Stability of freeze-dried glucagon for tracer preparations*

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INTRODUCTION

Glucagon lebeled with radioactive iodine is widely used as a tracer in radioimmunoassay studies. Since the glucagon for radioiodination must be weighed out and dissolved immediately before use and can't be stored in solution, it is of interest to know how long the same batch of hormone could be used for labeling when freeze-dried. Thus, the stability of freeze-dried glucagon aliquots was followed during a period up to 21 months of storage at −20ºC. The changes of the hormone, before and after labeling and purification were analyzed on poly-acrylamide gel electrophoresis (PAGE) and the binding capacity of ^{125}I-glucagon to its antiserum was also evaluated.

MATERIAL AND METHODS

Two mg of twice cristallized pork glucagon (NOVO, Lot Nº G 501575) were dissolved in 0.003N HCL, divided in several aliquots of 45μg each, freeze-dried and stored at −20ºC until use for radioiodination. One, four, nine, twenty and twenty-one months later, different aliquots were used for the iodination (10μg of glucagon) with 0.5mCi of ^{125}I except the first one which was performed with 0.75mCi, using a modification of the Chloramine T technique[8]. The labeled glucagon was purified by anion exchange chromatography in OAE-Sephadex A 25 according to Jørgensen and Larsen[8].

The main peak-related fraction, from the tracer purification, and one sample of the unlabeled hormone were submitted to PAGE analysis, using 10 or 15cm long gel tubes, according to a modification of the original method of Davis[5] by Bartolini et al.*[3]. The radioactive gel was cut into segments 0.7cm long and their activity was measured in a well-type gamma counter, while the cold gel was stained in Coomassie brilliant blue according to Chrambach* et al.*[4]. The mobility rate values (Rm) of the electrophoretic components were determined in both gels relative to the tracking dye (T.D.) bromophenol blue.*

Glucagon radioimmunoassay standard curves were made up in time-expired blood bank plasma according to the assay technique described by Alford et al.*[1] employing the antiserum specific for pancreatic glucagon, RCS 5, kindly supplied by Dr. S.R.Bloom, Hammersmith Hospital, London, and dextran-coated charcoal to separate bound and free hormone.*

RESULTS AND DISCUSSION

The anion−exchange chromatograms are shown in panel A (Fig. 1). The elution pattern of radioactivity from the purifications corresponding to the iodination of the glucagon stored until 20 months, exhibited a large peak near the fraction 50 and one or two smaller ones localized around the fraction 30 or 80 (panels A1 to A4). However, on 21 months of storage, the correspondent chromatographic profile was altered revealing the main peak in fraction number 30 and a smaller one in number 80 (panel A5). The fractions corresponding to the hatched area from the major peaks were pooled and stored at −20ºC (tracer preparations a, b, c and d). The ^{125}I-glucagon contained in these preparations presented binding of the order of 60% for the three first (a, b and c) and 40% for the fourth preparation (d) after a brief incubation with an excess of antiserum. The two fractions corresponding to tubes 30 and 80, which were predominant in the last iodination (panel A5) had no affinity for the antibody, binding only to about 5% which corresponds to the non-specific binding of 4-5% obtained with all preparations in the absence of antiserum.

The fractions from the QEA-Sephadex purifications, analyzed on PAGE, presented the electrophoretograms shown in panel B (Fig. 1). The electrophoretic patterns of the unlabeled glucagon are indicated in panel C (Fig. 1). The Rm values indicated in parenthesis under the main component correspond to the purified tracer peaks in B and to the more intensely stained bands in C.

In the panels B1 to B5 the fractions present another radioactive peak which moves together with the T.D. but does not represent free ^{125}I, which according to our and other observations[2] appears two segments ahead of the T.D. This peak is more evident in the fraction resulting from the first iodination, carried out with a greater amount of ^{125}I and containing 55% of the total radioactivity in the gel (panel B1) which was observed to be decreasing with time (panel E, Fig. 2). Besides, using the same PAGE as a bound-free separation system, we could show its binding to the antibody (panel F, Fig. 2). According to its position in the gel, it could represent either a more acidic form probably due to deamidation or a smaller fragment of the lebeled hormone. Finally, it could also represent a diiodinated side-product since the substitution with iodine in the tyrosine residues induces a reduction in the pK values for the phenolic hydroxyl[7], increasing thereby its electrophoretic mobility. The findings of Heding[6], showing that degraded pancreatic ^{125}I-glucagon with a molecular weight lower than the undamaged ^{125}I-glucagon which reacted with specific antisera would be favorable to the possibility of being a labeled glucagon fragment. Though the purification pro-

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cedure removes practically all diiodotyrosine cantaining material[8], the last proposition can be considered specially in the particular case of the first iodination, since the use of an excess of radioisotope greatly increases the probability of having polyiodinated molecules[9].

After one month of storage, the unlabeled glucagon aliquot revealed on PAGE, after staining, one major band and a faster-moving anionic component, the latter possibly representing deamidated glucagon (panel C1) already present in the native material supplied by the NOVO Research Institute. Afterwards, up to nine months storage besides the major component there were two additional bands appearing on its anodal side (panels C2 and C3). After 20 months, the electrophoretic pattern was the same, but the anionic components were enlarged (panel C4). One month later, four bands could be seen, the new one being localized on the cathodal side of the major component (panel C5). One could suggest that these glucagon components are the result from not only deamidation but also non-enzymic cleavage and/or aggregation occuring during the storage above nine months. However, chemical and physicochemical analysis for characterization of these bands were not done.

The decreased Rm of the ^{125}I-glucagon occurring with the unlabeled hormone stored during 20 months (panel B4) would indicate a change in the glucagon molecule related to a loss in immunoreactivity already observed when incubated with excess antibody.

The tracer preparation used in the radioimmunoassays (a, b, c and, fig. 1, panel A) provided the standard curves ilustrated in panel D (Fig. 1). All tracers were effectively displaced from the antiserum by the standard hormone, except the last one which presented a much lower binding (curve D4). The two initial preparations (curves D1 and D2) had a higher specific binding than the third one (curve D3).

It can be concluded that the use of freeze-dried glucagon aliquots for labeling purposes, proceeding from an unique bath, can be prolonged without affecting too much the sensivity of the assay. Thus, at least up to nine months of storage at −20°C, the hormone remains unaltered, yielding suitable tracers for radioimmunoassay. After it had been stored for a longer period of time, as 20 or 21 months, it becomes improper for radioiodination, presenting several components which appear during this time.

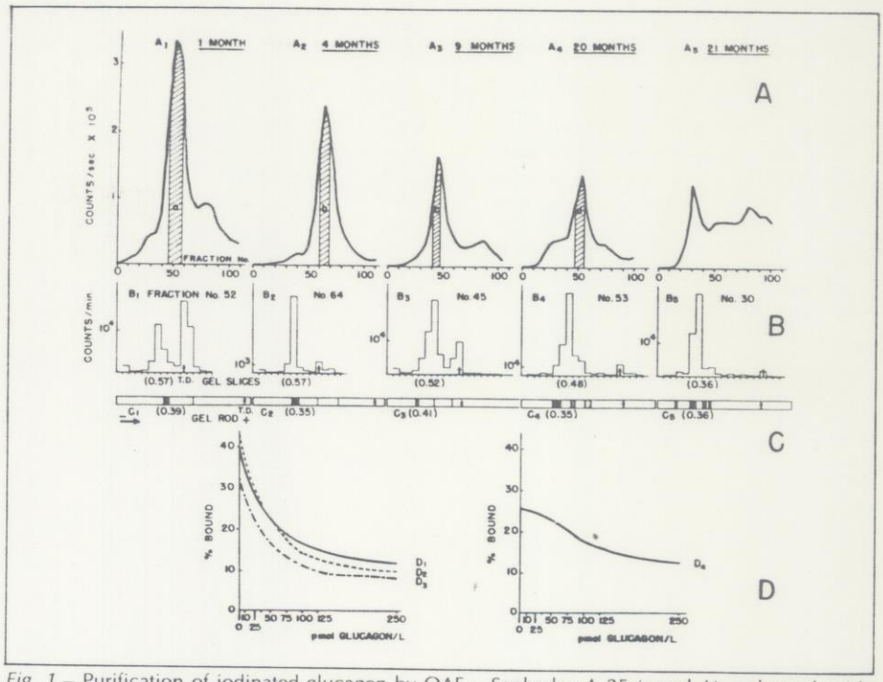

Fig. 1 – Purification of iodinated glucagon by QAE – Sephadex A 25 (panel A), polyacrylamide gel electrophoresis of the purified labeled (panel B) and correspondent unlabeled glucagon (panel C). The lower part of the figure (panel D) shows the glucagon radioimmunoassay standard curves.

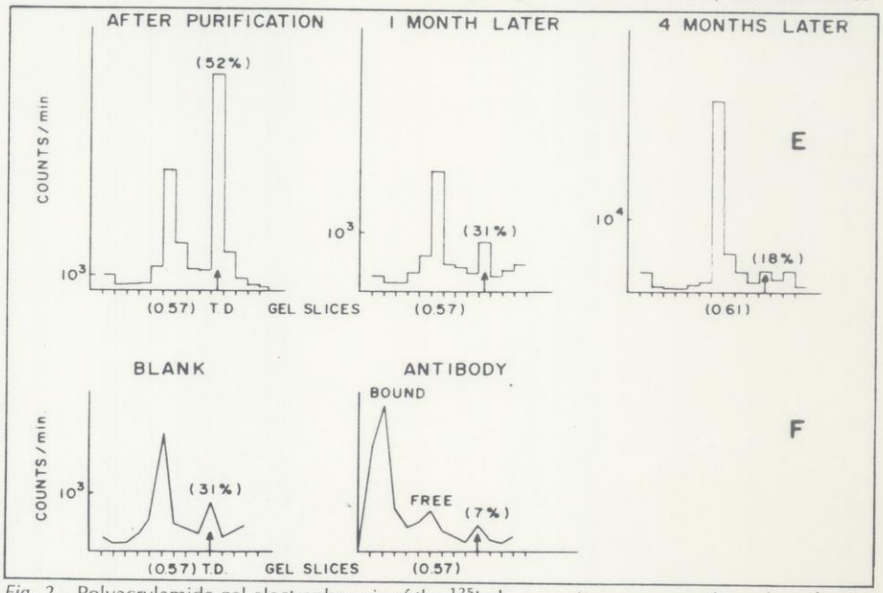

Fig. 2 – Polyacrylamide gel electrophoresis of the ^{125}I-glucagon (tracer preparation a from fig. 1): Immediately after purification, one and four months later (panel E) and separation of bound from free from the secondary component (panel F). The RM values of the main component are indicated in parenthesis and the percentual values of the secondary component relative to the total radioactivity in the gel are indicated in brackets.

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SUMMARY

The adequacy of the freeze-dried glucagon for radioiodination was evaluated during a period up to 21 months of storage at −20°C. During this period changes in the unlabeled and correspondent lebeled and purified hormones were analyzed on polyacrylamide gel electrophoresis. The immunological reactivity of the tracer was also investigated in a radioimmunoassay system. The results obtained indicate that up to the ninth months of storage the glucagon remains unchanged. After 20 months the glucagon molecule was altered with correspondent loss of tracer immunoreactivity.

RESUMO

Estabilidade de glucagon seco por congelamento para preparação de tracejadores.

A propriedade de glucagon seco por congelamento para a radioiodinação foi avaliada durante período de até 21 meses para armazenamento a −20ºC. Nesse período, alterações nos hormônios purificados rotulados e não rotulados foram analisados por eletroforese com gel de poliacrilamida. A reatividade imunológica do tracejador também foi observada num sistema de radioimunoensaio. Os resultados obtidos indicaram que até o nono mês de estocagem o glucagon permanece inalterado. Depois de 20 meses a molécula de glucagon se mostrou alterada, com correspondente perda da imunorreatividade.

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