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Determination of ^{125}I-labelled thyroxine glucuronide by reversed-phase high-performance liquid chromatography using on-line radiochemical detection to determine UDPglucuronosyltransferase activity

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ABSTRACT

A convenient, fast and highly sensitive high-performance liquid chromatographic method, using on-line radiochemical detection, is described for the determination of [^{125}I]thyroxine glucuronide. The method involves direct injection of the supernatant, a total analysis time of 30 min and a detection limit of 1 pmol. The results demonstrate that the method is suitable for the determination of UDPglucuronosyltransferase activity with thyroxine as substrate in native hepatic microsomes. The rate of thyroxine glucuronidation in microsomes from rats treated with Arodor 1254 was ten times higher than in control microsomes, indicating that with this method, increases of UDPglucuronosyltransferase thyroxine activities, often associated with hepatic induction process involved in thyroid hypertrophy, can be easily detected. This method could also be applied to all experimental biological systems that involve the separation and quantification of [^{125}I]thyroxine and [^{125}I]thyroxine glucuronide.

INTRODUCTION

Thyroid hormones play numerous and important roles in regulating metabolism, growth and development, and in the maintenance of homeostasis. Thyroxine (T4), the major hormone secreted from the thyroid, is converted into the more active 3,3′,5-triiodothyronine (T3) by 5′-monodeiodinase in a variety of peripheral tissues, including the liver. T4 is also metabolized to 3,3′,5′-triodothyronine (rT3) and subsequently to 3,5-diiodothyronine (T2), which are both hormonally inactive. Degradative metabolism of the thyroid hormones occurs primarily in

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the liver and involves conjugation with either glucuronic acid (mainly T4) or sulphate (mainly T3) through the phenolic hydroxyl group. The resulting conjugates are excreted in the bile into the intestine [1].

Of central importance in the homeostatic control of thyroid hormone synthesis and secretion is the feedback mechanism by which the thyroid-stimulating hormone (TSH) is secreted by the anterior pituitary in response to a decrease in circulating thyroid hormones. However, chronic stimulation of the thyroid by TSH can cause thyroid carcinogenesis [1]. It has been reported that increases in the biliary T4 elimination are involved in the thyroid gland hypertrophy observed in rats treated with phenobarbital [2], 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [3], clofibrate [4] and aminotriazole [5] through induction of hepatic microsomal UDPglucuronosyltransferases (UDP-GT). Thus, evaluations of UDP-GT activity with T4 as substrate are important in pharmacological and toxicological studies. To measure the enzyme activity, the [^{125}I]thyroxine glucuronide (T4G) formed *in vitro* from [^{125}I]T4 by hepatic microsomes are usually separated by thin-layer chromatography (TLC) and subsequently quantified by radioactivity counting of successive fractions [2,3,6]. A few reports describe the use of high-performance liquid chromatographic (HPLC) methods to separate labelled thyroid hormones in bile [7–9], but only one group has described its application to microsomal UDP-GT activity determination [7]. The time needed to separate [^{125}I]T4G from [^{125}I]T4 was 80 min and a fraction collector was necessary. Here we describe an HPLC method for rapid separation (30 min) and quantification of low concentrations of T4G (around 10 n*M*) using on-line radiochemical detection, and its application to *in vitro* measurements of microsomal T4 UDP-GT activities.

EXPERIMENTAL

Reagents

Methanol (HPLC grade) was supplied by Prolabo (Paris, France). Water was deionized and glass-distilled. Orthophosphoric acid (Normapur®), triethylamine (HPLC grade), acetic acid (HPLC grade) and sodium hydroxide (Normapur) were from Prolabo. Sodium acetate (Normapur), sodium chloride (Normapur), magnesium chloride (Rectapur®), sodium dihydrogenphosphate (Rectapur), disodium hydrogenphosphate (Rectapur), ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (DTT) were also purchased from Prolabo.

^{125}I-Labelled thyroxine (specific activity 40–60 μCi/μg thyroxine) was obtained from the Amersham Radiochemical Centre (Amersham, Les Ulis, France).

Uridine 5′-diphosphoglucuronic acid (UDPGA) was from Sigma (St. Louis, MO, U.S.A.). Bovine serum albumin (BSA) fraction was from U.S. Biochemical (Cleveland, OH, U.S.A.). β-Glucuronidase extract of *Helix pomatia* was purchased from Sigma. T4 was supplied by Janssen (Beerse, Belgium), and the other iodothyronines, T3, rT3 and T2, by Sigma.

Aroclor 1254 was purchased from Analabs (New Haven, CT, U.S.A.). The flow-cell scintillator was Quickszint Flow 302 (Zinsser Analytic, Elancourt, France).

Apparatus and chromatographic conditions

The chromatographic system consisted of two HPLC Model 420 pumps (Kontron, St. Quentin-en-Yvelines, France), a UV detector (Model 430, Kontron), a radiochemical detector LB 507A (Berthold, Elancourt, France) and a Gilson autoinjector Model 231 (Gilson, Villiers le Bel, France) equipped with a 100-μl Rheodyne loop. Chromatographic system control, data acquisition from the two detectors and integration were performed on a Kontron data system 400.

The flow-rate through the column at ambient temperature was 2.0 ml/min, which produced a back-pressure of 200 bar when starting the gradient. The solvent system for the separation was 35:65 (v/v) methanol–20 m*M* potassium phosphate buffer containing 1% triethylamine (v/v) adjusted to pH 7.0 with orthophosphoric acid (solvent A) and methanol (solvent B). A pH value of 7.0 was found to be the best compromise between T4 solubilization and peak resolution. Triethylamine aided good elution of the compounds. The buffer was filtered through a 0.22-μm Durapore® filter (Millipore, St. Quentin-en-Yvelines, France) and the solvents were degassed with helium. Elution started at 0% B and a linear gradient was run to 100% B in 15 min. Elution was completed using 100% B over 5 min.

The column chosen was a prepacked C_{18}-Ultrabase® 10-μm stainless-steel tube [250 mm × 4.6 mm I.D.; Société Française Chromato Colonne (SFCC), Neuilly-Plaisance, France], which, in contrast to the column first tested, μBondapak (Waters), was not damaged by the mobile phase. A cartridge system (10 mm × 4.6 mm I.D.) from SFCC was used as a guard column, with the same stationary phase.

Two methods of on-line radiochemical detection were tested: liquid scintillation and solid scintillation. Fig. 1 shows chromatograms of sample containing [^{125}I]T4 and [^{125}I]T4G, revealed by liquid scintillation using a 2-ml detection cell (Fig. 1a) or solid scintillation using a 1-ml detection cell equipped with a lithium scintillator glass (Fig. 1b). The background was more quiet and resolution better when liquid scintillation was used, and this can be explained by an effective detection cell volume of 570 μl. Fig. 2 shows that the resolution of the solid scintillation method could be markedly increased by using a 400-μl detection cell. Thus the convenient, non-destructive solid scintillation method, which avoids quenching variations, was chosen.

Under such conditions, the detection limit of the HPLC method was *ca.* 1 pmol. No memory effects were observed.

Preparation of rat liver microsomes

Male Sprague–Dawley CD-BR rats were obtained from Charles River (St.

Aubin-les-Elbeuf, France). Control and Aroclor 1254-treated rats (500 mg/kg intraperitoneally in olive oil; sacrifice five days later [10]) were decapitated. The liver was removed, and microsomes were prepared by differential centrifugation as described by Amri *et al.* [11]. The final microsomal pellets were resuspended in a 100 m*M* phosphate buffer (pH 7.4) containing 5 m*M* EDTA and 1 m*M* DTT, and stored at −80°C until analysis. The protein content of the hepatic microsomes was estimated by Bradford's method [12].

Hepatic microsomal [^{125}I]T4G formation

The incubation conditions for determination of hepatic UDP-GT activity with T4 as substrate were adapted from McClain *et al.* [2] and Comet *et al.* [6] with some modifications. The final assay mixture consisted of 1.3 mg/ml microsomal proteins, 50 m*M* phosphate buffer (pH 8.0), 10 m*M* magnesium chloride and 10 μM T4 [in 1% (w/v) BSA–sodium hydroxide to achieve complete solubilization] containing 0.5 μCi/nmol [^{125}I]T4. The reaction was initiated by the addition of 2 m*M* UDPGA. In blanks, UDPGA was omitted. Incubation was performed at 37°C with shaking, and stopped by the addition of a volume of methanol–or-

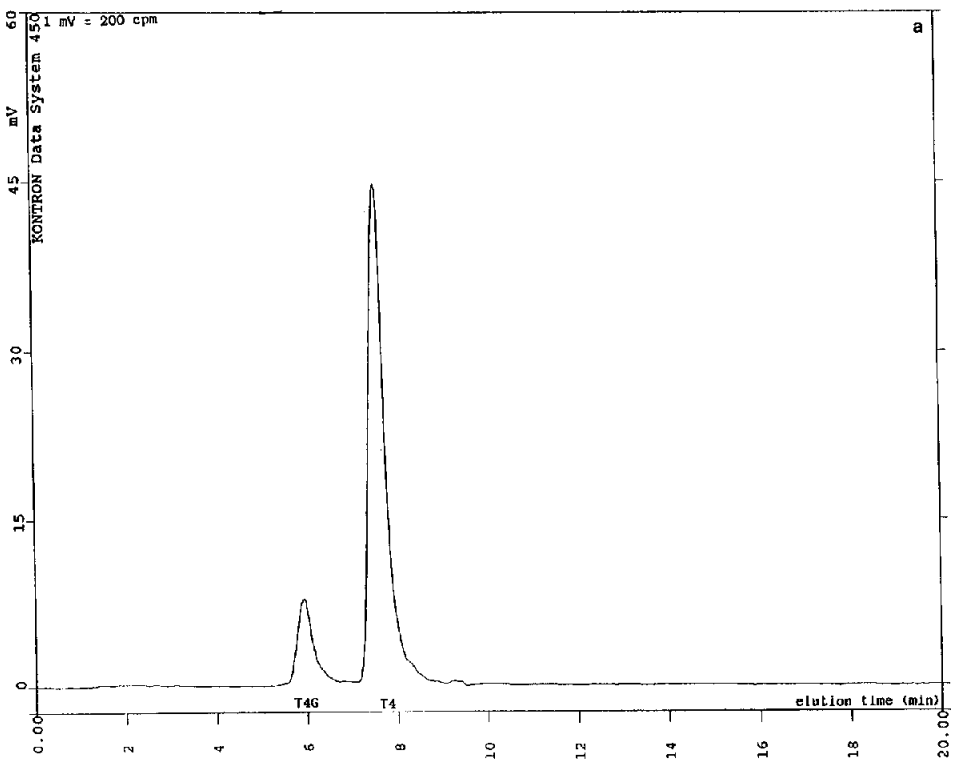

Fig. 1.

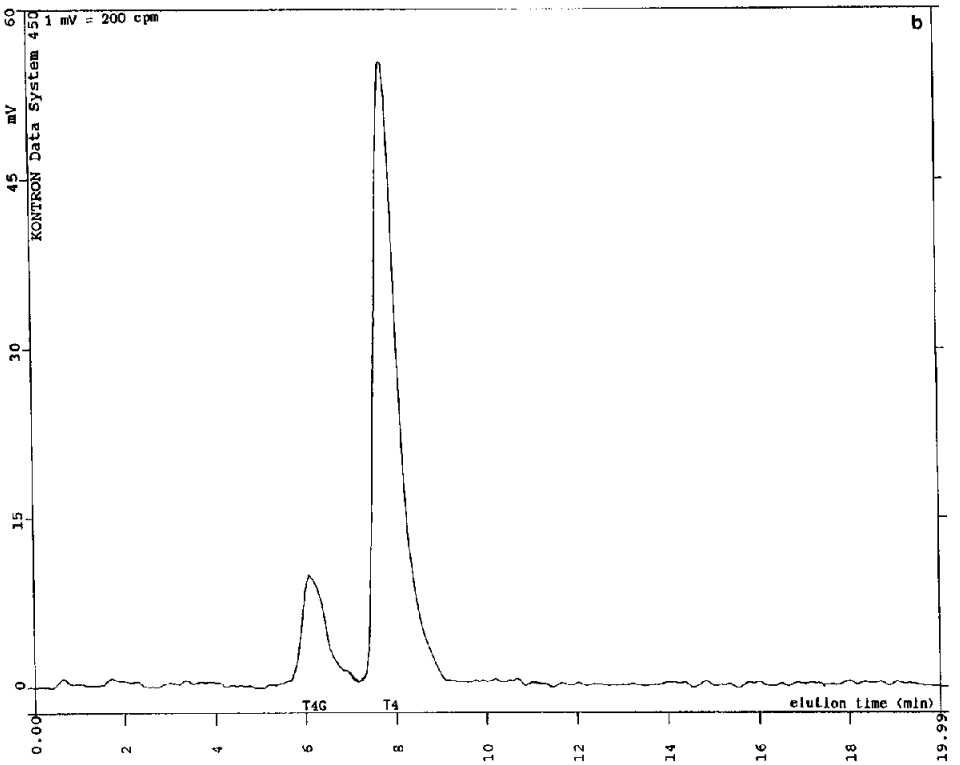

Fig. 1. Comparison of ^{125}I-labelled thyroxine glucuronide (T4G) and ^{125}I-labelled thyroxine (T4) elution profiles detected by (a) an on-line homogeneous method, liquid scintillation, and (b) an on-line heterogeneous method, solid scintillation. Aroclor 1254-induced microsomal incubation was stopped after 60 min. A 25-nCi aliquot of the supernatant fraction was injected. The solvent system for the separation was methanol 20 m*M* phosphate buffer (pH 8.0) (35:65, v/v) (solvent A) and methanol (solvent B). A 2-ml cell (effective volume 570 μl) was used for liquid scintillation. A 1-ml cell was used for solid scintillation (lithium scintillator glass).

thophosphoric acid (9:1, v/v) equal to the incubation volume. Proteins were precipitated by centrifugation, and the supernatant was kept at 4°C in the autoinjector until injection into the HPLC column. In the incubations followed by β-glucuronidase digestion, the enzymic reaction was stopped after 60 min with liquid nitrogen. An equal volume of a mixture of 60 m*M* acetate buffer (pH 4.5), 0.11 m*M* sodium chloride and 1500 U/ml β-glucuronidase was added, followed by a second 60-min incubation at 37°C. The reaction was then terminated by the addition of an equal volume of methanol–orthophosphoric acid (9:1, v/v), proteins were removed, and the supernatant was chromatographed.

As [^{125}I]T4 was the only detected product of T4 metabolism in our incubation conditions (data not shown), the formation of T4G during the reaction was calculated as follows: [T4G] = [T4G area/(T4G area + T4 area)] $[T4]_o$, where T4G area and T4 area are the areas under the curves on the chromatogram of

[^{125}I]T4G and [^{125}I]T4, respectively, $[T4]_0$ is the initial concentration of [^{125}I]T4 added to the incubation medium, and [T4G] is the concentration of [^{125}I]T4G formed. The rate of formation was expressed in pmol/min/mg microsomal proteins.

RESULTS AND DISCUSSION

Fig. 3 shows the elution profiles of hepatic microsomal incubations in which the formation of T4G was stopped after different periods of time. The main peak, with $k' = 3.97 \pm 0.02$ (k' is the capacity factor), corresponds to [^{125}I]T4. Some impurities were present in the commercial product. The areas under the curve of these impurities were constant. Free iodine was eluted in the dead volume. The elution profiles of incubation stopped after increasing periods of time show progressive increase in area of a peak with $k' = 2.66 \pm 0.03$ corresponding to a more polar compound than thyroxine.

To determine if the elution peaks with $k' = 2.66$ correspond to was thyroxine glucuronide, additional experiments were performed (Fig. 4). As shown in Fig. 4a, no peak with $k' = 2.66$ was observed in the elution profile of microsomal

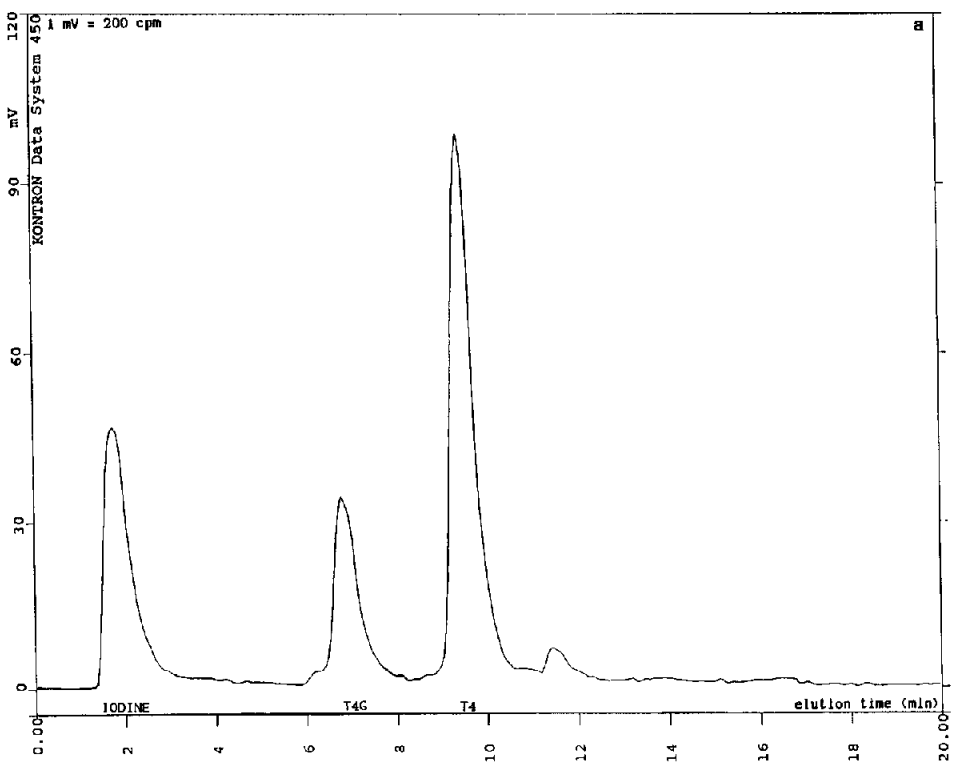

Fig. 2.

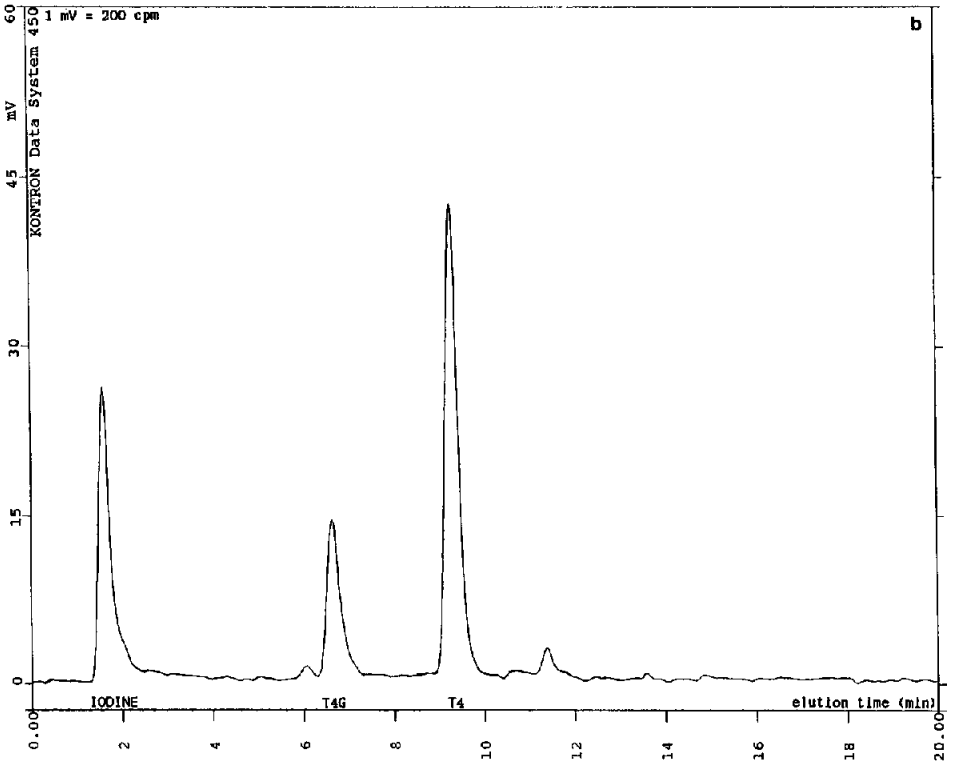

Fig. 2. Comparison of ^{125}I-labelled thyroxine glucuronide (T4G) and ^{125}I-labelled thyroxine (T4) elution profiles detected by an on-line heterogeneous method with (a) a 1000-μl cell and (b) a 400-μl cell. Aroclor 1254-induced microsomal incubation was stopped after 60 min. A 125-nCi aliquot of the supernatant fraction was injected. The solvent system for the separation was methanol–20 m*M* phosphate buffer with 1% (v/v) triethylamine (pH 8.0) (35:65, v/v) (solvent A) and methanol (solvent B).

incubations performed without the cosubstrate UDPGA. Furthermore, the elution profiles of other iodothyronines (Fig. 4b), obtained by UV detection [13] at 254 nm, show that their k' values, $k' = 2.79 \pm 0.03$ for T2, $k' = 3.60 \pm 0.01$ for T3, $k' = 3.85 \pm 0.01$ for rT3 and $k' = 3.96 \pm 0.02$ for T4, do not correspond to the k' value of T4G. Finally, after β-glucuronidase digestion, the peak of thyroxine glucuronide disappeared (Fig. 4c). All these observations strongly support the fact that the elution peak with $k' = 2.66 \pm 0.03$ corresponds to thyroxine glucuronide.

Thyroxine glucuronide in biological samples is usually determined by TLC [2,3,6]. This requires scraping of plates after elution, which is hazardous with radiolabelled compounds. The method described here is convenient: after the microsomal reaction and the precipitation of the protein, the supernatant is injected directly into the HPLC column.

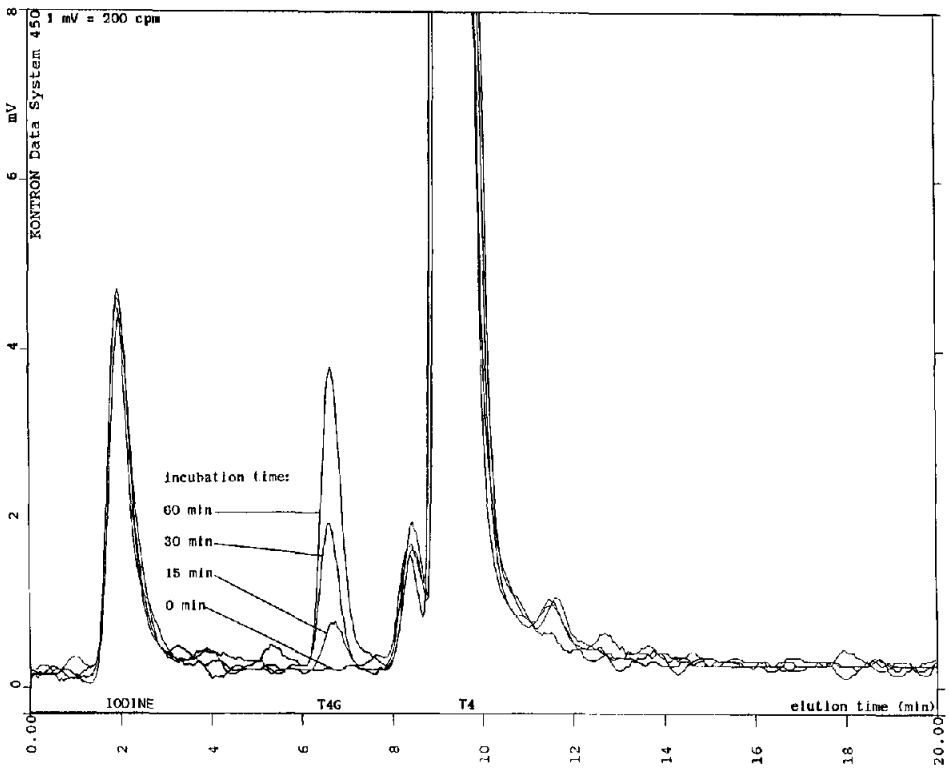

Fig. 3. Elution profiles of supernatant fractions after 0, 15, 30 and 60 min of native microsomal incubation. A 250-nCi aliquot of the supernatant fraction was injected. The solvent system for the separation was methanol–20 m*M* phosphate buffer with 1% (v/v) triethylamine (pH 7.0) (35:65, v/v) (solvent A) and methanol (solvent B). A 400-μl solid scintillation cell was used.

In most reported methods [2,3,6,8], fraction gamma-counters are used. This makes it necessary to collect and count fractions. The result is a non-continuous elution profile. The use of an on-line radiochemical detector as described gives a continuously registered signal on the computer during the elution. The heterogeneous method also has the advantage of being non-destructive.

The 30-min analysis time per sample is short. The analysis procedure can be fully automated.

Unlike other HPLC assays [8,9], this method was developed for the quantification of low concentrations of thyroxine glucuronide of *ca.* 10 n*M*. Furthermore, the reproducibility of the method is *ca.* ±8%.

All these qualities make this method suitable for routine assay of T4G in biological fluids.

Using this new methodology, the T4 UDP-GT activity was investigated in hepatic microsomes. Fig. 5 shows the kinetics of T4G formation in microsomes

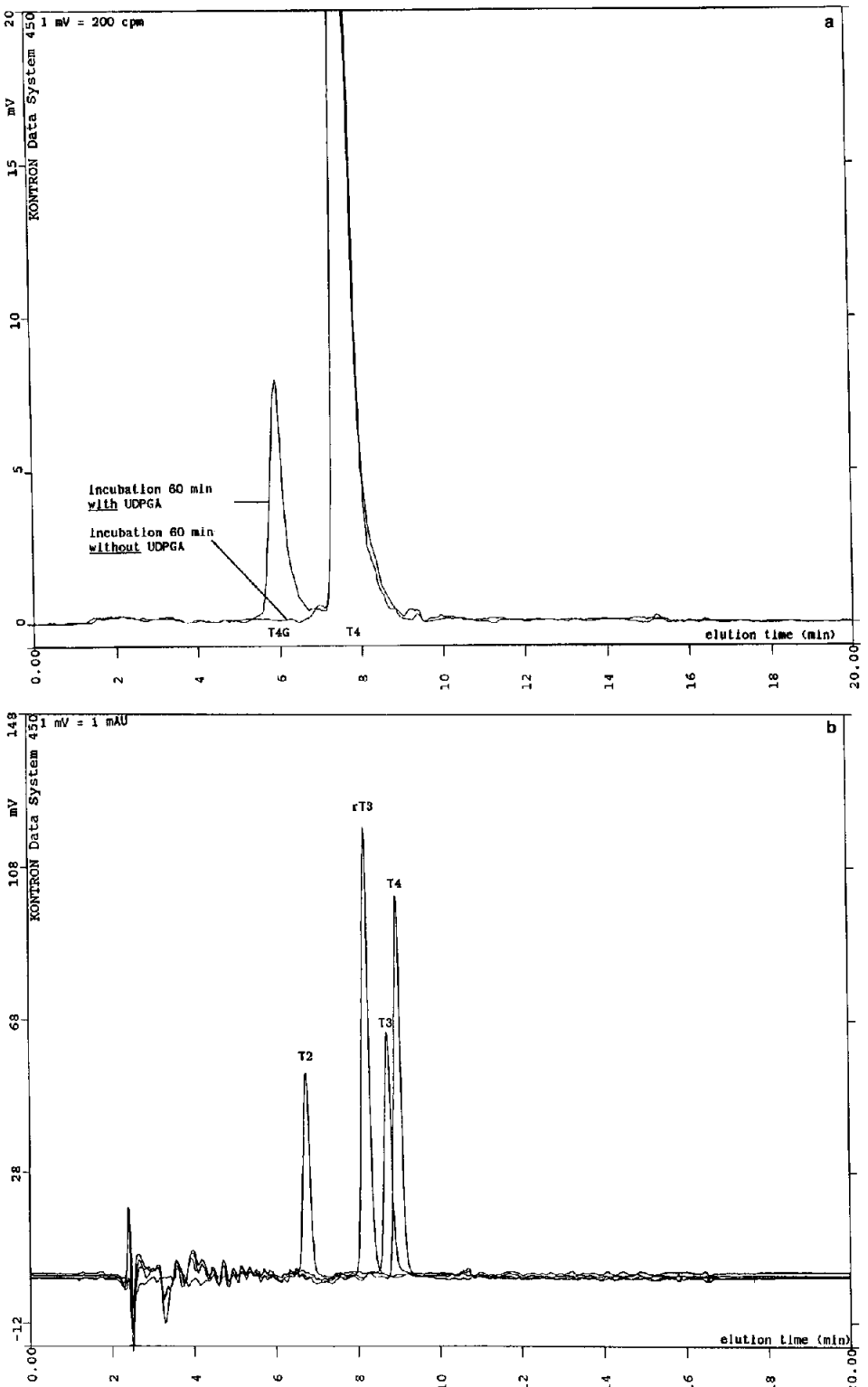

Fig. 4.

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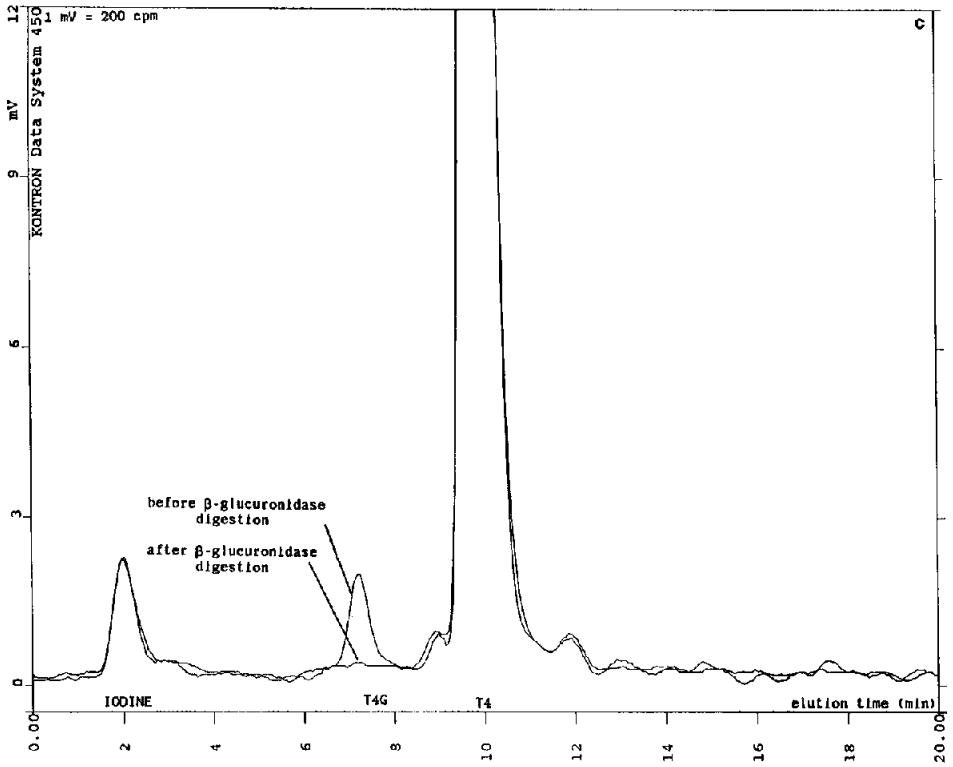

Fig. 4. (a) Elution profiles of supernatant fractions of Aroclor 1254-induced microsomal incubation with and without UDPGA. Reactions were stopped at 60 min. A 25-nCi aliquot was injected. The solvent system for the separation was methanol–20 m*M* phosphate buffer (pH 8.0) (35:65, v/v) (solvent A) and methanol (solvent B). A 2-ml liquid scintillation cell was used. (b) UV detection (254 nm) elution profiles of iodothyronine hormones: 10 μl of 1 m*M* solution were injected. The solvent system for the separation was methanol–20 m*M* phosphate buffer with 1% (v/v) triethylamine (pH 7.0) (35:65, v/v) (solvent A) and methanol (solvent B). The baseline was subtracted. Peaks: T2 = 3,5-diiodothyronine; T3 = 3,3′,5-triiodothyronine; rT3 = 3,3′,5′-triiodothyronine; T4 = thyroxine. (c) Elution profiles of supernatant fractions of native microsomal incubation before and after β-glucuronidase digestion. A 250-nCi aliquot of the supernatant fraction was injected. The solvent system for the separation was methanol–20 m*M* phosphate buffer with 1% (v/v) triethylamine (pH 7.0) (35:65, v/v) (solvent A) and methanol (solvent B). A 400-μl solid scintillation cell was used.

from control and Aroclor 1254-treated rats: the reaction was linear for 60 min in both cases, and the correlation factors were 0.968 and 0.999, respectively. From these curves, specific activities were calculated as 1.9 pmol/min/mg microsomal proteins in control rats and 18.4 pmol/min/mg microsomal proteins in treated rats.

The specific activity of T4 UDP-GT obtained in control microsomes was comparable with values previously described by Henry and Gasievicz [3] and McClain

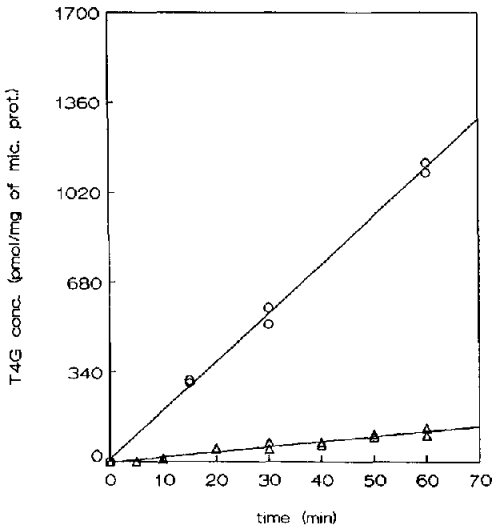

Fig. 5. Kinetics of ^{125}I-labelled thyroxine glucuronide (T4G) formation in microsomes from control (△) and Aroclor 1254-treated (○) rats. The incubation mixture contained 1.3 mg/ml microsomal proteins, 50 m*M* phosphate buffer (pH 8.0), 10 m*M* magnesium chloride and 10 μM thyroxine labelled at 0.5 μCi/nmol. The reaction was initiated by the addition of 2 m*M* UDPGA. The incubation was performed at 37°C under shaking and stopped by addition of an equal volume of methanol–orthophosphoric acid (9:1, v/v). After centrifugation, the supernatant was directly analysed by HPLC.

et al. [2] for male CD rat hepatic microsomes, using a TLC technique. It is noteworthy, however, that both groups incubated the microsomes with a detergent [Brij 58 (0.05%)] during the reaction to increase the enzyme activity. It has been well documented that the tightly membrane-bound UDP-GTs can be markedly activated *in vitro* by membrane perturbants, including detergents [14]. For convenience or sensitivity reasons, UDP-GT activities are often determined using detergent-treated microsomes [15,16], although it has been reported that the rate of glucuronide formation of various substrates *in vivo* most probably corresponded to that determined in native microsomes *in vitro* [17]. To our knowledge, the present study is the first report of T4 UDP-GT activity determination using non-detergent control microsomes. This was possible because of the high sensitivity of our method.

Aroclor 1254, a polychlorinated biphenyl mixture, is a potent hepatic microsomal enzyme inducer, increasing the levels of various P-450 and UDP-GT isozymes [18]. Aroclor 1254 has been reported to induce thyroid hypertrophy by enhancing biliary T4 excretion [19], through increases in hepatic T4G formation [20]. The rate of T4 glucuronidation in microsomes from rats treated with Aroclor 1254 was 9.7-fold higher than that in control microsomes. By using this convenient HPLC method, we confirmed the increase T4 glucuronidation in mi-

crosomes from Aroclor 1254-treated rats, most probably reflecting induction, *i.e.* synthesis of new UDP-GT proteins involved in T4 glucuronidation.

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