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High-performance liquid chromatographic separation of biotinylamide analogues used as substrates in biotinidase radioassays

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Abstract

A simple, one-step protocol for synthesizing biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]iodotyramine, which are used as tracer substrates in biotinidase radioassays, is presented. This synthetic protocol uses reversed-phase HPLC for the isolation of the biotinylamide analogues from the reaction mixture. The HPLC method developed can potentially be applied to a scaled-up synthetic protocol for non-radioactive biotinylmono- and diiodotyramine. In addition, it can be used for the identification of the above-mentioned compounds.

1. Introduction

Biotinidase (EC 3.5.1.12) is the enzyme that catalyses the removal of biotin from biocytin and small biotinyl peptides [1–3]. Biotinidase deficiency is the primary enzyme defect in late-onset multiple carboxylase deficiency, which is characterized by severe symptoms leading to death [4–6]. Early diagnosis of the deficiency state is crucial for survival [7,8].

We have previously described a radioassay system for determining biotinidase activity in biological samples [9,10], using a radioiodinated biotinylamide analogue, *i.e.*, biotinylmono [^{125}I]iodotyramine (abbreviated name of N-{β-(4-hydroxy - 3 - [^{125}I]iodophenyl)ethyl}biotinamide) or biotinyldi[^{125}I]iodotyramine (abbreviated name of N-{β-(4-hydroxy-3,5-di[^{125}I]iodophenyl)ethyl}biotinamide), as a biotinidase tracer substrate, suitably diluted either with biocytin or with the corresponding unlabelled molecule. The protocol we had followed for synthesizing the above radioiodinated biotinylamide analogues was based on a two-step procedure, including two discrete, tedious and time-consuming purification steps.

Here we present a simple, one-step synthetic protocol using an HPLC method for the direct isolation of biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]iodotyramine from the reaction mixture. The HPLC protocol developed can also

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be applied to the separation of the non-radioactive biotinylamide analogues.

2. Experimental

2.1. Materials

Carrier-free $Na^{125}I$, 17 kCi/g (radiochemical purity 99.9%, iodates <2%), was a product of Nordion Europe (Fleurus, Belgium). N-Hydroxysuccinimidobiotin was purchased from Pierce (Rockford, IL, USA). Biotin, tyramine, trifluoroacetic acid (TFA) (amino acid sequencing grade), acetonitrile, water (HPLC grade) and all the other reagents were obtained from Merck–Schuchard (Darmstadt, Germany).

2.2. Synthesis of the biotinidase tracer substrates biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]iodotyramine

A 10-μl volume of $Na^{125}I$ solution [250 Ci/l in 0.25 *M* phosphate buffer (pH 7.5)] and 5 μl of chloramine-T solution [3.55 m*M* in 0.25 *M* phosphate buffer (pH 7.5)] were incubated with 10 μl of aqueous tyramine solution (0.73 m*M*) for 70 s at 20–22°C, then 5 μl of sodium metabisulphite solution [5.26 m*M* in 0.25 *M* phosphate buffer (pH 7.5)] were added. After adjusting the pH to 8.5 with 30 μl of 0.2 *M* borate buffer (pH 9.0), 90 μl of N-hydroxysuccinimidobiotin solution (0.98 m*M* in anhydrous dioxane) were added and incubated for 90 min at 20–22°C. Aliquots of 20 μl volume of the reaction mixture (total volume 150 μl) were injected into a Waters Model 600E HPLC system equipped with a Beckman Model 171 radioisotope detector for β- or low γ-radioactivity. The HPLC separation method used is described below. The radioactive peaks corresponding to retention times of 18.6 min (biotinylmono-[^{125}I]iodotyramine) or 23.2 min (biotinyldi[^{125}I]-iodotyramine) were collected, concentrated to remove organic solvent, diluted to 1.4 mCi/l with 0.25 *M* phosphate buffer (pH 7.5), divided into 3-ml aliquots, lyophilized and stored at 4°C.

2.3. HPLC separation method

The chromatographic column used was LiChrospher RP-18 (250 × 4.6 mm I.D., 5-μm particle size) (Merck), protected by a μBondapak C_{18} (Waters) HPLC-precolumn insert. Solvent A was 0.05% TFA in water and solvent B was 60% acetonitrile in solvent A. Linear gradient elution was applied from 10 to 53% B in 15 min, isocratic at 53% B for 5 min and linear from 53 to 100% B in 10 min, at a flow-rate of 1.6 ml/min. All chromatographic procedures were performed at room temperature. Detection was by measurement of γ-radioactivity emission, except where indicated otherwise.

3. Results and discussion

In previous work [9,10] we recommended the synthesis of the two radioiodinated biotinylamide analogues and their use as tracer substrates in biotinidase radioassays. The synthesis of these analogues was performed in two discrete steps. First, tyramine was radioiodinated to mono[^{125}I]iodotyramine and di[^{125}I]iodotyramine, and then the radioiodinated tyramines were isolated by paper electrophoresis. Second, purified mono[^{125}I]iodotyramine or di[^{125}I]iodotyramine was separately reacted with N-hydroxysuccinimidobiotin to prepare biotinylmono-[^{125}I]iodotyramine or biotinyldi[^{125}I]iodotyramine, respectively, which was finally purified by thin-layer chromatography (TLC). Previous purification of mono[^{125}I]iodotyramine and di[^{125}I]iodotyramine and their separate biotinylation were necessary because the TLC and paper electrophoresis separation conditions used did not permit the separation of biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]-iodotyramine from each other or from substances that would have been present in the reaction mixture if the radioiodinated tyramine isolation step had been omitted. Thus, the R_F values for biotinylmono[^{125}I]iodotyramine (0.70) and biotinyldi[^{125}I]iodotyramine (0.67) were al-

most identical in the TLC system used, whereas biotinyltyramine was found to be co-chromatographed with the biotinylmono[^{125}I]iodotyramine [11]; in addition, partial overlap between biotinylmono[^{125}I]iodotyramine and di[^{125}I]iodotyramine was observed in the paper electrophoresis system used. On the other hand, complete separation of biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]iodotyramine from each other and from any possible contaminating substance is critical for developing a reliable and sensitive biotinidase radioassay, as slight contamination of these biotinidase tracer substrates may alter the apparent enzyme K_m and/or their specific radioactivity.

In this work, the previous two-step synthetic protocol was replaced with a one-step, simpler and faster procedure with higher overall yield. Using this procedure, the separation of mono[^{125}I]iodotyramine and di[^{125}I]iodotyramine was eliminated and the reaction proceeded directly to the formation of biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]iodotyramine, which were isolated from the reaction mixture using the HPLC method described under Experimental.

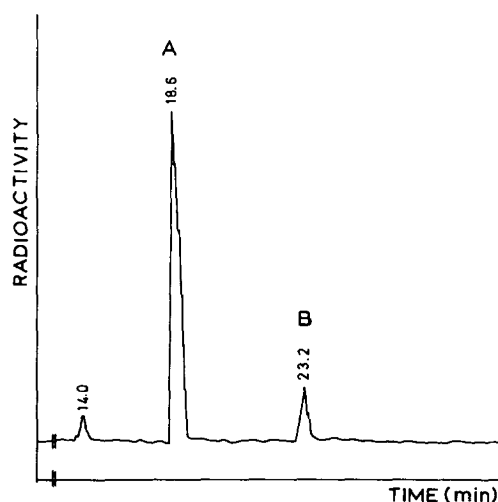

Fig. 1. Isolation of biotinylmono[^{125}I]iodotyramine (A, retention time 18.6 min) and biotinyldi[^{125}I]iodotyramine (B, retention time 23.2 min) from their synthetic reaction mixture by HPLC. The small peak preceding A (retention time 14.0 min) corresponds to unreacted mono[^{125}I]iodotyramine. For conditions, see Experimental.

Fig. 1 shows the chromatographic profile of a 20-μl sample of the reaction mixture. The radioactive peaks corresponding to retention times of 14.0, 18.6 and 23.2 min were unreacted mono[^{125}I]iodotyramine, biotinylmono[^{125}I]iodotyramine and biotinyldi[^{125}I]iodotyramine, respectively. No unreacted di[^{125}I]iodotyramine was detected under the conditions used. A small radioactive peak (not shown) at the beginning of the chromatogram was attributed to free radioiodine. The above peaks were identified using the corresponding non-radioactive compounds as standards, which were synthesized according to Evangelatos *et al.* [10]. The detection of non-radioactive peaks was achieved by means of a Waters Model 484 UV absorbance detector at 220 nm.

In order to examine further the effectiveness of the HPLC method in separating the synthesized biotinylamide analogues from any possible contaminating substances, we also determined the retention times of substances such as tyramine, biotin, N-hydroxysuccinimidobiotin and biotinyltyramine, which may be present in the reaction mixture. Fig. 2 shows the HPLC profile and the retention times of a mixture of the above substances together with the non-radioactive biotinylmonoiodotyramine and biotinyldiiodotyramine; non-radioactive monoiodotyramine and diiodotyramine were also injected. As can be seen, the biotinylamide analogues were completely separated from all the substances tested under the chromatographic conditions used.

If one is interested exclusively in the biotinylmonoiodotyramine, which is considered a better biotinidase substrate than biotinyldiiodotyramine [10], then one can use an alternative gradient elution system (linear gradient from 10 to 50% B in 10 min and from 50 to 53% B in 5 min, isocratic at 53% B for 5 min, then linear from 53 to 60% B in 5 min and from 60 to 100% B in 5 min). This alternative system increases even more the difference between the retention times of biotinylmonoiodotyramine (15.4 min) and diiodotyramine (18.0 min) while keeping

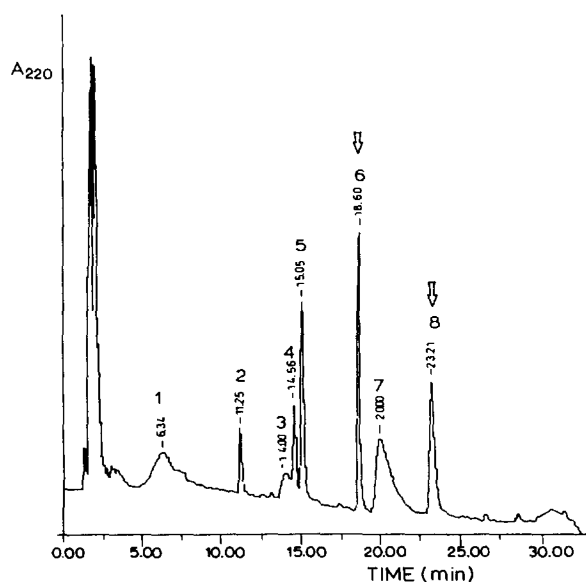

Fig. 2. Chromatographic profile obtained for a mixture of (1) tyramine, (2) biotin, (3) non-radioactive monoiodotyramine, (4) biotinyltyramine, (5) N-hydroxysuccinimidobiotin, (6) non-radioactive biotinylmonoiodotyramine, (7) non-radioactive diiodotyramine and (8) non-radioactive biotinyldiiodotyramine. For conditions, see Experimental.

almost unaffected the difference between those of biotinylmonoiodotyramine and biotinyldiiodotyramine (20.1 min). However, using this system, the difference between the retention times of biotinyldiiodotyramine and diiodotyramine decreases. Hence the above system is suitable for isolating biotinylmonoiodotyramine, but it is not recommended for the isolation of biotinyldiiodotyramine.

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