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Short communication

Double purification of radiolabelled D-fructose by high-performance liquid chromatography for tracing its metabolism

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Abstract

This paper describes the microsynthesis and the purification of various ^{14}C- or ^{3}H-labelled tracers of D-fructose. These tracers were prepared from the corresponding ^{14}C- or ^{3}H-tracers of D-glucose by interconversion in alkaline media, and then purified by HPLC using a carbohydrate column constituted of a calcium-loaded sulfonated polymer. This work also documents that the purification step is essential to investigate in a reliable manner the metabolism of D-fructose in isolated rat pancreatic islets.

Keywords: Fructose; Carbohydrates; Glucose

1. Introduction

Our recent finding that D-glucose confers to glucokinase positive cooperativity towards D-fructose phosphorylation by purified human B-cell glucokinase draws attention to new potential modalities for the reciprocal effects of D-glucose and D-fructose upon their respective metabolism in pancreatic islets [1]. Since various ^{14}C- or ^{3}H-labelled tracers of D-fructose are not commercially available, the aim of this work was to prepare and purify such tracers in order to investigate in greater detail the metabolism of the ketohexose in intact islets.

The alkali-catalyzed epimerization of aldoses to ketoses, referred to as the Lobry de Bruyn-Alberda van Ekenstein transformation, has been known for more than a century [2]. This reaction has been used for isomerisation of different sugars under various conditions (see Ref. [3] for review). The interconversion of D-glucose into D-fructose in aqueous alkali represents one of these epimerizations. However the yield of this transformation was rather low, e.g., after 20 days incubation in 0.035 *M* sodium hydroxide at 35°C the interconversion of a molar solution of D-glucose into D-fructose did not exceed 30% [4]. Mendicino [5] was maybe the first to develop a method giving yields higher than 50%. However, the low amount of hexose, ranging from micro- to millimoles, used in this method made it unsuitable for industrial use. On the contrary, this became very interesting for the preparation of radiolabelled tracers which are generally synthesized in low amounts. In the present work, this method was used with slight modifications.

2. Experimental

2.1. Chemicals

Sodium hydroxide, sodium borate, water (HPLC grade) were supplied by Merck (Darmstadt, Ger-

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many); Dowex resin (50W-X8 200–400, H^+ form) and methanol (HPLC grade) by Aldrich (Milwaukee, WI, USA). D-[1-^{14}C]glucose, D-[3,4-^{14}C]glucose, D-[U-^{14}C]glucose, D-[5-^{3}H]glucose and D-[U-^{14}C]fructose were purchased from NEN (Boston, MA, USA); D-[2-^{14}C]glucose and D-[5-^{3}H]fructose from Biotrend Chemikalien (Köln, Germany) and D-[6-^{14}C]glucose from Amersham (Nycomed Amersham, Little Chalfont, UK).

2.2. Equipment

The high-performance liquid chromatography (HPLC) apparatus consisted of a Model 307 pump (Gilson, Middelton, WI, USA) equipped with a 100-μl sample loop valve injector (Rheodyne, Cotati, CA, USA). Two carbohydrate columns and their respective pre-columns were used. The Polyspher CH CA column (300×6.5 mm, 8 μm) was purchased from Merck and the Sugar Pak I column (300×6.5 mm, 10 μm) was a gift from Waters (Milford, MA, USA). Both were made of sulfonated styrene–divinylbenzene resins in calcium form. The columns were maintained at the required temperature by use of a L-7350 column oven (Merck–Hitachi, Darmstadt, Germany). The fractions were collected by a 2112 Redirac fraction collector (LKB, Bromma, Sweden).

2.3. Synthesis

In order to prepare the corresponding ^{14}C- or ^{3}H-labelled tracers of D-fructose, 0.05 to 3 μmol of radioactive D-glucose were placed in a 3.5-ml borosilicate glass tube. As these tracers are in ethanol–water solution (9:1, v/v), they were first evaporated to dryness under nitrogen flux. To the desiccate, 40 μl of water, 80 μl of 0.1 *M* sodium borate and 80 μl of 0.5 *M* sodium hydroxide were successively added and mixed. The tube was immediately heated to 90–92°C in a water bath for 90 s and was kept thereafter on ice for 3 min. The solution was then passed on a Dowex 50W-X8 (H^+) column constituted by 1 ml of a water suspension of the resin (1:1, v/v) placed in a Pasteur pipette. The reaction products were eluted with 1.5–2 ml of an water–methanol solution (1:1, v/v). The eluate was evaporated to dryness in a Speedvac concentrator (Savant, Farmingdale, NY, USA) and the resulting material resolubilized in 0.5 ml of pure methanol. This procedure was repeated twice and the final product solubilized in 200 μl of water for separation of hexoses by HPLC.

2.4. Chromatography

The final product was injected in the HPLC system and eluted with water (HPLC grade) at a flow-rate of 0.5 ml/min and a column temperature of 70°C. Fractions were collected at a rate of one test tube per 18 s during 15 min. At the end of the run, aliquots (1–2 μl) from each tube were taken and placed in counting vials containing 5 ml of scintillation liquid. Radioactivity of each fraction was assessed in a Tri-Carb 2100 TR counter (Packard, Meriden, CT, USA) and chromatograms were obtained from these results.

3. Results and discussion

For purely preparative synthesis, Mendicino only used millimolar amounts of D-glucose [5]. The addition of the HPLC technique to this synthesis allowed us to synthesize and to purify very low amounts of D-fructose and to apply this technique to the preparation of radioactive tracers of the ketohexose otherwise unavailable, except by very expensive customized synthesis.

For the carbohydrate columns used, the mode of separation is based on hydroxyl coordination to the metal cation [6]. The different configuration of the hydroxyl groups on the carbohydrates induces different interaction strengths and thus allows the separation of the different sugars. As shown in Fig. 1, the separation of D-fructose from D-glucose is very effective. Although the analytical columns used are based on ion-exchange resins, the elution of the analytes is performed with water. The simplicity of the eluent and the absence of any salt or buffer allowed to collect the different analytes without any further manipulation like desalting or other. After the separation of D-glucose and D-fructose, the test tubes corresponding to each hexose were pooled together. The two fractions obtained were then evaporated to

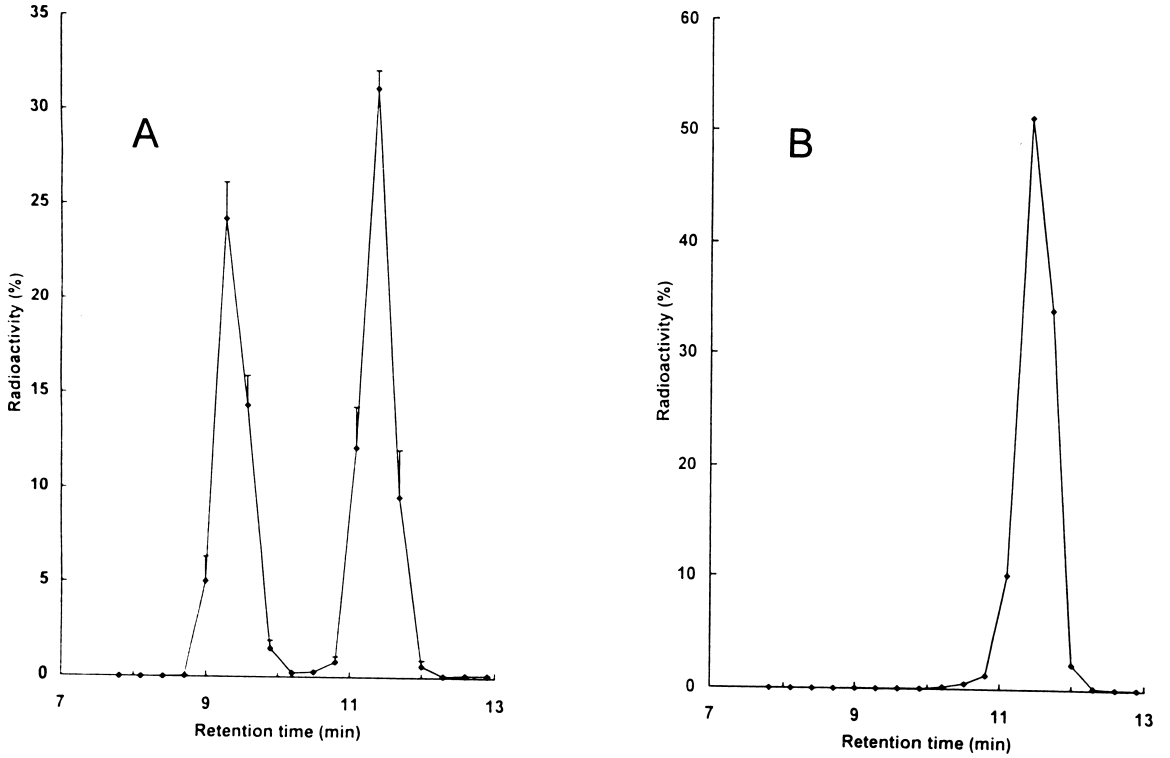

Fig. 1. (A) High-performance liquid chromatogram of a mixture of D-[U-^{14}C]glucose and D-[U-^{14}C]fructose standards in a close to 4:6 ratio. D-Glucose is eluted first (retention time=9 min 18 s) and D-fructose second (retention time=11 min 24 s). The ^{14}C content of each carbohydrate being expressed relative to the total amount of the hexose radioactivity. Mean values refer to five individual experiments. Conditions are described in Section 2. (B) Typical purity test of a tracer of D-fructose (D-[U-^{14}C]fructose in this case) after the purification steps. Same presentation as (A).

dryness in a Speedvac concentrator. The desiccate corresponding to D-glucose was kept to be used in new synthesis and the one corresponding to D-fructose was submitted to a second purification step following the same conditions of chromatography. After this second purification, the test tubes corresponding to D-fructose were pooled together, evaporated to dryness and dissolved with an ethanol–water solution (4:6, v/v). The radioactivity of the solution was assessed by counting an aliquot of the final solution. The overall yield ranged from 30 to 45% which is in good agreement with the 50% value given by Mendicino for the synthesis of larger amounts of the ketohexose.

The purification is the most important step in this procedure. Indeed, after the first purification of the synthesized radioactive D-fructose, small amounts of the corresponding tracer of D-glucose are still present. Relative to the total radioactivity, the level of this contaminant was a few tenths of percent. Similar results were found with commercial tracers of D-fructose as reported earlier [1].

However, even such a small contamination may lead to incorrect conclusions. The oxidation of 10 m*M* D-[U-^{14}C]fructose in isolated rat pancreatic islets is a good illustration of this potential problem. Table 1 reveals that the addition of D-glucose (10 m*M*) increased the oxidation of D-[U-^{14}C]fructose up to twice of the mean value found in the absence of the aldose. However, when the same experiment was repeated with a commercial preparation of D-[U-^{14}C]fructose contaminated with a tracer amount of radioactive D-glucose (less than 1% of the total radioactivity), the results were quite different. First, the oxidation of D-fructose was overestimated and, second, the enhancing effect of added D-glucose was

Table 1
Oxidation of a commercial preparation of D-[U-^{14}C]fructose (10 m*M*) examined before and after purification in isolated rat pancreatic islets incubated in the absence or in the presence of 10 m*M* D-glucose

	D-[U-^{14}C]Fructose oxidation[a]	
	Commercial	Purified
Control (no D-glucose)	6.93±0.48 (39)	4.43±0.37 (40)
D-glucose (10 m*M*)	7.71±0.47 (19)[b]	8.84±0.43 (19)[c]

[a] Results (±S.E.M.) expressed as pmol of D-fructose equivalent/islet per 120 min.
[b] n.s. for the effect of D-glucose upon D-[U-^{14}C]fructose oxidation.
[c] $p<0.001$ for the effect of D-glucose upon D-[U-^{14}C]fructose oxidation.

no more observed. These differences are easily explained by the fact that D-glucose is better metabolized in pancreatic islets than D-fructose. The small amount of radiolabelled D-glucose is thus very quickly and completely metabolized, so that the absolute amount of $^{14}CO_2$ produced is now doubled. This experiment thus illustrates the absolute necessity to use very pure products in such metabolic studies.

Using preparations of D-[5-^{3}H]fructose, D-[U-^{14}C]fructose, D-[1-^{14}C]fructose, D-[2-^{14}C] fructose, D-[3,4-^{14}C]fructose and D-[6-^{14}C]fructose, we have reevaluated the metabolism of the ketose in isolated rat pancreatic islets and the influence of D-glucose thereupon. Table 2 provides the absolute values for the conversion of D-fructose (10 m*M*) labeled with ^{14}C or ^{3}H to $^{14}CO_2$ or $^{3}H_2O$ in isolated rat pancreatic islets. It also documents the effect of D-glucose (10 m*M*) relative to the corresponding values (no D-glucose) recorded within the same experiments. D-Glucose, whilst decreasing both the overall rate of D-[5-^{3}H]fructose utilization and the fractional contribution of the pentose pathway to the generation of CO_2 and D-glyceraldehyde-3-phosphate, stimulates preferentially the oxidative, as distinct from anaerobic, modality of D-fructose metabolism by glycolysis (for complete metabolic study see Ref. [7]).

In conclusion, this work proposes a cheap and simple method for the preparation and purification of radiolabelled D-fructose. The availability of these tracers allowed us to reevaluate the metabolism of the ketohexose in pancreatic islets, and to illustrate the importance of the purity of such tracers. Since the interconversion of carbohydrates is not limited to glucose and fructose, this method could find other interesting applications.

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Table 2
Effect of D-glucose upon the production of $^{3}H_2O$ or $^{14}CO_2$ by islets exposed to 10 m*M* D-fructose in the presence of distinct ^{3}H- or ^{14}C-labelled tracers of the ketohexose

D-Fructose	Control (no D-glucose)[a]	D-Glucose (10 m*M*)[b]
D-[5-^{3}H]fructose	22.01±0.64 (176)	85.8±2.5 (120)
D-[U-^{14}C]fructose	4.40±0.29 (81)	200.7±6.1(50)
D-[1-^{14}C]fructose	6.8±0.3 (50)	98.1±4.4 (38)
D-[2-^{14}C]fructose	4.7±0.4 (28)	131.3±7.8 (16)
D-[3,4-^{14}C]fructose	7.0±0.4 (28)	175.3±4.7 (15)
D-[6-^{14}C]fructose	4.7±0.2 (47)	132.5±6.9 (35)

[a] Results expressed as pmol of D-fructose equivalent/islet per 120 min.
[b] Results expressed in percent of the corresponding control value.

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