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Short communication

Ion-pair high-performance liquid chromatographic separation of two thyroxine glucuronides formed by rat liver microsomes

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Abstract

A simple reversed-phase ion-pair high-performance liquid chromatographic separation method has been developed for thyroxine (T4) and its glucuronide metabolites formed by liver microsomes of untreated and 3-methylcholanthrene-treated rats. Besides the phenol-T4-glucuronide, another, probably acyl-T4-glucuronide, formation has been detected. The effect of pH and temperature on the stability of the acyl-T4-glucuronide was also investigated. The lowering of pH to 2 and cooling the samples to 5°C is necessary to prevent the hydrolysis of acylglucuronide, while both pH and temperature do not affect the stability of the phenol-T4-glucuronide. The retention times of T4 and phenol-T4-glucuronide are highly influenced by the pH of the mobile phase, but not that of acyl-T4-glucuronide.

Keywords: Thyroxine; Thyroxine glucuronides

1. Introduction

Conjugation by the hepatic UDP-glucuronyltransferase (UDPGT) system is one of the major metabolic pathways of the thyroid hormone, thyroxine (T4) [1,2]. The resulting glucuronides are excreted into the bile. Some chemicals, like 3-methylcholanthrene, that induce some UDPGT-s activity have been known to enhance the metabolic clearance of T4, resulting in the decrease of serum T4 concentration [3,4]. The low plasma T4 level increases the secretion of thyroid-stimulating hormone, and chronic exposure of rats or humans to UDPGT inducers may cause morphological changes in the thyroid [5].

Many methods have been described to separate and determine T4-glucuronide, most of them are based on the separation of T4 from T4-glucuronide according to radioactivity measurements of ^{125}I-labelled T4 [1,6]. This paper describes a reversed-phase ion-pair HPLC method capable of separating T4 and its glucuronides. This method made possible to demonstrate that two different T4-glucuronides are formed by rat liver microsomes.

2. Experimental

2.1. Reagents

Thyroxine and Triton X-100 were purchased from Reanal (Budapest, Hungary); UDP-glucuronic acid (UDPGA) and 3-methylcholathrene were from Sigma (St. Louis, MO, USA), Sodium dodecyl

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sulfate (SDS) was from Merck (Darmstadt, Germany). Other chemicals were of the highest purity available. Methanol and acetonitrile were HPLC grade from Chemolab (Budapest, Hungary).

2.2. Animals

LATI male Wistar rats (Gödöllô, Hungary), weighing 100 g and having free access to food and water, were either untreated or treated with 3-methylcholanthrene (i.p. 20 mg/kg for three days), liver microsomes were prepared and stored at −80°C [7]. Protein content was determined according to the method of Lowry et al. [8] by using bovine serum albumin as standards.

2.3. Enzyme assays

The incubation medium for determination of T4-glucuronide formation contained microsomes (4 mg protein/ml), in 0.1 *M* Tris–HCl pH 7.4, 6 m*M* $MgCl_2$, 1 m*M* UDPGA, 75 μM T4 in a final volume of 1 ml. Microsomes were preincubated with 0.05% of Triton X-100 for 30 min at 0°C. Incubations were performed at 37°C for 60 min, and were terminated by adding 0.2 ml of 5% trichloroacetic acid. Incubations with samples in which T4 or UDPGA were omitted served as control samples.

2.4. Sample preparation

Samples were mixed with 3 ml of 0.5 *M* ammonium sulfate (pH 9.3) just before being passed through the Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA). The cartridge was washed with 20 ml of 0.005 *M* of ammonium sulfate (pH 9.3) and 0.5 ml of distilled water. T4 and its glucuronides were eluted with 3 ml of 40% acetonitrile–38% methanol in 0.02 *M* potassium phosphate buffer (pH 2.1).

2.5. Reversed-phase ion-pair HPLC

The chromatographic equipment consisted of 2 Isco Model 2350 pumps, an Isco V^4 type UV detector (Isco, Lincoln, NE, USA), a Hypersil ODS 150×4 mm I.D. reversed-phase column with particle size of 3 μm (BST, Budapest, Hungary). The mobile phase consisted of 40% (v/v) acetonitrile–10% methanol in 0.02 *M* potassium phosphate buffer (pH 1.8)–2.5 m*M* SDS. The flow-rate was 0.5 ml/min for 5 min and 1.2 ml/min for 15 min. The temperature was ambient (22–25°C) and the detector wavelength was set at 234 nm.

3. Results and discussion

Representative chromatograms are shown in Fig. 1 and Fig. 2. The retention time of T4 was 18.3 min, of phenol-T4-glucuronide ($T4Gl_1$) 5.7 min, and of the other glucuronide ($T4Gl_2$) 4.1 min. Both metabolites can be shown only in samples which contained the entire incubation mixture. The formation of both metabolites is linear with the protein content of the microsomes (Fig. 3). Methylcholanthrene treatment enhances the formation of both $T4Gl_1$ and $T4Gl_2$, 6.4- and 3.0-times respectively.

Hydrolysis with β-glucuronidase was used to confirm that the products of metabolism were indeed glucuronides. An aliquot of the eluate after Sep-Pak cartridge treatment was evaporated to dryness and was diluted in 0.1 *M* sodium acetate buffer of pH 4.5. One part of the sample was incubated with 4000 units of β-glucuronidase, and another part was incubated without the enzyme at 37°C for 24 h. After β-glucuronidase hydrolysis both $T4Gl_1$ and $T4Gl_2$ disappeared. In the sample incubated without the enzyme the amount of $T4Gl_1$ was preserved, while $T4Gl_2$ was not detected (Fig. 4).

T4 molecules have two potential places for glucuronide conjugation, the phenol-OH and the carboxylic group of the amino acid. Many reports describe the formation of the phenol-T4Gl, the effects of different inducing agents on its formation [3,4], but no publication appeared on other metabolites produced by UDPGTs. It is well documented that the products of acyl glucuronidation are very unstable when the pH is higher than 4, and the temperature is higher than 4°C [9–11]. Acyl glucuronides are readily hydrolyzed back to the aglycones, or rearranged by intramolecular transesterification of the hydroxyl groups of the glucuronic acid, resulting in β-glucuronidase resistant conjugates [12].

In our experiments 24 h at pH 4.5 and 37°C were enough for complete hydrolysis of $T4Gl_2$. The stability of this metabolite was measured at pH 8 at

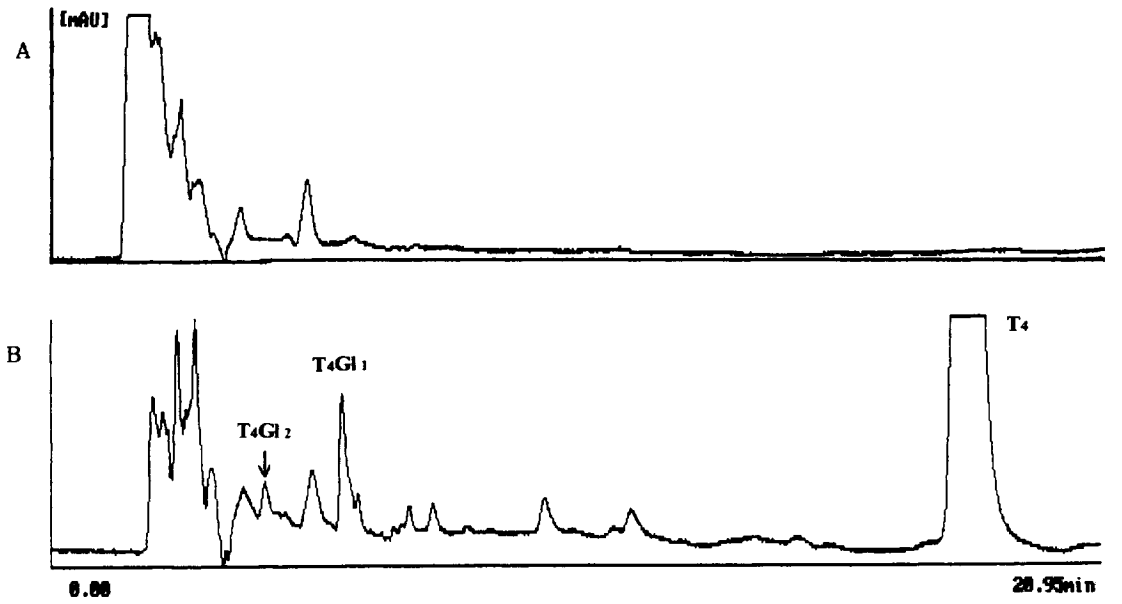

Fig. 1. Chromatograms of T4 incubations with untreated rat liver microsomes. Peaks: T4 = thyroxine; $T4Gl_1$ = phenolic-thyroxine-glucuronide; $T4Gl_2$ = acyl-thyroxine-glucuronide. (A) Blank incubation without of T4; (B) whole system.

−20°C also. After 8 h the total amount of $T4Gl_2$ disappeared.

The dependence of retention time of $T4Gl_1$, but not of $T4Gl_2$ on the pH value of the mobile phase proves that the site of conjugation is very critical for the chromatographic behaviour of the two glucuro-

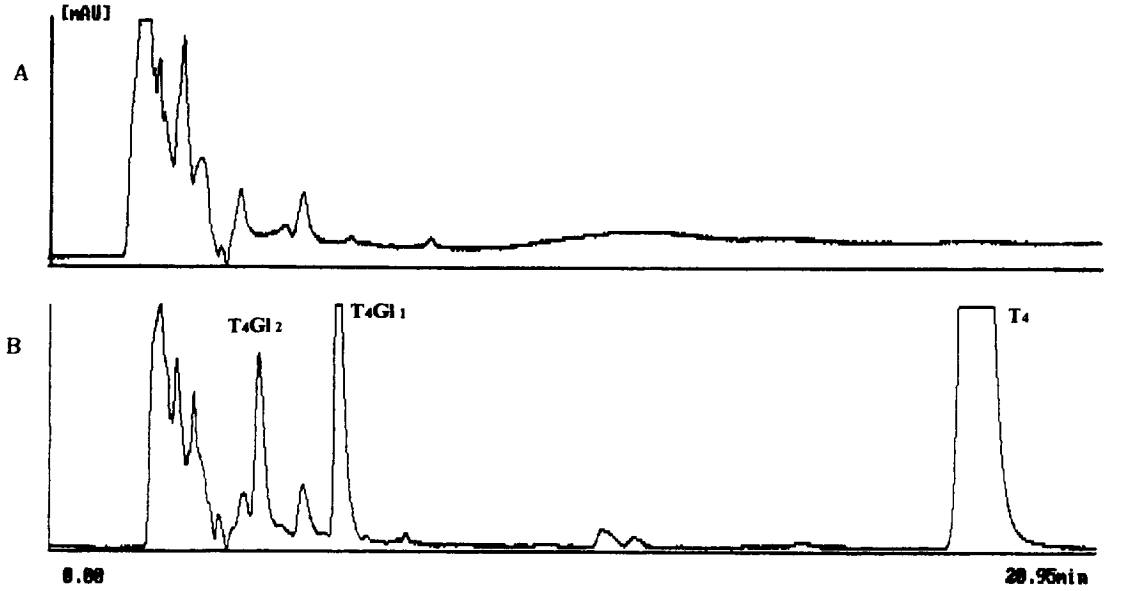

Fig. 2. Chromatograms of T4 incubations with 3-methylcholanthrene induced rat liver microsomes. Peaks: T4 = thyroxine; $T4Gl_1$ = phenol-thyroxine-glucuronide; $T4Gl_2$ = acyl-thyroxine-glucuronide. (A) Blank incubation without T4; (B) whole system.

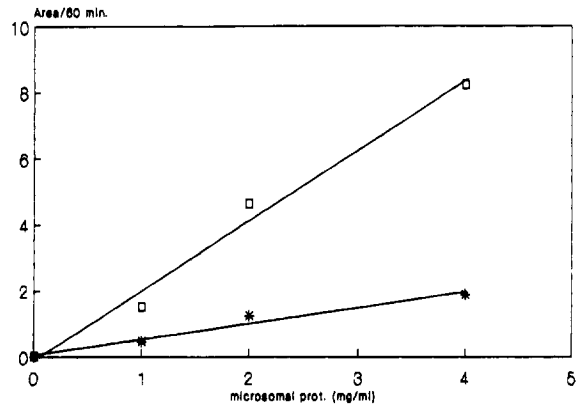

Fig. 3. The formation of T4 glucuronides by MC-treated rat liver microsomes in the function the amount of microsomal protein. (□) Phenol-T4-glucuronide; (*) acyl-T4-glucuronide.

nides. The retention time of $T4Gl_1$ is altered with the pH of the eluent which proves that its carboxylic group is free. The retention time of $T4Gl_2$ remained

Table 1
Effect of pH upon the HPLC retention times of T4 and its glucuronide conjugates measured by HPLC

pH	Retention time (min)		
	T4	$T4Gl_1$	$T4Gl_2$
3.0	8.2	3.1	4.1
2.5	10.5	3.7	n.s.[a]
2.1	16.1	5.2	4.1
2.0	16.6	5.5	4.1
1.8	18.3	5.8	4.1

[a] n.s. = not separated; $T4Gl_2$ could not be separated from $T4Gl_1$ at this pH value.

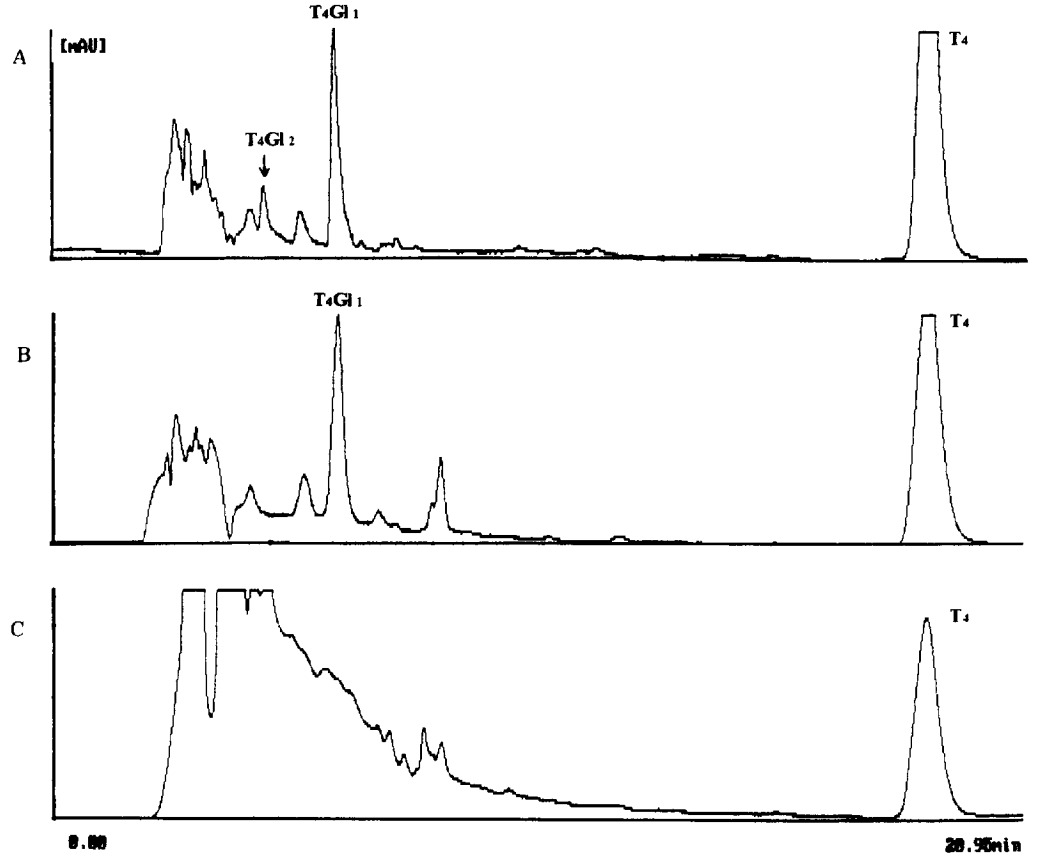

Fig. 4. Chromatograms of β-glucuronidase treatment of T4-glucuronides formed by 3-methylcholantrene treated rat liver microsomes. Peaks: T4 = thyroxine; $T4Gl_1$ = phenol-thyroxine-glucuronide; $T4Gl_2$ = acyl-thyroxine-glucuronide. (A) T4-glucuronides before hydrolysis; (B) incubation in 0.1 *M* sodium acetate buffer pH 4.5 for 24 h a 37°C in the absence of β-glucuronidase enzyme; (C) incubation with the whole system at 37°C for 24 h.

unchanged, which also indicates acyl-glucuronide conjugation (Table 1).

The method presently described proved to be sensitive and simple, makes possible to avoid the use of radiolabelled substrate, and the detection of a new T4-glucuronide metabolite that has not been separated up to now.

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