Isolation of the Glucuronic Acid Conjugate of N-Hydroxy-4-aminobiphenyl from Dog Urine and Its Mutagenic Activity

Jack L. Radomski, William Lee Hearn, Teresa Radomski, Henry Moreno, and William E. Scott

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SUMMARY

The glucuronic acid conjugate of N-hydroxy-4-aminobiphenyl (NOH-4-ABP) has been isolated in relatively pure form from the urine of dogs given 4-aminobiphenyl, utilizing molecular size, ion exchange, adsorption, and partition chromatography. This conjugate is an active mutagen in Salmonella typhimurium strains TA1538 and TA98 but not in TA1535 or TA1537. NOH-4-ABP and 4-nitrosobiphenyl are also highly active in TA1538 and TA98 and inactive in TA1535 and TA1537. These observations support the concept that this conjugate is the water-soluble carrier that delivers the active metabolite to the bladder.

A substance of identical chromatographic and spectral properties to the conjugate isolated from dog urine has been synthesized in low yield by the direct condensation of NOH-4-ABP with glucuronic acid. This substance yields NOH-4-ABP on dilute acid hydrolysis. Sodium (N-4-biphenyl-N-hydroxy-D-glucuronosylamine) uronate, the N-O-C isomer, was also synthesized. It was found to have differing chromatographic and chemical properties to the natural conjugate. This evidence suggests that the urinary conjugate is the compound in which conjugation has occurred with the nitrogen atom of the hydroxyamine group rather than the oxygen atom.

INTRODUCTION

Bladder cancer is induced in man and in the beagle dog by a number of aromatic amines including 4-ABP and 2-naphthylamine (5, 10, 11, 21). Since the demonstration of the urogenic nature of the induction of this disease (14), much research, over a span of more than 20 years, has been expended in an attempt to identify the active urinary metabolite. A considerable body of data has accumulated implicating N-hydroxylation as the key process involved (3, 5, 6, 16–19).

More than 3 years ago we published evidence for the existence of a glucuronic acid conjugate of NOH-4-ABP (20). This conjugate is believed to be the water-soluble carrier that transports the NOH-4-ABP from its site of formation in the liver to its site of action in the bladder. Since that time a great deal of research effort has been devoted to the isolation and final identification of this metabolite. This effort has been continually frustrated by the unstable nature of this substance. It is highly sensitive to the effects of oxygen in the atmosphere, water, acids, bases, light, and heat.

Our primary objective was to isolate enough of the metabolite in pure form to obtain a satisfactory IR spectrum which would, we believed, give us critical clues as to its identity. All that we really knew about the structure of this conjugate was that, when hydrolyzed in dilute acid solution, it liberated a molecule of glucuronic acid and a molecule of 4-NOBP as measured by gas chromatography. The formation of 4-NOBP is the result of the oxidation of NOH-4-ABP in aqueous solutions at pH 3 (17).

This paper deals with the procedures we have developed which finally enabled us to isolate a few mg of this conjugate, the chromatographic and spectrophotometric evidence we have which is suggestive of its identity, and its mutagenicity to 4 strains of Salmonella typhimurium bacteria.

MATERIALS AND METHODS

Preparation and Purity of Compounds. 4-ABP was obtained from Aldrich Chemical Co., Milwaukee, Wis., and redistilled twice followed by recrystallization from aqueous ethanol. NOH-4-ABP (m.p., 161–162°C) was prepared by the method of Willstatter and Kubli (22). 4-NOBP (m.p., 73–74°C) was prepared by the diethyldiazodicarboxylate oxidation of NOH-4-ABP and purified on a silica gel column (8).

Sodium (N-4-biphenyl-N-hydroxy-β-D-glucuronosylamine) (Chart 1, Structure a) was synthesized by a reaction analogous to the synthesis of N-glucuronides from aromatic amines (7). d-Glucuronolactone (1.7 g) was dissolved in 15 ml H₂O, and 0.81 g NaHCO₃ was added. The solution was allowed to stand at room temperature overnight. Three ml of this solution were added to 100 ml of a solution of 0.35 g NOH-4-ABP. To this freshly prepared solution, 33 ml of a solution containing 350 mg ascorbic acid neutralized to pH 7.2 with NaOH were added. Triethylamine (0.2 g) was also added as a catalyst. The reaction was stirred under nitrogen for 48 hr. The reaction mixture was then filtered and concentrated to 10 ml in a flash evaporator. This solution was filtered again to remove insoluble material and then chromatographed on Sephadex G-10. A total of 4.5 mg of the glucuronic acid conjugate was obtained in impure form when the fractions collected were pooled and lyophilized. Final purification was obtained by chromatography on...
Chart 1. Structures of possible glucuronic acid conjugates of NOH-4-ABP. Structure a, sodium (N-4-biphenyl-N-hydroxy-D-glucuronosylamine); Structure b, sodium (N-4-biphenylhydroxyIamine-@-D-glucopyranosid) uronate; Structure c, the nitrone of Structure a.

DEAE-Sephadex and Amberlite XAD-2 as described below for the isolation of the natural conjugate.

Methyl(N-trifluoroacetyl-N-4-biphenylhydroxylamine-2-, 3,4-tri-O-acetyl-@-p-glucopyranosid) uronate was prepared via a modified Koenigs-Knorr (9) synthesis using methylene chloride as the solvent. The reaction mixture, containing 0.14 g of N-trifluoroacetyl-N-4-biphenylhydroxylamine, 0.4 g of methyl(tri-O-acetyl-a-D-glucopyranosylbromide) uronate, 0.17 g of cadmium carbonate, and 50 ml of methylene chloride, was refluxed for 48 hr. The mixture was filtered through a pad of Celite, and the solvent was removed from the filtrate under reduced pressure. The residue was dissolved in the minimum amount of acetone and poured, with stirring, into 20 ml of water. The mixture was filtered through a pad of Celite, and the precipitate was washed on the filter with water and then dissolved in methylene chloride. This solution was dried and evaporated to give 0.25 g (83%) of tan crystals, m.p. 76.3°. This material, without further purification, was subjected to alkaline hydrolysis.

Sodium (N-4-biphenylhydroxyIaminogluco-@-p-glucopyranosid) uronate (Chart 1, Structure b) was prepared by dissolving the above O-glucuronide in 15 ml of absolute methanol. To this stirred solution at room temperature was added 0.2 ml of 10 N sodium hydroxide. After 1 hr, during which time a white precipitate formed, the methanol was removed under reduced pressure, 2 ml of water were added, and the pH was adjusted to about 8. The reaction mixture was filtered to remove a dark gummy precipitate and washed with 3 ml of water. The resulting filtrate was pale yellow and syrupy. It was purified by chromatography on Sephadex G-10. Fractions containing the conjugate determined by acid hydrolysis, extraction, and gas chromatography were lyophilized. The slightly off-white amorphous material was chromatographed on paper. The compound was very unstable and decomposed completely within a few hr despite storage in the cold under N2.

**Dosage Administration and Urine Collection.** 4-ABP (10 mg/kg) was administered to dogs in dry, powdered form by capsule. Higher doses are apt to produce fatal methemoglobinemia. Usually, dogs (both pure bred beagles and mongrels) were used that had been pretreated for 2 weeks with phenobarbital (20 mg/kg/day given p.o.). Urine was collected every 4 hr by catheter. Approximately 50% of the dose was excreted in phenobarbital-treated dogs. Both male and female dogs were used.

**Column Chromatography.** The initial separation step usually utilized was a Sephadex G-10 column packed with N2-treated water, pH adjusted with NaOH to between 7.2 and 7.8. Urine (10 to 15 ml) was chromatographed on a 3- x 60-cm column. Elution was with N2-treated, pH-adjusted water. Fifteen-ml fractions were collected through a LKB UV absorption meter (280 nm) until no further UV absorption was noted. These columns usually ran for about 16 hr at an elution rate of 1 ml/min. The fractions were collected in a refrigerated fraction collector (20). Recoveries ranging from 35 to 55% of the conjugate based on the liberation of NOH-4-ABP by acid hydrolysis were obtained.

A 2nd column utilized ion exchange. This column consisted of a 3- x 60-cm glass column packed with DEAE-Sephadex A-25 in the sodium form. The column was washed with 0.1 M Tris buffer, pH 7.6, to constant pH. Aqueous fractions containing the conjugate were applied to the top of the column. The column was then eluted with 0.1 M Tris to 0.1 M Tris-2 M NaCl gradient. The eluate from the column was monitored by UV (280 nm) and was collected in 15-ml fractions in a refrigerated centrifuge. The conjugate did not begin to appear until close to the end of the gradient and required approximately 24 hr to be eluted. Recoveries of 20 to 35% were observed with this column.

The 3rd chromatographic procedure utilized was an Amberlite XAD-2, 4- x 100-cm column. Fractions containing the conjugate were applied to the column. The column was then washed with 6 liters of N2-treated, pH-adjusted water and then allowed to run dry. Elution of the conjugate was accomplished with 600 ml of methanol. Fifty-ml fractions were collected and analyzed. At this point the fractions containing the conjugate were dried by lyophilization. Recovery of the conjugate ranged from 30 to 50% from the Amberlite XAD-2 column.

The 4th procedure utilized for purification was a partition column utilizing n-butyl alcohol saturated with water. A 2- x 40-cm column was packed with Silica Gel Woeim (0.063 to 0.2 mm, containing 20% water for partition chromatography). It was then washed with 100 ml of water-saturated n-butyl alcohol. The lyophilized conjugate was dissolved in 5 ml of the water-saturated butyl alcohol and added to the column; the column was then developed with this eluant. Twenty-ml fractions were collected. Recoveries of 30 to 50% were observed with this column.

These chromatographic procedures were always carried out in a minimum of light, and care was taken to maintain, as much as possible, an atmosphere of nitrogen.

Due to continuous, gradual decomposition, overall recovery of the conjugate was poor and difficult to determine accurately since we were never certain of the recovery of our analytical procedure, having no primary standard. Our usual procedure was to dose 2 dogs with 10 mg/kg on 2 consecutive days, pooling the fractions from the Amberlite XAD-2 column, and lyophilizing before putting it on the final n-butyl alcohol partition column. We usually obtained 1 to 2 mg of pure material overall.

**Paper Chromatography.** In the latter purification steps, detection of the conjugate and the determination of the presence of impurities were carried out by horizontal chromatography utilizing circles of Whatman No. 1 filter paper. The solvent was introduced into the bottom Petri dish. The compounds to be compared were applied with a finely drawn glass pipet in arcs within a few mm surrounding the
with strains TA1535, TA1537, TA1538, and TA98 obtained from Dr. Bruce N. Ames. The assays were conducted according to his recommended procedures (1, 2). Since our objective was to evaluate the activity of the conjugate at the target site, activating microsomal enzymes were not added. In addition to conducting the test at the usual pH of 6.8, a 2nd series of plates was run at pH 5.5, a common pH observed in dog urine. This was achieved by the addition of dilute HCl to the usual phosphate buffer. No reduction of the lawn (an indication of toxicity) was observed at the lower pH or after exposure to the compounds.

High-Pressure Liquid Chromatography. High-pressure liquid chromatography was carried out using: (a) a Pelaminodon (0.5-m x 2.6-mm inside diameter) stainless steel column with 2-propanol:H2O (1:4) at a flow rate of 0.4 ml/min and 700 psi; (b) a Sil-X-1 (0.5-m x 2.6-mm inside diameter) stainless steel column with methanol:H2O (70:30) and a 0.6 ml/min flow rate; and (c) Glycophase G (cpg 100; 0.5-m x 2.6-mm inside diameter) with H2O at 100 psi and a 0.4 ml/min flow rate.

RESULTS AND DISCUSSION

Purification of the Conjugate of NOH-4-ABP. The initial step in purification was, as previously reported (20), chromatography on Sephadex G-10. However, several major improvements have been made which have contributed significantly to our ability to isolate the conjugate. The 1st of these was the observation that phenobarbital stimulation increased the concentration of the conjugate in the urine. The 2nd major improvement was the development of an ion-exchange column that was capable of resolving most of the contaminants remaining after the initial Sephadex G-10 chromatography (Chart 2). This column has the additional advantage of permitting the addition of fractions from the Sephadex G-10 column without concentrating them. Flash evaporation and other concentrating procedures, despite all precautions, invariably cause high losses when carried out on impure samples. Previous attempts to utilize ion-exchange chromatography were unsuccessful primarily because of the extreme sensitivity of the conjugate to pH's deviating from neutrality. The 2nd problem encountered with ion exchange was the fact that large amounts of inorganic salts were introduced into the purification procedure.

Determination of Glucuronic Acid. Glucuronic acid concentrations were measured by the method of Bitter and Muir (4) without modification. Measurement of the color developed in the carbazole reaction was made on a Beckman Model DBG spectrophotometer.

Mutagenesis Assay. Mutagenesis tests were conducted with strains TA1535, TA1537, TA1538, and TA98 obtained from Dr. Bruce N. Ames. The assays were conducted according to his recommended procedures (1, 2). Since our objective was to evaluate the activity of the conjugate at the target site, activating microsomal enzymes were not added. In addition to conducting the test at the usual pH of 6.8, a 2nd series of plates was run at pH 5.5, a common pH observed in dog urine. This was achieved by the addition of dilute HCl to the usual phosphate buffer. No reduction of the lawn (an indication of toxicity) was observed at the lower pH or after exposure to the compounds.

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This difficulty was overcome by a 3rd column, Amberlite XAD-2, which is capable, because of its polystyrene composition, of adsorbing aromatic compounds from aqueous solutions. The fractions from the DEAE-Sephadex column were added to the Amberlite XAD-2 column without concentrating them; the NaCl and other nonadsorbed water solubles they contained were washed away by water, and the conjugate was eluted with methanol. The methanol was concentrated until freezing occurred, followed by lyophilization which provided a semipure product. Final purification was achieved by paper chromatography utilizing n-butyl alcohol (water saturated). The conjugate was visible as a dark purple band in UV (254 nm) which was excised and eluted with water. The eluate was lyophilized yielding light pink, needle-like crystals of the conjugate. Additionally, the conjugate was purified on a silica gel column utilizing the same solvent system (n-butyl alcohol, water saturated). Using this procedure, the conjugate was usually recovered as a white amorphous material upon flash evaporation. Chromatography of this substance by horizontal circular paper chromatography in n-butyl alcohol:n-propyl alcohol:water (2:1:1) produced a single dark purple band visible by UV (254 nm). Chromatography by high-pressure liquid chromatography gave single, symmetrical peaks on 3 different columns, Pellamidon, Sil-X-1, and Glycophase G (Rf = 4.2, 6.1, and 3.0 min, respectively).

Attempts at Chemical Synthesis. We had been attempting to prepare the conjugate by chemical synthesis for as many years as we have been trying to isolate it. Because of the extreme difficulty of obtaining anything more than a very few mg from dog urine, the difficulty of holding on to what we have already isolated, and its incompatibility with such analytical techniques as nuclear magnetic resonance and mass spectrometry, we had felt all along that chemical synthesis provided the best hope of its identification.

There are 3 possible structures for this conjugate (Chart 1). Compound a, sodium (N-4-biphenyl-N-hydroxy-D-glucuronosylamine), an N-C conjugate, could arise by conjugation with the nitrogen atom of the hydroxylamine group in a manner analogous to the formation of the N-glucuronides of 2-naphthylamine and other aromatic amines. Compound b, sodium (N-4-biphenylhydroxylaminol-β-D-glucopyranoside) uronate, an N-O-C conjugate of the N-hydroxy group, could arise through the glucuronyl transferase mechanism which conjugates phenolic hydroxyl groups. Compound c could arise by oxidation of Compound a to form a nitrene. All 3 structures have almost the same molecular weight and could liberate NOH-4-ABP under acidic conditions. All 3 structures represent new types of compounds, the properties of which are virtually unknown.

We have finally succeeded in synthesizing Compound b (see "Materials and Methods"). Although it has properties similar to those of the urinary conjugate, its Rf values in butyl alcohol-containing solvent systems are slightly different. Recently, Kadlubar et al. (13) have reported the in vitro production by liver microsomes of glucuronic acid conjugates of N-hydroxy-2-naphthylamine, N-hydroxy-1-naphthylamine, and NOH-4-ABP. These results have strengthened our concept of the importance to bladder carcinogenesis of these glucuronides as carriers of the active N-hydroxy metabolites. The possibility occurred to us that the urinary conjugate of NOH-4-ABP we were seeking to identify might be related to an N-glucuronide of the type observed by Boyland et al. (7) with the aromatic amines themselves. By a modification of the procedure of Boyland et al. (7), utilizing triethylamine as a catalyst and using sodium ascorbate as an antioxidant, we have succeeded in preparing small quantities of what we believe to be Compound a by direct condensation of glucuronic acid with NOH-4-ABP. This compound matches in every respect the chromatographic properties of the conjugate isolated from dog urine. When the 2 compounds were chromatographed together by horizontal circular chromatography (see "Materials and Methods"), a perfect circle of dark purple fluorescence (254 nm) was observed at the identical Rf (0.6). Both compounds gave a blue color when sprayed with pentacyanoamine ferrate, and a deep red color was seen with dimethylaminocinnamaldehyde (Table 1). An interesting phenomenon was noted with this spray; a 2nd band, very light orange in color, appeared in both the synthetic conjugate portion and the natural conjugate portion of the chromatogram. Its Rf (0.4) was lower than that of the band reacting with pentacyanoamine ferrate. Dimethylaminocinnamaldehyde is a very sensitive spray reagent. Apparently, both compounds, the synthetic conjugate and the naturally occurring conjugate, have a small amount of the same impurity or decomposition product, an observation which adds additional credence to the chromatographic identification.

The synthetic conjugate (Chart 1, Compound a) was purified by column chromatography with n-butyl alcohol (water

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Rf a</th>
<th>Rf b</th>
<th>Rf c</th>
<th>UV (254 nm)</th>
<th>PCAF*</th>
<th>PMAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-NOBP</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
<td>Purple</td>
<td>Blue</td>
<td>Red</td>
</tr>
<tr>
<td>Urine conjugate</td>
<td>0.60</td>
<td>0.40</td>
<td>0.80</td>
<td>Dark purple</td>
<td>Blue</td>
<td>Red</td>
</tr>
<tr>
<td>Conjugate a</td>
<td>0.60</td>
<td>0.40</td>
<td>0.80</td>
<td>Dark purple</td>
<td>Blue</td>
<td>Red</td>
</tr>
<tr>
<td>Conjugate b'</td>
<td>0.65</td>
<td>0.65</td>
<td>0.90</td>
<td>Dark purple</td>
<td>None</td>
<td>Red</td>
</tr>
</tbody>
</table>

* For n-butyl alcohol:n-propyl alcohol:water (2:1:1).
* For n-butyl alcohol (H2O saturated).
* For ethanol:water (3:1).
* PCAF, pentacyanoamine ferrate; PMAC, p-dimethylaminocinnamaldehyde.
* Sodium (N-4-biphenyl-N-hydroxy-D-glucuronosylamine).
* Sodium (N-4-biphenylhydroxylaminol-β-D-glucopyranoside) uronate.
Mutagenic Glucuronide of NOH-4-ABP

The IR spectrum was taken of this product and compared with that of the urinary conjugate (Chart 3). These spectra are virtually identical. It is clear that we have succeeded in synthesizing the dog urinary conjugate. Attempts to recrystallize either the natural or synthetic compound for elemental analysis have consistently resulted in decomposition. We are attempting to synthesize the methyl ester and the silylated derivatives of the conjugate which may enable us to apply nuclear magnetic resonance and mass spectrometry to the unequivocal identification of the conjugate. However, all attempts thus far to derivatize the conjugate have resulted in its decomposition.

The IR spectra appear to be that of a glucuronide of a biphenyl compound. The carboxylate group is clearly present. In addition, the broad peak between 1000 and 1100 cm\(^{-1}\) is indicative of a glucosiduronate; all such compounds prepared in this laboratory have this peak. The 3 peaks around 800 cm\(^{-1}\) are characteristic of biphenyl compounds. Absorption between 1170 and 1280, which is characteristic of nitrones, seems to be totally absent. In addition, the fact that we were able to synthesize the conjugate in a reaction mixture containing an antioxidant (sodium ascorbate) tends to eliminate consideration of Structure c (Chart 1). The fact that the N-O-C conjugate sodium (N-4-biphenylhydroxyamino-\(\beta\)-D-glucopyranosid) uronate prepared by an unequivocal synthesis has significantly different chromatographic characteristics eliminates this structure from consideration. The fact that we have prepared the conjugate by a method analogous to the synthesis of an N-glucuronide points toward Structure a as the conjugate.

Mutagenic Activity of the Conjugate. In order to evaluate the potency of the conjugate as the urinary carcinogen, a purified preparation recently isolated from dog urine was tested for mutagenic activity by using \textit{S. typhimurium} tester strains obtained from Dr. Bruce Ames. These tests have shown a remarkable correlation between the mutagenic activity detected and the carcinogenicity of a large series of compounds (12, 15). Since we were interested in evaluating the conjugate as a proximate carcinogen, the tests were conducted without the addition of liver microsomes for activation. Because the pH of dog urine is frequently as low as 5.5, tests were conducted at this pH in addition to the normal pH of 6.8. NOH-4-ABP and 4-NOBP, which have been shown to be mutagenic in the Ames Test (15), were included at 1 dosage as positive controls. 4-ABP was a negative control. The conjugate showed a marked mutagenic activity in TA1538 and TA98 at a dose level of 25 \(\mu\)g/plate and significant activity at 5 \(\mu\)g/plate (Table 2). There was a clear-cut dosage response at the 3 dosages tested. In strain TA98 but not in TA1538 an increased mutagenic activity was observed at pH 5.5. This would be consistent with the possibility that hydrolysis at the lower pH favored the release of the N-hydroxy and/or nitroso compounds. However, if this were true, the same pH effect would have occurred in TA1538. It is clear that more definitive experiments must be done before it is possible to determine whether the conjugate reacts directly with the bacterial genome or whether hydrolysis occurred first, liberating the N-hydroxy compound that produced the effect. At any rate, these results demonstrate the mutagenic activity of the conjugate without microsomal activation and lend further support to the importance of the biological role of the glucuronic acid conjugate of NOH-4-ABP in aromatic amine carcinogenesis.

Chart 3. IR spectra of glucuronic acid conjugate of NOH-4-ABP isolated from the urine of dogs given 4-ABP (upper curve) and synthetic conjugate prepared by direct reaction of NOH-4-ABP and glucuronic acid (lower curve).

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Table 2
Mutagenesis assay of the conjugate from dog urine in S. typhimurium tester strains (supplied by Dr. Ames) compared to parent amine and N-hydroxy and nitroso metabolites

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose/plate (µg)</th>
<th>Media pH</th>
<th>TA1535</th>
<th>TA1537</th>
<th>TA1538</th>
<th>TA98</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-ABP</td>
<td>10</td>
<td>6.8</td>
<td>11, 9</td>
<td>23, 25</td>
<td>11, 19</td>
<td>13, 15</td>
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<tr>
<td>4-NOBP</td>
<td>10</td>
<td>6.8</td>
<td>24, 29</td>
<td>5, 9</td>
<td>141, 127</td>
<td>149, 131</td>
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<tr>
<td>NOH-4-ABP</td>
<td>10</td>
<td>6.8</td>
<td>13, 16</td>
<td>30, 33</td>
<td>224, 204</td>
<td>800</td>
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<tr>
<td>Urine conjugate</td>
<td>25</td>
<td>6.8</td>
<td>21</td>
<td>166, 178</td>
<td>237, 224</td>
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<tr>
<td>Urine conjugate</td>
<td>5</td>
<td>6.8</td>
<td>22, 29</td>
<td>21, 24</td>
<td>44, 51</td>
<td>48, 50</td>
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<tr>
<td>Urine conjugate</td>
<td>0.5</td>
<td>6.8</td>
<td>28, 22</td>
<td>15, 20</td>
<td>25, 27</td>
<td>6, 4</td>
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REFERENCES

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