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IN VITRO GLUCURONIDATION OF THYROXINE AND TRIIODOTHYRONINE BY

LIVER MICROSOMES AND RECOMBINANT HUMAN UDP-

GLUCURONOSYLTRANSFERASES

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Abbreviations: T3, triiodothyronine; T4, thyroxine; UGT, UDP-glucuronosyltransferase;

UDPGA, UDP-glucuronic acid or Uridine 5′-diphosphoglucuronic acid; LC/MS, liquid

Chromatography/Mass Spectrometry; HPLC, high performance liquid chromatography; ESI,

electrospray ionization; MRM, multiple reaction monitoring; SRM, selected reaction monitoring;

CID, collision-induced dissociation; NMR, nuclear magnetic resonance spectroscopy; gCOSY,

gradient selected correlation spectroscopy; TOCSY, total correlation spectroscopy; gHSQC,

gradient heteronuclear single quanta correlation; gHMBC, gradient selected heteronuclear

multiple bond correlation; TFA, trifluoroacetic acid.

ABSTRACT:

Glucuronidation, which may take place on the phenolic hydroxyl and carboxyl groups, is a major pathway of metabolism for thyroxine (T4) and triiodothyronine (T3). In this study, an LC/MS method was developed to separate phenolic and acyl glucuronides of T4 and T3. The method was used to collect the phenolic glucuronide of T4 for definitive characterization by NMR and to determine effects of incubation pH, species differences and human UDP-glucuronosyl-transferases involved in the formation of the glucuronides. Formation of T4 phenolic glucuronide was favored at pH 7.4, while formation of T4 acyl glucuronide was favored at pH 6.8. All UGTs examined catalyzed the formation of T4 phenolic glucuronide except UGT1A4; the highest activity was detected with UGT1A3, 1A8 and 1A10, followed by UGT1A1 and 2B4. Formation of T3 phenolic glucuronide was observed in the order of UGT1A8>1A10>1A3>1A1; trace activity was observed with UGT1A6 and 1A9. UGT1A3 was the major isoform catalyzing the formation of T4 and T3 acyl glucuronides. In liver microsomes, phenolic glucuronidation was the highest in mice for T4 and in rats for T3, while lowest in monkeys for both T4 and T3. Acyl glucuronidation was highest in humans and lowest in mice for T4 and T3. Phenolic glucuronidation was higher than acyl glucuronidation for T4 in humans; in contrast, the acyl glucuronidation was slightly higher than phenolic glucuronidation for T3. UGT activities were lower toward T3 than T4 in all species. The LC/MS method was a useful tool in studying glucuronidation of T4 and T3.

Thyroxine (T4) is the main secretory product of the thyroid follicular cells but has little biological activity (Visser, 1996). T4 is converted to the most active form of thyroid hormone triiodothyronine (T3) by outer ring deiodination in peripheral tissue and the inactive product 3,3',5'-triiodothyronine (rT3) by inner ring deiodination (Visser, 1996). Deiodination and conjugation were the principal pathways of thyroid hormone metabolism. Deiodination regulates the thyroid hormone bioactivity, while conjugation, which involves sulfation and glucuronidation, is to increase the water-solubility of the substrates and, thus, to facilitate their urinary and biliary clearance(Visser, 1996). Glucuronidation by hepatic UGTs is one of the major metabolic pathways of T4 and T3 (Kohrle et al., 1987; Saito et al., 1991; Visser, 1996). T4 and T3 glucuronides are cleared from the body through biliary excretion (Visser, 1996). Studies with recombinant human UGTs and rat hepatocytes demonstrated that human UGT1A1 and 1A9, and rat UGT1A1 and 1A6 were responsible for T4 conjugation, while rat UGT2B2 was responsible for T3 glucuronidation (Visser et al., 1993a; Findlay et al., 2000; Vansell and Klaassen, 2002).

Glucuronidation of T4 and T3 may take place on the phenolic hydroxy and carboxyl groups in the molecules to give phenolic and acyl glucuronides, respectively (Jemnitz and Vereczkey, 1996). However, there is little documented information about separation of phenolic and acyl glucuronides of thyroid hormone and their formation by UGT isoforms, especially the acyl glucuronides of these two hormones. In the present study, an LC/MS method was developed to separate and characterize the phenolic and acyl glucuronides of T4 and T3. This method was used to evaluate formation of phenolic and acyl glucuronides of T4 and T3 by recombinant human UGT isoforms and hepatic microsomes of animals and humans.

Materials and Methods

Materials. Fexofenadine, L-thyroxine, triiodothyronine, 4-nitrophenol, 4-nitrophenol

glucuronide, UDP-glucuronic acid, β-glucuronidase (363,400 units/g, type H-1 from *Helix*

pomatia) and deuterated methanol (CD_3OD) were purchased from Sigma-Aldrich (Milwaukee,

WI). [Glucuronyl-^{14}C(U)]-uridine diphosphate glucuronic acid (292.8 mCi/mmol), with a purity

of 97%, was purchased from PerkinElmer Life And Analytical Sciences, Inc. (Boston, MA).

Baculovirus-insect cell-expressed human UGT1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, 2B4, 2B7,

2B15 and 2B17 supersomes (Table 1) were obtained from BD Biosciences (San Jose, CA).

Liver microsomes from mice, rats, dogs, monkeys and humans listed in Table 2 were purchased

from Xenotech, LLC (Lenexa, Kansas). Monobasic potassium phosphate, reagent grade

chemicals, HPLC grade water and solvents were purchased from EMD Chemicals (Gibbstown,

NJ) or Mallinckrodt Baker (Phillipsburg, NJ).

Incubations. Incubations with liver microsomes or recombinant human UGTs consisted of T4

or T3 (100 µM), magnesium chloride (10 mM), microsomes (2 mg/mL) or supersomes (0.5

mg/mL) and UDPGA (6 mM) in 0.5 mL of 0.1 M Tris-HCl buffer (pH 6.8 or 7.4). Alamethicin

(50 µg/mg protein) was mixed with liver microsomes for use in incubations. T4 or T3 in 5 µL of

methanol was added to the incubation tubes containing buffer, magnesium chloride solution,

alamethicin and microsomes or UGT isoforms. After mixing, the tubes were pre-incubated for

3 minutes in a shaking water bath at 37°C. The reactions were initiated by the addition of 30 µL

of 100 mM UDPGA solution in water. Control incubations were conducted under the same

conditions without UDPGA or microsomes. All incubations were performed in duplicate.

Reactions were stopped by the addition of 100 µL ice-cold methanol containing

5% trichloroacetic acid.   Fexofenadine in 30 µL of 10 µM solution in methanol was added to

each incubation as an internal standard, followed by the addition of 600 µL of methanol.

Samples were vortex-mixed. Denatured proteins were separated by centrifugation at 4300 rpm

and 4°C for 10 minutes in a Sorvall Model T21 super centrifuge. An aliquot of the supernatant

was analyzed by LC/MS.

To evaluate the effect of pH on the formation of T4 glucuronides, T4 was incubated with human

liver microsomes at pH 6.8 and 7.4 in the presence of UDPGA at 37 $^{\circ}$C for 5, 10, 15, 20, 30 and

60 minutes. All other incubations were conducted at pH 6.8 for 30 minutes.

Hydrolysis of T4 and T3 Glucuronides. The sample prepared from incubations with human

liver microsomes in the presence of UDPGA was dried under a nitrogen stream in a TurboVap

LV evaporator (Zymark Corp., Hopkinton, MA). The residue was reconstituted in 1 mL of 0.1

M sodium acetate buffer, pH 5.0. Approximately 560 units of β-glucuronidase in 100 µL of 0.1

M sodium acetate buffer, pH 5.0, was added to 0.5 mL of the reconstituted solution, and the

mixture was incubated at 37 $^{\circ}$C for 30 minutes with gentle shaking. The reaction was stopped by

the addition of 1 mL of acetonitrile and the precipitated enzyme was removed by centrifugation.

The supernatant was evaporated under nitrogen to remove acetonitrile. An aliquot of the

aqueous residue was analyzed by HPLC. A control incubation was prepared under the same

conditions, but without β-glucuronidase.

For alkaline hydrolysis, the sample was adjusted with 2N sodium hydroxide to about pH 11 and

incubated at 37 $^{\circ}$C for 60 minutes. After adjustment with acetic acid to about pH 5, an aliquot

was analyzed by HPLC. A control incubation was conducted at pH 6.8. All samples were

analyzed by LC/MS to monitor the glucuronides and the internal standard.

Liquid Chromatography/Mass Spectrometry. A Waters model 2690 HPLC system (Waters

Corp., Milford, MA) with a built-in autosampler and a diode array UV detector was used for

analysis. The UV detector was set to monitor 200 to 400 nm. Separations were accomplished on

a Luna C18(2) column (150 x 2.0 mm, 5 μm) (Phenomenex, Torrance, CA). The sample

chamber in the autosampler was maintained at 4°C, while the column was at ambient

temperature of about 20°C. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in

water (A) and 0.1% TFA in acetonitrile (B) and was delivered at 0.2 mL/min. The mobile phase

B linearly increased from 25% to 65% over the first 5 minutes, to 85% over ten minutes, and

then to 95% over five minutes. The first 7 minutes of flow was diverted away from the mass

spectrometer.

The HPLC system was coupled to a Micromass Quattro Ultima triple quadrupole mass

spectrometer (Micromass, Inc., Beverly, MA), which was equipped with an electrospray

ionization (ESI) interface and operated in the positive ionization mode. The capillary and cone

voltages were set at 4000 and 20 Volt, respectively. The cone and the desolvation gas flows

were 100 and 1100 L/hr, respectively. The source block and the desolvation gas temperatures

were set at 105 and 200 ^{o}C, respectively. Mass resolution for multiple reaction monitoring

(MRM) was 15 for MS1 and MS2. The scan mode used selected reaction monitoring (SRM)

with ion transitions of m/z 953.5 to 777.5 for T4 glucuronides, m/z 827.7 to 651.7 for T3

glucuronides and m/z 502 to 466 for fexofenadine at collision energy of -25 eV. Micromass

MassLynx software (version 4.0) was used for collection and analysis of LC/MS data. The peak

area ratio of thyroxine glucuronides to the internal standard was used for comparison of enzyme activities.

To evaluate the limit of detection and linearity of MS response, T4 was incubated with human liver microsomes in the presence of a mixture of [^{14}C]UDPGA and non-labeled UDPGA (1:5) as described in the Incubations section. The phenolic glucuronide was isolated by HPLC using the same conditions described above, except that a 250 x 4.6 mm column was used and the mobile phase was delivered at 0.6 mL/min. The isolated fractions were concentrated under nitrogen and reconstituted with the control incubation mixture. An aliquot of the supernatant was analyzed by LC/MS after protein precipitation. The amount of T4 glucuronide was calculated by the specific radioactivity of [^{14}C]UDPGA in the incubation and the radioactivity in the isolated fractions after concentration.

NMR Analysis of T4 Phenolic Glucuronide. T4 phenolic glucuronide was isolated as described in the above section from incubations of T4 with human liver microsomes in the presence of non-radiolabeled UDPGA. The isolated fractions were dried under a nitrogen stream and reconstituted in approximately 160 μl of deuterated methanol (CD$_3$OD) and transferred to a 3 mm OD NMR tube. For the purpose of comparison, a saturated T4 solution was also prepared in CD$_3$OD. Data were collected on Varian Inova 500 MHz and Bruker Avance 600 MHz spectrometers, each equipped with a 3mm z-gradient indirect detection probe. One-dimensional (1D) proton (^{1}H) NMR data and two-dimensional (2D) gradient selected correlation spectroscopy (gCOSY), total correlation spectroscopy (TOCSY), heteronuclear ^{1}H-^{13}C gradient heteronuclear single quanta correlation (gHSQC) and ^{1}H-^{13}C gradient selected heteronuclear multiple bond correlation (gHMBC) (Croasmun and Carlson, 1994) NMR data were acquired. All spectra were referenced to the signal of CHD$_2$OD at 3.32 ppm in ^{1}H and 48.1 ppm in ^{13}C

spectra. Because of limited quantity of metabolite the ^{13}C chemical shifts were extracted from

heteronuclear gHSQC and gHMBC.

RESULTS

Separation and Detection of T4 and T3 Glucuronides. The LC/MS method used in the

present study separated T4 glucuronides, with the T4 phenolic and acyl glucuronides eluting at

13.2 and 14.8 minute, respectively (Figure 2), and T3 phenolic and acyl glucuronides eluting at

11.0 and 12.2 minute, respectively (data not shown). The internal standard fexofenadine had a

retention time of 15.7 minute. T4 and T3 were separated from their respective glucuronides and

eluted at 13.9 and 13.2 minute, respectively (data not shown). Limit of detection for T4 phenolic

glucuronide was 0.1 ng on column. The linear range of mass spectrometric response was 0.1 to

46 ng (data not shown). Samples analyzed by LC/MS had lower response than that for 46 ng of

T4 phenolic glucuronide, suggesting that the samples analyzed were within the linear range of

mass spectrometric response.

Identification of T4 and T3 Glucuronides. The identities of the T4 glucuronides were

elucidated by hydrolysis, LC/MS and NMR. After hydrolysis with β-glucuronidase for 30

minutes, both glucuronide peaks completely disappeared (Figure 2). After hydrolysis under

alkaline conditions for 1 hour, the second glucuronide peak complete disappeared, while the first

glucuronide peak was intact (Figure 3), suggesting that the first glucuronide was stable while the

second glucuronide was labile under alkaline conditions. These findings suggested that the first

peak was a phenolic glucuronide and the second peak was an acyl glucuronide of T4.

LC/MS analysis showed that both T4 metabolites formed in human liver microsomes in the

presence of UDPGA had a molecular ion at m/z 953.5. Figure 4 shows the product ions of

m/z 953.5 obtained from collision-induced dissociation (CID). Loss of 176 Da generated the

molecular ion of T4, indicating that both products were T4 glucuronides. The ion at m/z 731

was a fragmentation product of T4.

NMR results for T4 phenolic glucuronide are summarized in Table 3 and Figures 5 and 6. From changes in chemical shifts of iodinated carbons, C-9 and C-13 (Figure 5) going from thyroxine to thyroxine glucuronide, more than 6 ppm downfield shifts it is evident that glucurodination took place on the phenolic part of thyroxine. The downfield chemical shift 0.08 ppm is also observed on protons H-10 and H-12 (Figure 6). The signal of anomeric proton H-2 at 5.27 ppm also supports the presence of aromatic ether.

T3 glucuronides were identified by LC/MS and hydrolysis. The molecular ions for T3 glucuronides were observed at m/z 827.7, which was 176 Da larger than the molecular ion for T3. Neutral loss of 176 Da from the molecular ions of T3 glucuronides produced m/z 651.7, identical to T3 molecular ion, indicating that the metabolites were T3 glucuronides (data not shown). Similar to T4 glucuronides described above, both T3 glucuronides were hydrolyzed by β-glucuronidase, while only acyl glucuronide was hydrolyzed under alkaline conditions (data not shown).

Effect of pH on the Formation of T4 Glucuronides. Formation of the phenolic and acyl glucuronides of T4 was evaluated at pH 6.8 and 7.4 (Figure 7). At both pH conditions, formation of the phenolic glucuronide of T4 increased over incubation time up to 60 minutes. Formation of the acyl glucuronide of T4 reached the highest level at 20 minute of incubation at pH 7.4 and 30 minute at pH 6.8. Formation of phenolic glucuronide was favored by pH 7.4, while formation of the acyl glucuronide was favored by pH 6.8. Therefore, incubations were carried out at pH 6.8 for 30 minutes in the present study to ensure detection of the acyl glucuronide. Under these conditions, formation of the acyl glucuronide was about 60% higher, while formation of the phenolic glucuronide was about 40% lower compared with incubations at pH 7.4.

Glucuronidation of T4 and T3 by Recombinant Human UGTs. Recombinant human UGT1A1, 1A3, 1A4, 1A6, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15 and 2B17 were incubated with T4 and T3 in the presence of UDPGA (Figures 8 and 9). The results were normalized with the enzyme activities towards probe substrates described in the Materials section by arbitrarily setting the UGT1A1 activity as 100%. All UGT isoforms catalyzed the formation of the phenolic glucuronide of T4 except for UGT1A4. The activity catalyzing the formation of T4 phenolic glucuronide was in the order of 1A3>1A8>1A10>2B4>1A1. Trace activity was observed with UGT1A6, 1A9, 2B7, 2B15 and 2B17. Formation of the acyl glucuronide of T4 catalyzed by UGTs was in the order of 1A3>1A4, 2B7>1A1; T4 acyl glucuronide was not detected with other UGT isoforms. Lower activity was observed with T3 as the substrate. Formation of T3 phenolic glucuronide followed the order of 1A8>1A10>1A3>1A1. Trace activity was observed with UGT1A6 and 1A9; T3 phenolic glucuronide was not formed by 1A4 or 2B isoforms. Formation of T3 acyl glucuronide was observed with only four UGT isoforms in the order of 1A3>1A4>1A1, 2B7. UGT2B7 catalyzed the formation of both phenolic and acyl glucuronides of T4, while only the acyl glucuronide of T3 was detected with UGT2B7.

Species Differences in Glucuronidation of T4 and T3. Species differences were observed in glucuronidation of T4 and T3 (Figures 10 and 11) by liver microsomes. The microsomal UGT activities across species were normalized with P450 contents described in the Materials section. Formation of T4 phenolic glucuronide was in the order of mouse>rat, human>dog>monkey, while formation of T4 acyl glucuronide was in the order of human>dog>mouse, rat, monkey. Female rats had slightly higher activity than male rats in T4 phenolic glucuronidation. Formation of T3 phenolic glucuronide was observed in the order of rat>mouse>dog>monkey and human, while formation of T3 acyl glucuronide was observed in the order of human>dog> rat,

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monkey>mouse. Female rats had higher activity than male rats in formation of T3 phenolic

glucuronide. Humans generated the highest amounts of acyl glucuronides of T4 and T3. In

human liver microsomes, phenolic glucuronide was formed in higher amounts than acyl

glucuronide for T4; in contrast, the acyl glucuronide was formed in slightly higher amounts than

phenolic glucuronide for T3. Humans had the highest ratios of the acyl glucuronide of T4 or T3

to the phenolic glucuronide of T4 or T3. UGT activities toward T3 were lower than those toward

T4 in all species.

DISCUSSION

Formation of the phenolic glucuronides of thyroid hormones has been well studied (Visser et al., 1993a; Visser et al., 1993b; Visser, 1996). Study of the acyl glucuronides of T4 and T3 could be hindered by their low formation and instability. For example, Jemnitz and Vereczkey reported that T4 acyl glucuronide was completely hydrolyzed at 37 $^{\circ}$C in an overnight incubation even at pH 4.5 (Jemnitz and Vereczkey, 1996). It is well documented that acyl glucuronides are labile and may undergo a number of reactions including hydrolysis, rearrangement and covalent binding to proteins, although the reactivity varies largely depending on their structures (Spahn-Langguth and Benet, 1992; Sallustio et al., 2000). Degradation of acyl glucuronides is pH-dependent (Dickinson and King, 1989; Smith and Liu, 1993; Sekikawa et al., 1995; McGurk et al., 1996), and lower than physiological pH conditions have been used to favor the stability of acyl glucuronides (Shipkova et al., 2001). In the present study, a sensitive LC/MS method was developed for the first time to separate and detect the phenolic and acyl glucuronides of T4 and T3, and the structure of the phenolic glucuronide of T4 was unequivocally elucidated by LC/MS and NMR.

Formation of the phenolic and acyl glucuronides of T4 and T3 is catalyzed by multiple human UGT isoforms. UGT1A1, 1A3, 1A8, 1A10 and 2B4 are the major isoforms responsible for the formation of the phenolic glucuronide of T4. UGT1A1, 1A3, 1A8 and 1A10 are also the major isoforms catalyzing the formation of the phenolic glucuronide of T3, with lower activity than T4. UGT1A3 was the major isoform catalyzing the formation of the acyl glucuronide of T4 and T3; minor activity was observed with UGT1A1, 1A4 and 2B7. Our results are consistent with literature reports, and offer some new insights into the UGTs involved in glucuronidation of

these two compounds. Previous studies have showed that multiple UGT isoforms are involved in

the formation of the phenolic glucuronides of T4 and T3 (Visser, 1996). T4 is predominantly

glucuronidated in rat liver by both bilirubin and phenol UGTs, while T3 is primarily conjugated

by androsterone UGT. In the V79 cell lines transfected with cDNA of different human UGTs,

Visser et al reported significant conjugation of T4 by bilirubin UGT HP3 (1A1) as well as the

phenol UGT HP4 (1A9) (Visser et al., 1993a). However, the planar phenol UGT isoform HP1

(1A6) had little or no glucuronidation of iodothyronines. Similar findings were also reported by

Findlay et al with human liver microsomes (Findlay et al., 2000). Vansell and Klaassen reported

in rat hepatocytes that T4 glucuronidation was catalyzed by UGTs 1A1 and 1A6, while

conjugation of T3 was catalyzed by UGT 2B2 (Vansell and Klaassen, 2002). That activity was

detected with multiple human UGT isoforms in the present study might be attributed to

overlapping substrate specificity for UGTs (Radominska-Pandya et al., 1999). Formation of the

acyl glucuronides of thyroid hormones by UGT isoforms has not previously been reported.

However, at least six human UGTs, 1A1, 1A3, 1A8, 1A9, 1A10 and 2B7, have been identified

catalyzing the glucuronidation of carboxylic drugs (Gall et al., 1999; Mackenzie, 2000; Sallustio

et al., 2000). These UGTs also catalyze the glucuronidation of a number of non-carboxylic acid

compounds, including both endogenous and xenobiotic chemicals, and have overlapping but

different substrate specificities (Sallustio et al., 2000). Significant differences in relative

specificities toward the same carboxylic acid substrates have been shown (Sallustio et al., 2000).

Formation of acyl glucuronides of T4 and T3 by UGT1A8, 1A9 or 1A10 were not observed in

the present study. UGT1A4, which conjugates a structurally diverse group of compounds

including drugs, xenobiotics, hydroxysteroids, and particularly primary, secondary, and tertiary

amine containing compounds (Green et al., 1995; Green and Tephly, 1996), yielded the acyl but

not the phenolic glucuronide of T4 and T3. Since UGT1A7, 1A8 and 1A10 are extra-hepatic

enzymes (Strassburg et al., 1997; Strassburg et al., 1999), UGTs 1A1 and 1A3, which are

expressed in human liver, may play major roles in T4 glucuronidation in humans.

Species differences in glucuronidation of T4 and T3 were also observed under the experimental

conditions. Mice have much higher activity toward T4 than any other species, while rats have

the highest activity toward T3 among all the species examined. Female rats show higher T3

UGT activity than male rats. The yields of T3 phenolic glucuronide were lower, while the acyl

glucuronides of both T4 and T3 were formed in higher amounts in humans among the species

examined. Species and sex differences in UGT activities toward thyroid hormones have not

been well documented, although species differences in response to chemical inducers have been

reported (Craft et al., 2002; Kato et al., 2003).

In summary, a novel LC/MS method was developed to separate and detect the phenolic and acyl

glucuronides of T4 and T3. This method was used to evaluate a number of UGT isoforms for

formation of the phenolic and acyl glucuronides of T4 and T3, to study species differences in T4

and T3 glucuronidation in liver microsomes of animals and humans. Among the eleven UGT

isoforms examined, UGT1A1, 1A3, 1A8, 1A10 and 2B4 were the major isoforms responsible for

the formation of T4 phenolic glucuronide, while UGT1A1, 1A3, 1A8 and 1A10 catalyzed the

formation of T3 phenolic glucuronide. UGT1A3 was the major isoform responsible for the

formation of the acyl glucuronides of T4 and T3. The activity in formation of T4 and T3

phenolic glucuronide was lower, while the activity in formation of T4 and T3 acyl glucuronides

were higher in human liver microsomes than in other species.

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Footnotes

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Legends for Figures

Figure 1. Formation of the phenolic glucuronide and acyl glucuronide of thyroxine (T4, R=I) and 3,3´,5´-triiodothyronine (T3, R=H).

Figure 2. T4 glucuronides after incubation (A) without and (B) with β-glucuronidase.

Figure 3. T4 glucuronides after incubation (A) at pH 6.8 and (B) at pH 11.0.

Figure 4. Product Ions of m/z 953.5 for peaks eluted at 13.2 and 14.8 minutes.

Figure 5. Comparison of the proton NMR spectra of thyroxine with that of phenolic glucuronide of thyroxine.

Figure 6. Gradient selected heteronuclear multiple bond correlation NMR spectrum of phenolic glucuronide of thyroxine.

Figure 7. Effect of pH on the formation of the phenolic and acyl glucuronides of T4. Data represent the average of duplicate experiments.

Figure 8. Glucuronidation of T4 by recombinant human UGTs. Data represent the average of duplicate experiments.

Figure 9. Glucuronidation of T3 by recombinant human UGTs. Data represent the average of duplicate experiments.

Figure 10. Species comparison of T4 glucuronidation. Data represent the average of duplicate experiments.

Figure 11. Species comparison of T3 glucuronidation. Data represent the average of duplicate experiments.

Table 1. UGT Isoforms Used in the Present Study.

Isoforms	Lot Number	Activity (pmol/min/mg)
1A1	10	1,000
1A3	11	150
1A4	2	1,450
1A6	1	6,970
1A8	15467	510
1A9	1	8,740
1A10	23954	110
2B4	16905	700
2B7	11	1,300
2B15	15468	2,100
2B17	15397	2,200

UGT1A1 and 1A3 activity was measured as estradiol 3-glucuronidation. UGT1A4 activity was determined as trifluoperazine glucuronidation. UGT2B17 activity was measured as eugenol glucuronidation. Activity of other UGT isoforms were determined as 7-hydroxy-4-trifluoromethylcoumarin glucuronidation.

Table 2. Liver Microsomes Used in the Present Study.

Species	Sex	Number of Subjects for Pool	P450 Content (nmol/mg protein)
Mouse	Male	750	0.99
	Female	200	0.84
Rat	Male	191	0.88
	Female	25	0.62
Dog	Male	4	0.53
	Female	4	0.86
Monkey	Male	10	1.17
	Female	9	1.31
Human	Mix	50	0.43

Table 3. Table of chemical shifts for thyroxine and thyroxine glucoronide

Number	Thyroxine		Thyroxine Glucuronide	
	^{1}H	^{13}C	^{1}H	^{13}C
2			5.27	103.14
3			3.75	
4			3.5	76.14
5			3.69	71.9
6			3.69	76.06
8		151.1		150.1
9		84.4		90.65
10	7.15	125.9	7.23	126.7
11		150.4		152.7
12	7.15	125.9	7.23	126.7
13		84.4		90.65
22		153.14		153.01
23		90.4		90.4
24	7.93	141.5	7.93	141.2
25		141.5		141.1
26	7.93	141.5	7.93	141.2
27		90.51		90.4
31	3.04/3.27	35.32	3.11/3.31	35.0
32	3.81	55.71	4.16	55.0

Figure 1.

Phenolic glucuronide   Thyroid hormone   Acyl glucuronide
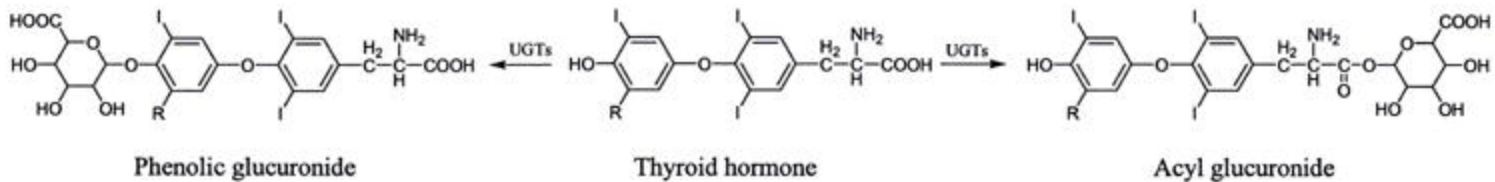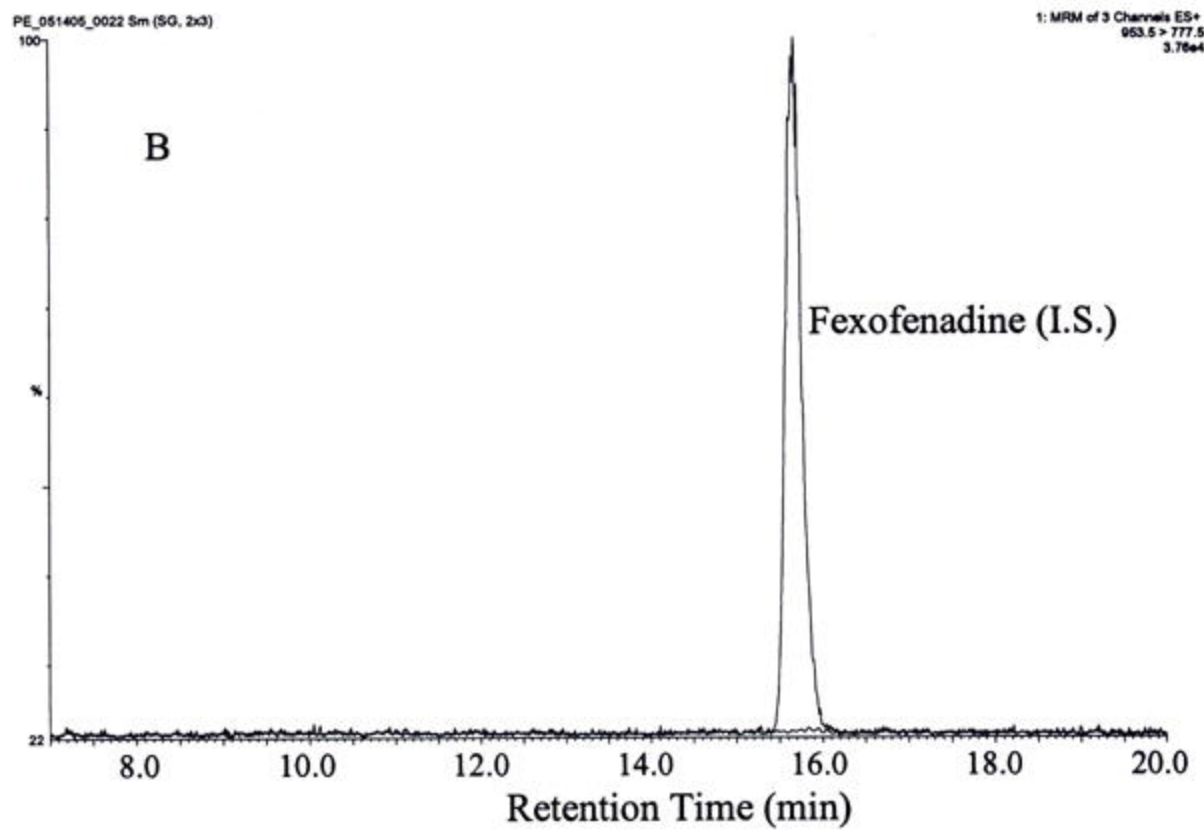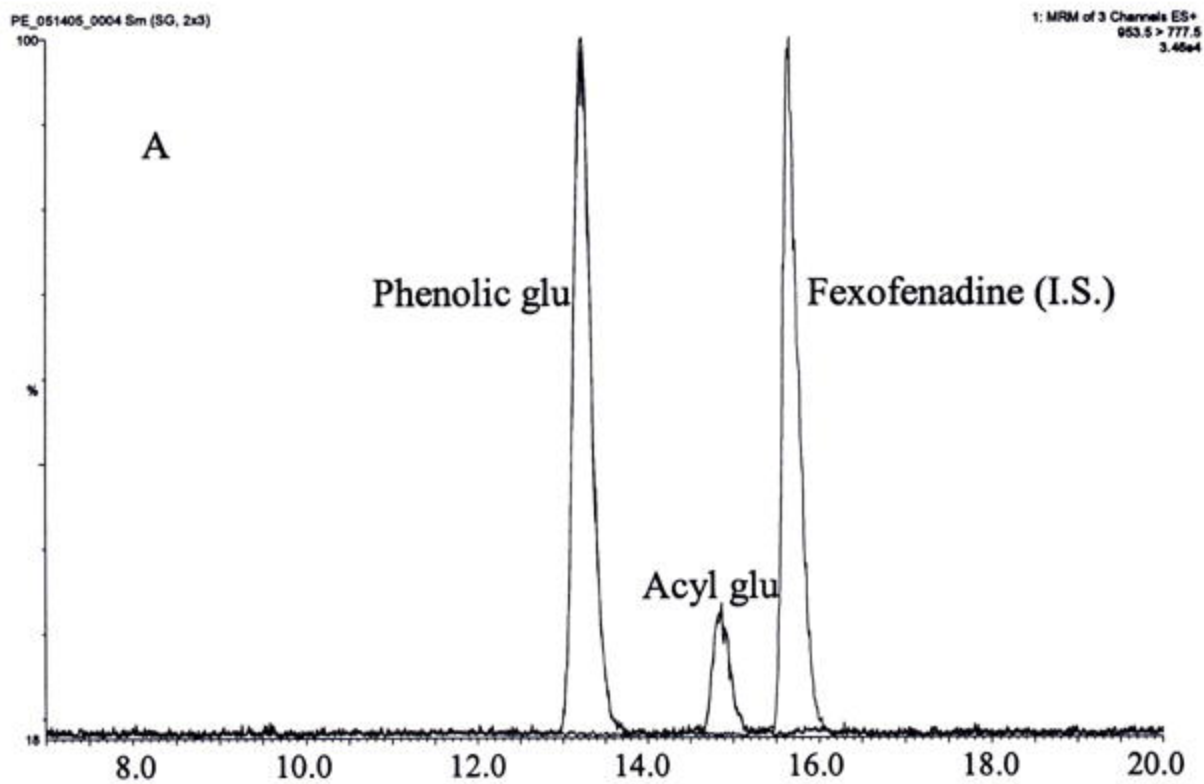
Figure 2.

Figure 3.

Figure 4.
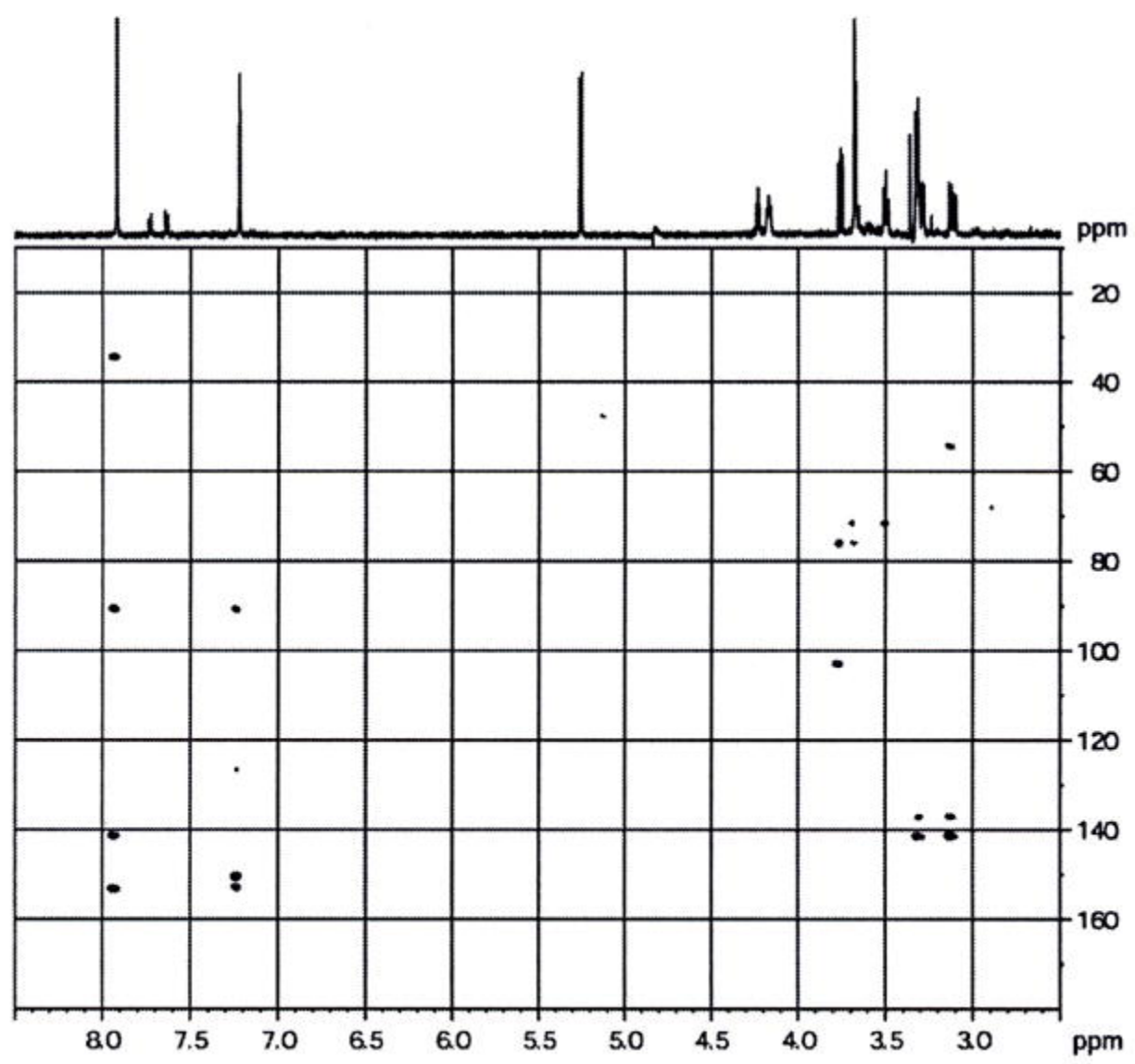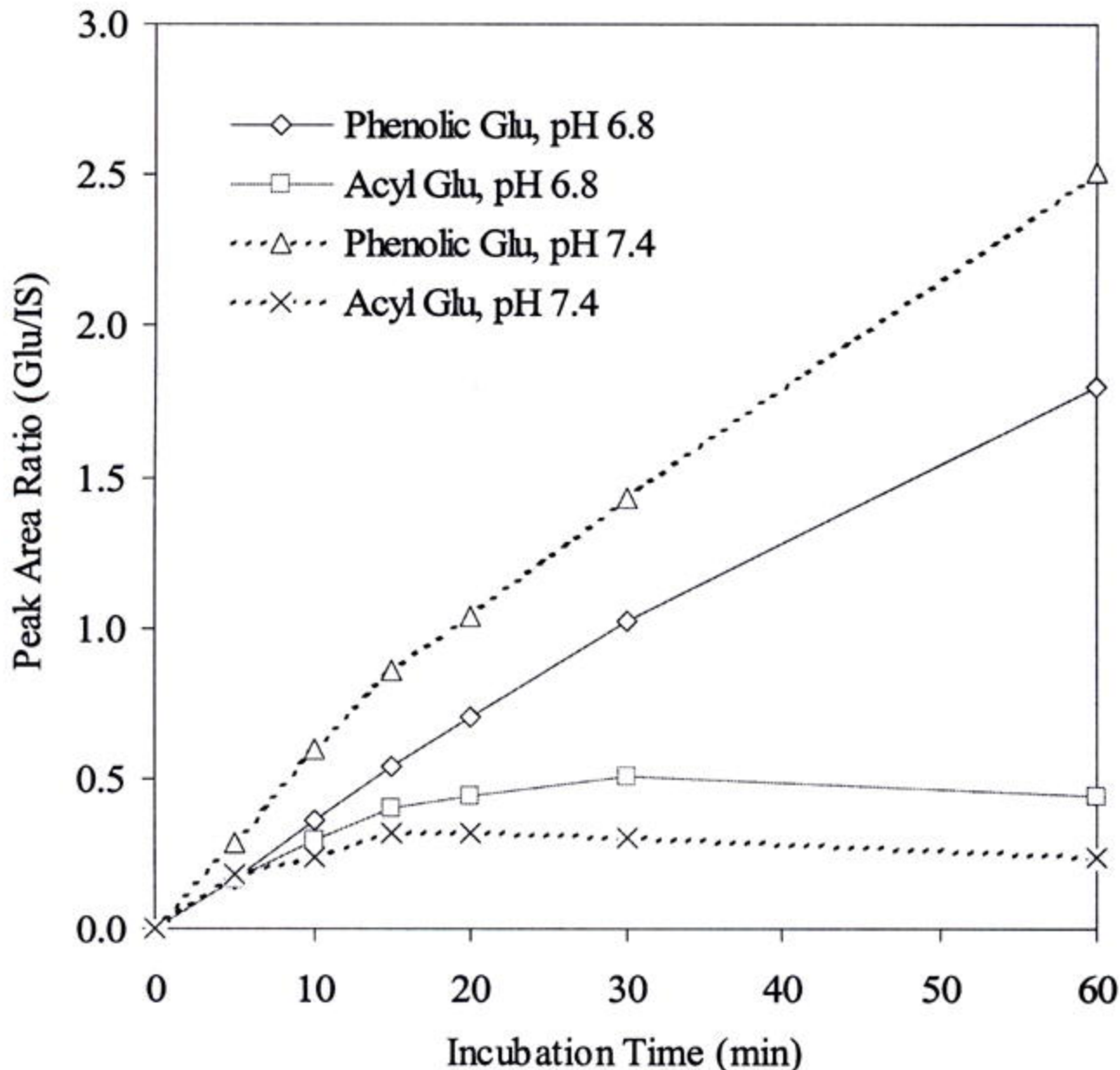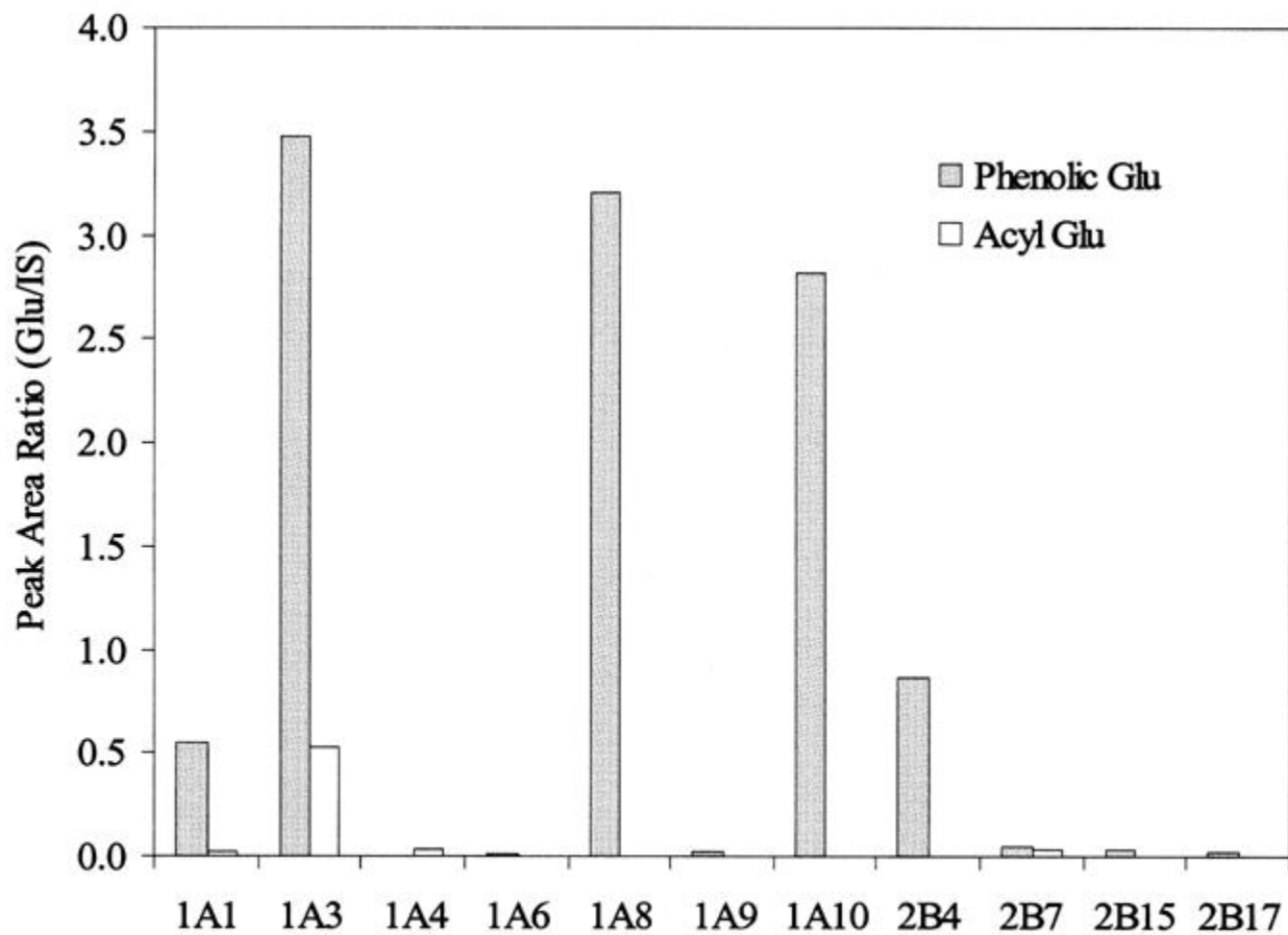
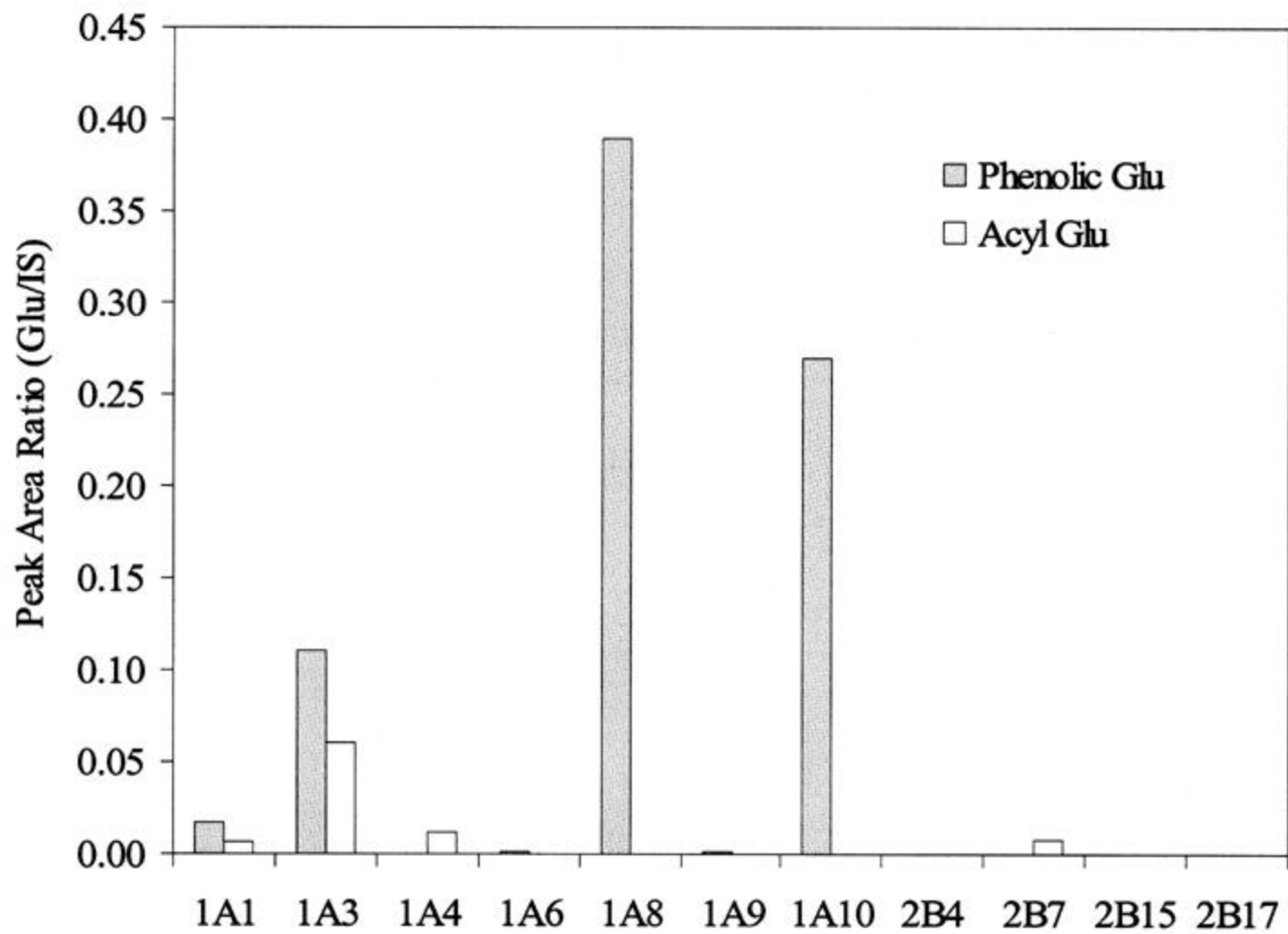# Figure 5.

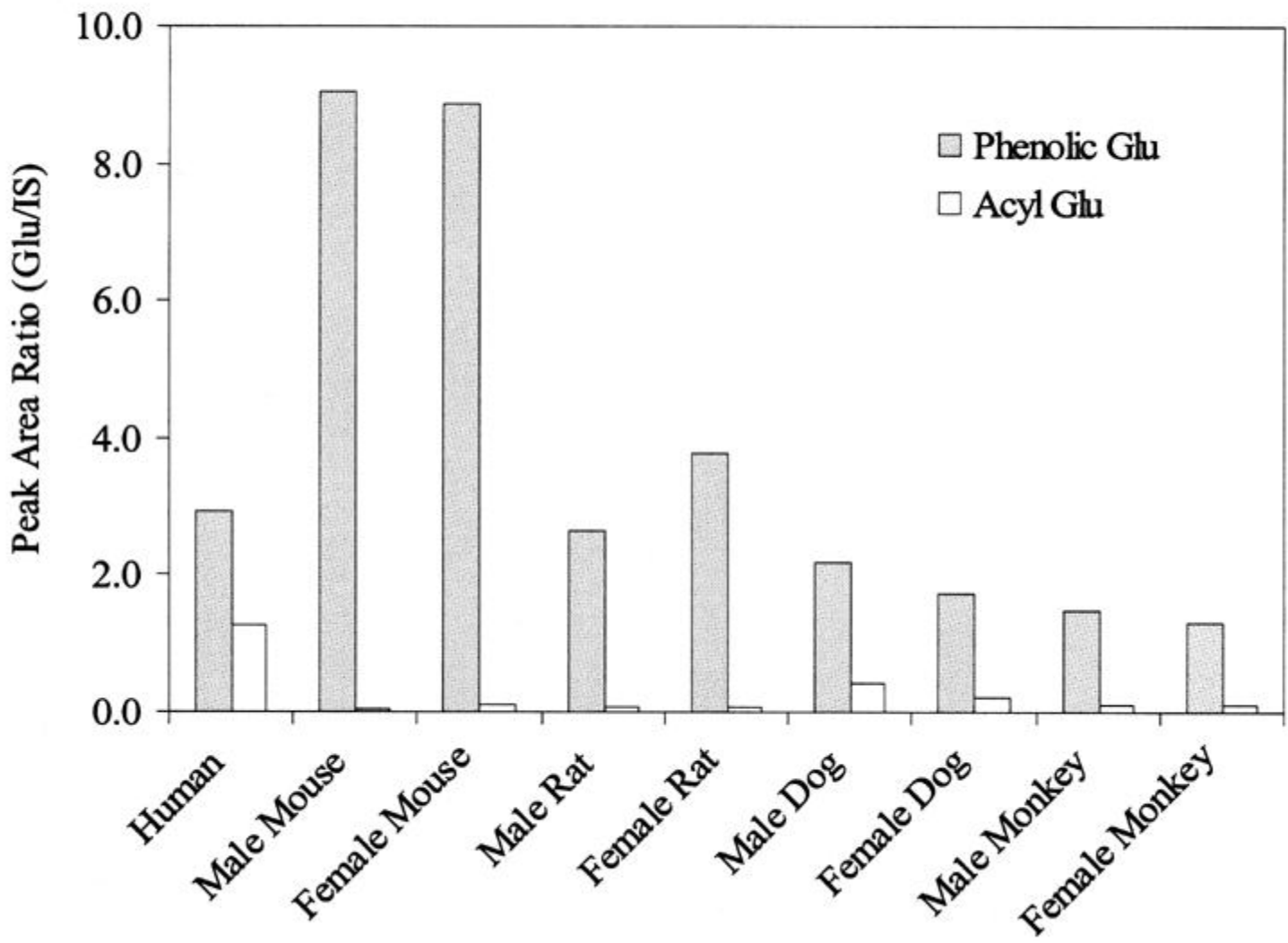**Thyroxine Glucuronide**

Thyroxine
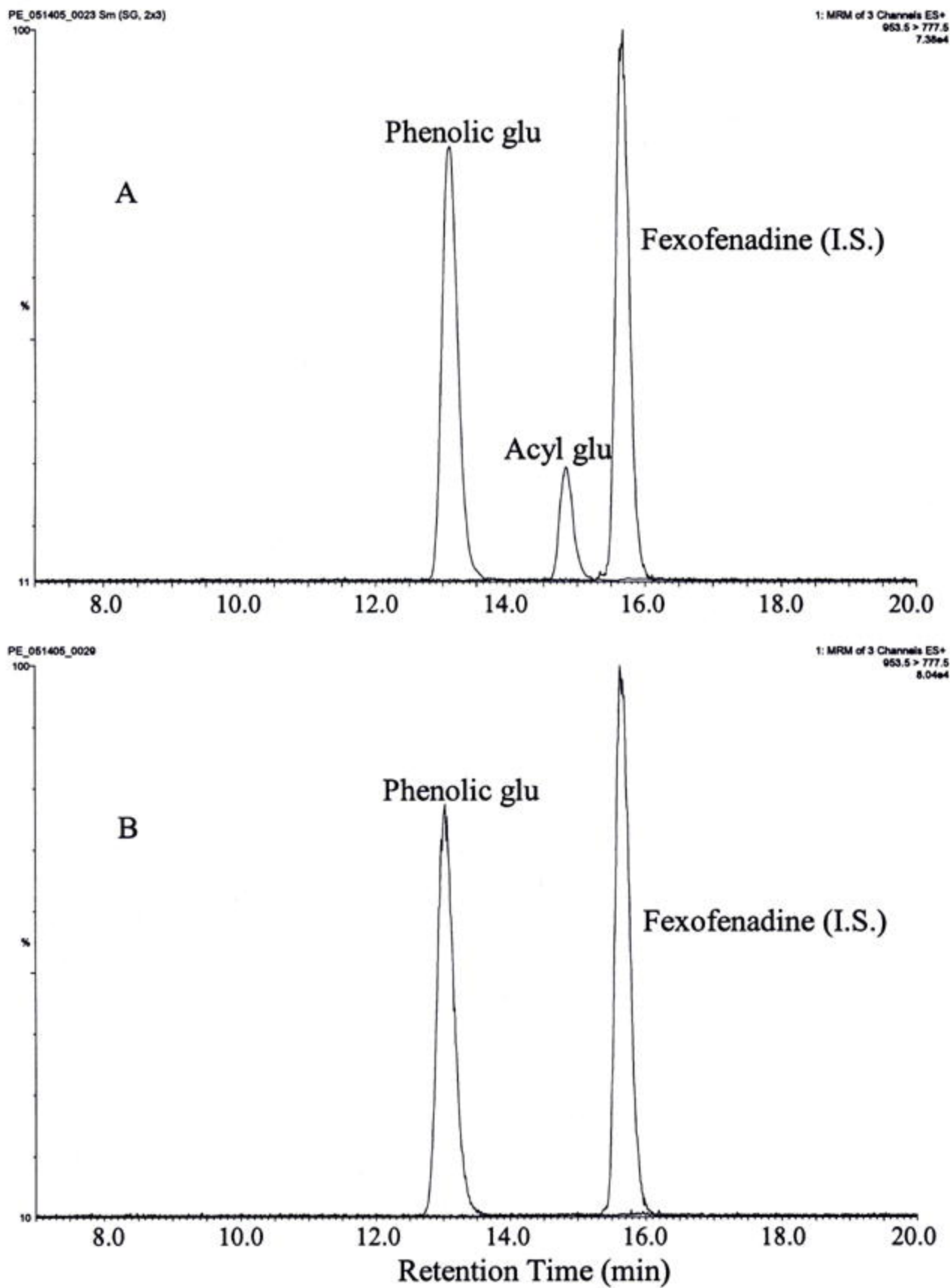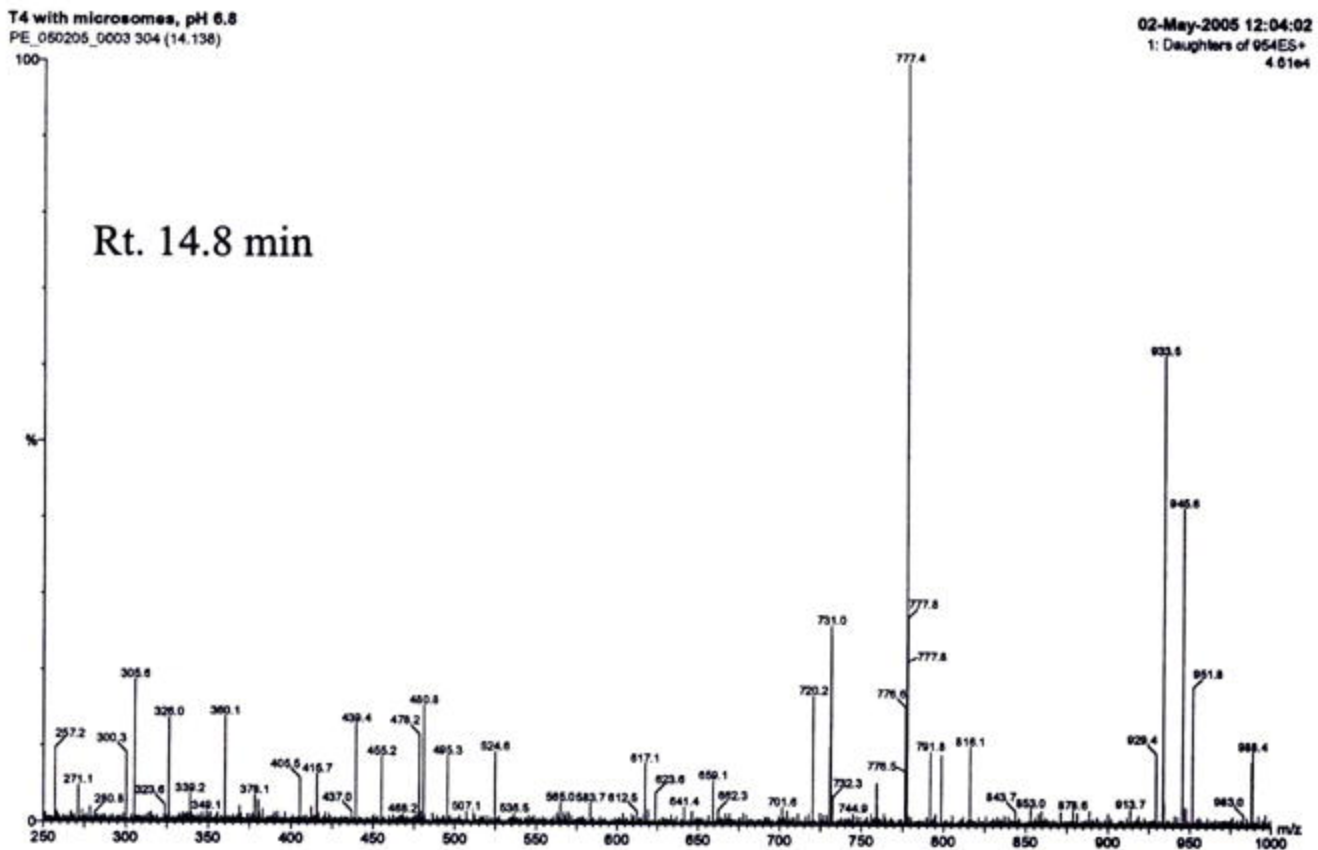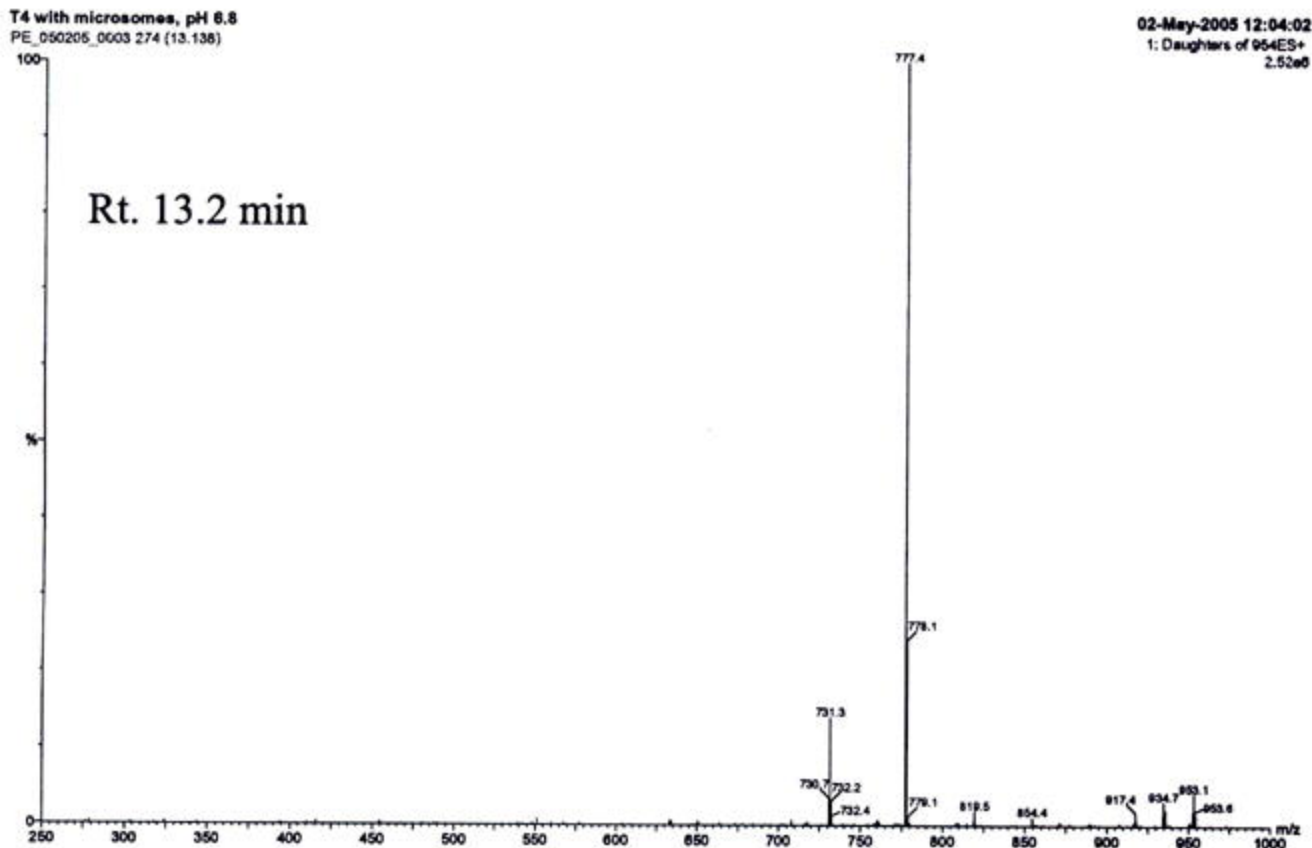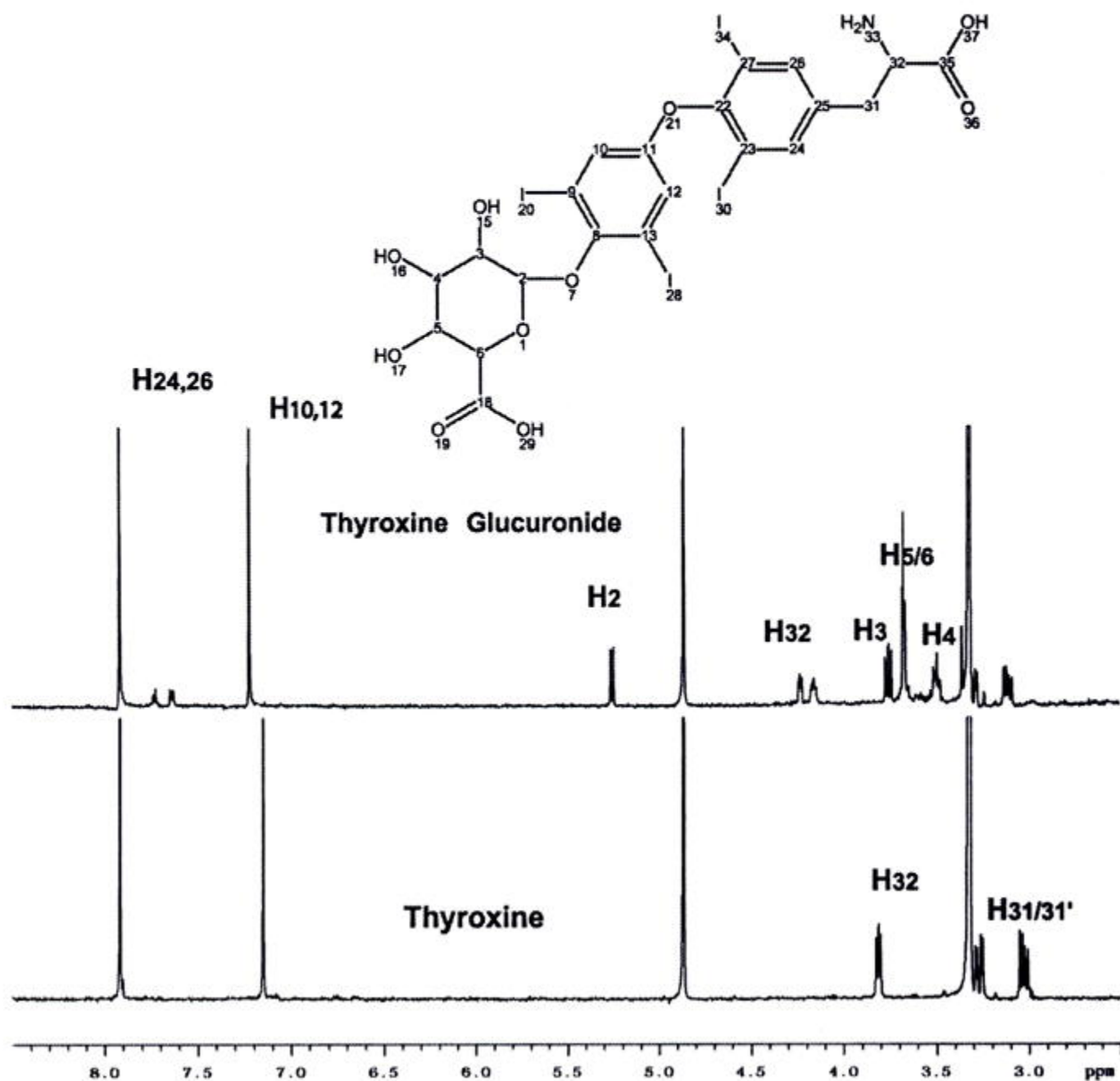
Figure 6.

Figure 7.

Figure 8.

Figure 9.

Figure 10.

Figure 11.