with 9.25 MBq/2.5 μl $Na^{125}I$. The labelling procedure had been complete over 2.5 min at room temperature and to prevent the peptide from further oxidative damage 0.1 % (v/v) TFA was administered to stop the reaction. Finally, the mixture was loaded onto a chromatographic column for separation.

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Techniques of separation

Ion-exchange chromatography

A modified anion exchange chromatographic method was employed for purification of the hG-17 labelled according to the chloramine-T method. The ion exchange DEAE sephadex gel was treated with at first 0.5 mol/l hydrochloric acid then 0.5 mol/l sodium hydroxide for activation, subsequently it was washed several times with distilled water, finally equilibrated with 0.005 mol/l (pH 7.2) ammonium carbonate. The dimensions of the column were 10 x 0.6 cm. After being loaded, the labelled gastrin was eluted at a flow rate of 12 ml/h by using an ammonium carbonate gradient, the concentration and pH of which ranged from 0.005 to 0.5 mol/l and from 7.2 to 8.5, respectively. The concentration gradient was created in a container of constant volume (30 ml). Fractions with a volume of 2 ml were collected and counted on a gamma counter (Gamma NZ 310).

Reversed-phase HPLC

The high-performance liquid chromatograph comprised a Waters 6000 A pump and a universal liquid chromatograph injector (Waters U6K) connected to an LKB UV monitor. The type of chromatographic column applied was Hypersil 5 ODS (250 x 4.6 mm). The column was washed with methanol or acetonitrile and equilibrated with 0.1 % (v/v) TFA prior to use. The gradient of methanol or acetonitrile as organic component together with aqueous solution of 0.1 % (v/v) TFA was employed to elute the labelled gastrin at a flow rate of 1 ml/min; fractions (1ml) were taken and the radioactivity was determined.

Identification of the various radioactive products eluted

Free-iodine and mono- or di-iodinated forms of hG-17 were separated with radiochromatography (detailed above). These substances appeared as three distinct peaks and their identification was based on the binding of labelled gastrin forms to polyclonal antibodies raised against the C-terminal of hG-17 (13). Determination of specific radioactivity was performed by the self-displacement method described by Morris (14).

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Testing the influence of storage period on non-specific binding

Both the ion-exchange chromatography and HPLC purified mono-iodinated hG-17-s were stored at -20 °C for 8 weeks. For evaluation of the unspecific binding of the tracer to assay buffer proteins etc. except the antibody, the non-specific binding values were determined at intervals of 2 weeks in a RIA system and expressed in % of total radioactivity measured in the RIA tubes.

RESULTS

Ion-exchange chromatography

After iodination using the chloramine-T method, followed by DEAE ion-exchange chromatography three distinct radioactive peaks were observed (Fig. 1.)

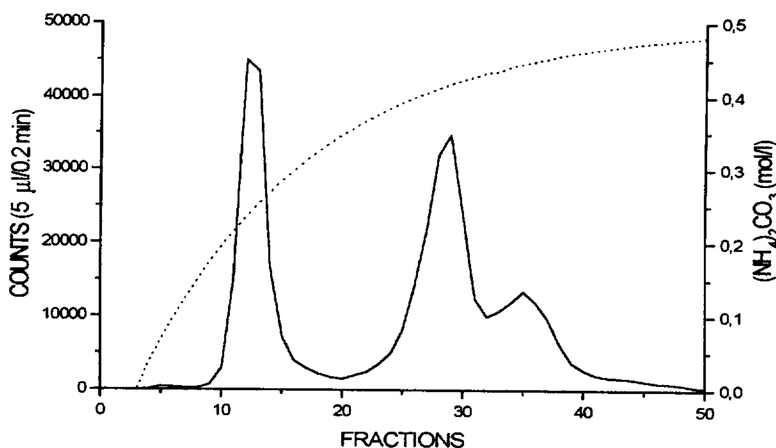

Fig. 1. Radiochromatogram of the ^{125}I-labelled hG-17 forms separated by means of ion-exchange chromatography. Dotted line designates concentration values of the ammonium carbonate gradient, continuous line shows radioactivity values of fractions collected

The first one denotes radioactivity of unincorporated iodine which has not been bound to hG-17 during the labelling process - this amounts to approximately 45 % of the total activity. The second peak reflects the radioactivity of the mainly mono-iodinated hG-17 with a specific activity of 95.4 TBq/mmol. The third peak gives the radioactivity of the predominantly di-iodinated hG-17 with a specific activity of

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116.1 TBq/mmol. The second and third peaks are responsible for approximately 40 % and 15 % of the total activity used for the iodination, respectively.

Reversed-phase HPLC (I)

After iodination of hG-17 using the chloramine-T method, the radioactive reaction mixture was separated on a reversed-phase HPLC column by employing the methanol gradient. According to the radioactivity values three distinct peaks occurred with varying retention times (Rt) (Fig. 2).

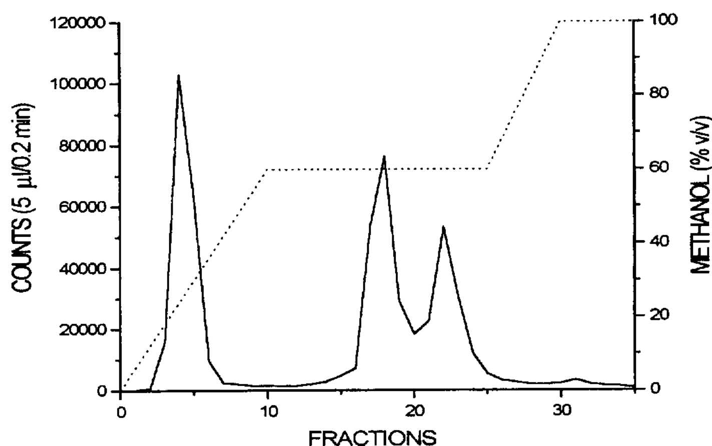

Fig. 2. Radiochromatogram of ^{125}I-labelled hG-17 forms separated by reversed-phase HPLC. Dotted line denotes concentration values of the methanol gradient, continuous line represents radioactivity values of fractions collected.

The first one represents the activity of the unincorporated iodine (Rt: 4 min), the second (Rt: 18 min) and the third (Rt: 22 min) peaks demonstrate the activity of the mono- and di-iodinated forms of hG-17, respectively. The specific radioactivity values of the mono- and di-iodinated forms of hG-17 were 73.1 and 143.4 TBq/mmol, respectively. The activity of the gastrin bound iodine accounted for approximately 60 % of the total. A methanol concentration of 60 % was found to be optimal to separate the mono- and di-iodinated gastrin forms.

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Reversed-phase HPLC (II)

Following iodination of hG-17 using the Iodogen method, the radioactive reaction mixture was separated by means of a reversed-phase HPLC employing an acetonitrile gradient. Based on the radioactivity measurements of the fractions collected the mono-iodinated gastrin (Rt: 19 min) contributes approximately 80 % of the total radioactivity. The di-iodinated gastrin occurs at a retention time of 23 min and accounts for 15 % of the entire radioactivity.

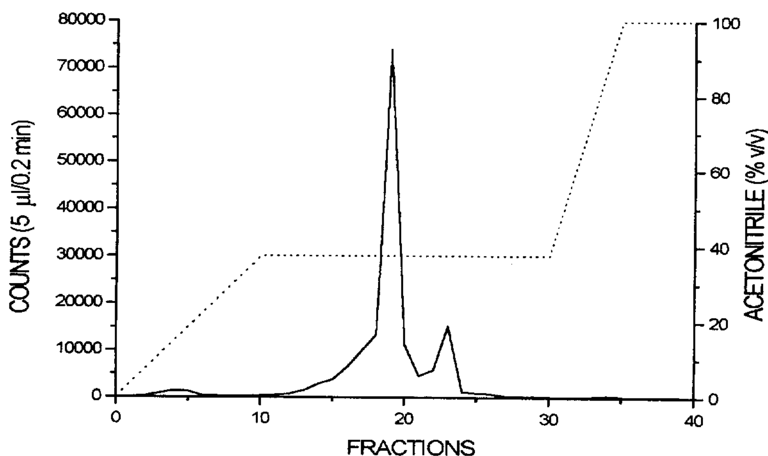

Fig. 3. Radiochromatogram of ^{125}I-labelled hG-17 forms separated by reversed-phase HPLC. Dotted line indicates concentration values of the acetonitrile gradient, continuous line demonstrates radioactivity values of fractions collected.

The activity of unincorporated iodine (Rt: 4 min) is negligible (Fig. 3). The specific activity of the mono- and di-iodinated forms of the hG-17 were 75.2 and 145.3 TBq/mmol. The optimal concentration of acetonitrile to make distinction between the mono- and di-iodinated forms of hG-17 was 37.5 %.

Impact of storage on non-specific binding

Table 1. shows non-specific binding of the tracers purified by ion-exchange chromatography and HPLC at intervals of 2 weeks. These values of mono-iodinated gastrin separated by ion-exchange chromatography reached the critical 5

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% limit over 4-week period of storage, by contrast the tracer purified by HPLC was usable as long as a 6-week storage period.

Storage time (week)	0	2	4	6	8
NSB (%) ion-exchange	1.08	3.51	6.39	9.69	15.97
NSB (%) HPLC	0.31	0.96	2.01	4.83	8.05

Table 1. Non-specific binding (NSB) values of the tracers purified by ion-exchange chromatography and reversed-phase HPLC.

CONCLUSION

The results of the present work indicate that the iodination process of hG-17 with iodogen is more effective than using chloramine-T. This latter compound is a highly potent oxidising agent (4), which can be allowed to progress for only a very restricted time (in range of seconds) to avoid oxidation of the methionine to methionine sulfoxide at position 15 (12). The efficacy of incorporation of the radioactive isotope (18.5 MBq) is only 60-65 % due to very short reaction time. By contrast, reaction time of peptide labelling by iodogen, a solid-phase oxidising agent (6,7), takes minutes without profound oxidative damage. Under optimal conditions, radioactive incorporation can be as high as 95-98 %, meaning that practically no unincorporated iodine can be found. Owing to the higher efficacy of incorporation, the activity of the applied radioactive isotope can be considerably decreased to 9.25 MBq. Moreover, the ratio of mono- and di-iodinated gastrin forms is considerably higher than that obtained with the chloramine-T method.

Separation of the mono- and di-iodinated forms of hG-17 is clearer by HPLC than by ion-exchange chromatography. Specific activity of pure mono- and di-iodinated gastrins are 78.63 and 157.26 TBq/mmol (15). The results obtained with ion-exchange chromatography markedly differ from these theoretical values. The second peak represents predominantly mono-iodinated gastrin and to a smaller extent di-iodinated form, whereas the third peak denotes mainly di-iodinated hG-17 and to a smaller extent mono-iodinated form. In contrast, specific activity of mono- and di-iodinated hG-17-s separated by HPLC are very close to the theoretical

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values evidencing a clearer separation between the two forms. Acetonitrile (37.5 % v/v) is as effective as methanol (60 % v/v) at eluting labelled hG-17-s.

The non-specific binding of mono-iodinated hG-17 purified by HPLC attains the critical limit after a longer storage period which can be attributed to higher degree of purity and the scavenging property of the eluting medium.

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