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Identification of radiolabeled metabolites of nicotine in rat bile

Synthesis of *S*-(−)-nicotine N-glucuronide and direct separation of nicotine-derived conjugates using high-performance liquid chromatography

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ABSTRACT

Four metabolites of nicotine, including two glucuronides, have been separated by high-performance liquid chromatography. This separation was applied to identification of biliary metabolites of radiolabeled nicotine by radiometric detection. *S*-(−)-Nicotine N-glucuronide was synthesized and used as a standard in method development.

INTRODUCTION

Recent studies of nicotine disposition in humans and macaques revealed that glucuronides of nicotine, cotinine, and 3-hydroxycotinine were major urinary metabolites [1–4]. In those studies, indirect methods, involving incubation with β-glucuronidase, were employed for identification of conjugates [5–8]. A direct method would be expected to provide better estimates of rates of glucuronide formation and excretion since hydrolysis with β-glucuronidase is incomplete. Therefore, for the present study we synthesized nicotine N-glucuronide and used cotinine N-glucuronide synthesized previously [9]. They served as standards and enabled development of a high-performance liquid chromatographic (HPLC) method for separation of these conjugates. Using radiochromatography, this method has been applied to the identification of nicotine metabolites in rat bile following administration of racemic [^{14}C]nicotine.

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EXPERIMENTAL

Materials

D-Glucurono-6,3-lactone was purchased from Aldrich (Milwaukee, WI, USA) and used to synthesize methyl (2,3,4-trio-O-acetyl-α-D-glucopyranosyl bromide) uronate [10]. All other chem-

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icals used in synthesis and purification of the heteroaromatic type N^+-glucuronide metabolite of *S*-(−)-nicotine were purchased from Sigma (St. Louis, MO, USA). *S*-(−)-Cotinine N-glucuronide (inner salt), synthesized by a published method [9], was a gift from Dr. Gary Byrd (Winston-Salem, NC, USA). *S*-(−)-Cotinine was purchased from Sigma. *trans*-3-Hydroxycotinine was generously provided by Dr. Georg Neurath (Hamburg, Germany). Racemic nicotine [2′-^{14}C]pyrrolidine, stored in ethanol under argon with a specific activity of 50.0 mCi/mmol was purchased from New England Nuclear (Boston, MA, USA). Acetonitrile and methanol were of analytical grade, and water was distilled, filtered and degassed.

Synthesis of the heteroaromatic type N^+-glucuronide of S-(−)-nicotine

S-(−)-Nicotine (0.5 g, 3.1 mmol) was treated with methyl (2,3,4-tri-O-acetyl-α-D-glucopyranosyl bromide) uronate (1.0 g, 2.5 mmol), according to a published procedure [11]. The work-up was modified as follows. The aqueous solution obtained was loaded on an XAD-2 resin column (200 g, packed in a 2.4 cm I.D. column), and the product was eluted with distilled water. The combined aqueous eluate was applied to a strongly acidic cation-exchange column (Dowex-50W, 200 g packed in a 2.4 cm I.D. column). The bound material was washed with distilled water (four bed volumes) and then eluted with 2 *M* aqueous ammonia. Collection and evaporation of the appropriate fraction of eluate yielded a light brown amorphous solid product (0.388 g, 37%).

^{1}H NMR δ 9.13–9.09 (m, 2H, pyridinium H-2 and H-6; *cf.* (−)-nicotine δ 8.50–8.43), 8.67 (d, J = 8.0 Hz, 1H, pyridinium H-4; *cf.* (−)-nicotine δ 7.85), 8.12 (t, J = 6.2 Hz, 1H, pyridinium H-5; *cf.* (−)-nicotine δ 7.42), 5.72 (d, J = 8.8 Hz, 1H, anomeric proton), 2.26 (s, 3H, NCH_3; *cf.* (−)-nicotine δ 2.16).

^{1}H NMR and mass spectrometry

The ^{1}H NMR spectra of the starting material and product were taken on a Bruker AM-300 spectrometer (Bruker, Milton, Ont., Canada) in CD_3OD; chemical shifts were recorded in parts per million downfield from $(CH_3)_4Si$. Only the diagnostic peaks are reported. The mass spectrum was obtained in the positive-ion fast-atom bombardment (FAB) mode, with argon as the source of fast atoms at 7 kV and 1 mA current on a VG Analytical 7070HE instrument (VG/Fisons, Altrincham, UK) connected to a DEC PDP 11-250J data system. Glycerol was used as sample matrix for mass spectrometric analysis with a direct probe.

The product of our synthesis was identified as *S*-(−)-nicotine N-glucuronide by FAB mass spectrometry and ^{1}H NMR spectroscopy. The characteristic ions in the FAB mass spectrum included the molecular cation and its sodium and potassium adducts, as well as protonated nicotine resulting from cleavage of the glycosidic bond, with transfer of a proton from the glucuronic acid moiety to the aglycone [12] (*m/z* (relative intensity, %) 377 ($[M - H + K]^+$, 12), 361 ($[M - H + Na]^+$, 2), 339 (M^+, 7), 163 ([aglycone + H]$^+$, 67)). The use of ^{1}H NMR spectroscopy comfirmed that the product was an aromatic, rather than an aliphatic, N^+-glucuronide metabolite. First, compared with the ^{1}H NMR spectrum of nicotine, there was a large downfield shift of signals assigned to protons of the pyridine ring, but not of the N–CH_3 group. Also, the chemical shift value (δ 5.72) for the anomeric proton (C_1–H) of the glucuronic acid moiety was in the range reported for aromatic N^+-glucuronide metabolites (δ 5.3–6.0) [9,13,14], but not for aliphatic N^+-glucuronide metabolites (δ 4.3–5.0) [11]. Finally, since the coupling constants for α- and β-anomers range from 2 to 4 Hz and from 7 to 12 Hz, respectively [15], the magnitude of coupling between H_1 and H_2 of the glucuronic acid moiety (8.8 Hz) indicates that nicotine N^+-glucuronide has a β-anomeric configuration.

High-performance liquid chromatography

High-performance liquid chromatography was performed on a Waters liquid chromatography system (Millipore, Bedford, MA, USA) comprising a WISP 710B autosampler and two Model

510 solvent-delivery systems controlled by an M680 automated gradient controller. Metabolite standards were detected by a 490 programmable multiwavelength absorbance detector. Absorbance at 254 nm was monitored on a 3390A integrator (Hewlett-Packard) with sensitivity set at 0.1 AUFS. Radiolabeled biliary metabolites were detected using a Berthold 505 radioactivity flow monitor system (Berthold Analytical, Nashua, NH, USA). The radioactivity signal was stored and integrated by an LB 510 Berthold chromatography data station. Separation was achieved using a 10 μm 300 mm × 3.9 mm I.D. μBondapak C_{18} reversed-phase column preceded by a Guard-Pak C_{18} insert and holder (Millipore).

Mobile phases were acetonitrile (A) and water (B), and the flow-rate was 1.5 ml/min. The column was equilibrated with 100% acetonitrile. After injection of standards or bile samples, conditions were changed to 97% A and 3% B for 4 min, followed by 94% A and 6% B for 2 min, and then 92% A and 8% B for 4 min. Final conditions were 70% A and 30% B for 2 min. The column was re-equilibrated with 100% A for 22 min. Chromatography was performed at room temperature.

Sample preparation

After the common bile duct was cannulated, male Sprague-Dawley rats received, as a 10-min infusion via the tail vein, 10 μCi of racemic [^{14}C]nicotine in saline. Bile was collected at regular intervals. Samples were lyophilized, reconstituted in methanol, centrifuged to remove undissolved material, concentrated under nitrogen and injected into the chromatograph.

For identification of glucuronide metabolites, individual peaks were incubated for 20 h at 37°C with 10 000 units β-glucuronidase from bovine liver (Sigma) in sodium acetate buffer (pH 4.5). After incubation, samples were centrifuged, and supernatants were injected into either the present HPLC system or the system previously described [1].

Prior to HPLC analysis, standards of cotinine N-glucuronide, nicotine N-glucuronide, cotinine and 3-hydroxycotinine were dissolved in methanol.

RESULTS AND DISCUSSION

A direct separation of nicotine-derived glucuronides in bile depends on the availability of appropriate standards. Authentic cotinine N-glucuronide was provided by the group that synthesized it [9]. We synthesized *S*-(−)-nicotine N-glucuronide using a previously described method [11] with slight modifications as described above. The third conjugate, 3-hydroxycotinine glucuronide, has not as yet been synthesized, so for its identification we had to rely on the indirect methods described above.

The optimum solvent system for separating standards of cotinine, 3-hydroxycotinine, nicotine N-glucuronide and cotinine N-glucuronide was determined through extensive systematic trials of different conditions, flow-rates, and linear and non-linear gradients. Fig. 1 presents separation of the four unlabeled nicotine metabolites. The more polar conjugates are well retained on the C_{18} matrix, in seeming disagreement with general principles of reversed-phase chromatography. This apparent contradiction results from the predominantly organic nature of the mobile phase employed. Under these conditions, very polar chemicals are more closely associated with

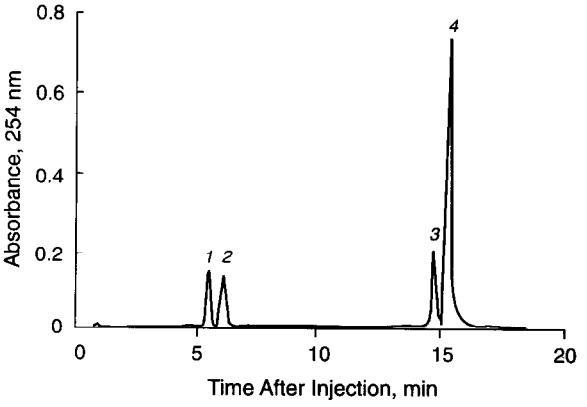

Fig. 1. HPLC separation of unlabeled standards of nicotine metabolites on a 300 mm × 3.9 mm I.D. 10 μm Waters μBondapak C_{18} reversed-phase column. See text for details of solvent program. Peaks: 1 = 3-hydroxycotinine (1.5 μg); 2 = cotinine (1.5 μg); 3 = cotinine N-glucuronide (0.3 μg); 4 = nicotine N-glucuronide (1.5 μg). Elution times: peak 1, 5.4 min; peak 2, 6.3 min; peak 3, 14.5 min; peak 4, 15.4 min.

the column matrix than are less polar compounds. In the present situation, the effect is that of normal phase separation, as the less polar aglycone metabolites elute first.

Prior to development of this method of separation, nicotine conjugates had been detected only in urine [1–6]. One of the eight metabolite peaks identified in urine of human subjects and stumptailed macaques [1,2] contained glucuronidated forms of nicotine, cotinine and 3-hydroxycotinine. Isolation of that peak required extensive extractions, which resulted in poor recovery (unpublished results). Because the biliary route of excretion is reserved primarily for conjugated products, extensive extraction to isolate glucuronides is unnecessary. Therefore, identification of nicotine metabolites in bile could reveal an additional route of excretion for, and facilitate the study of, nicotine-derived glucuronides.

When injected into the present HPLC system, bile samples collected from rats after administration of racemic [^{14}C]nicotine revealed three metabolite peaks (Fig. 2). Using an authentic standard, we identified peak 1 as cotinine. Identification was confirmed using a previously described HPLC system [1].

The retention time of peak 2 did not correlate with either the nicotine N-glucuronide standard or the cotinine N-glucuronide standard. Following incubation of peak 2 with β-glucuronidase,

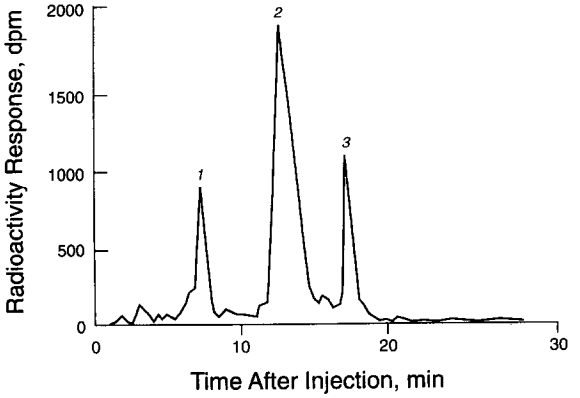

Fig. 2. HPLC separation of [^{14}C]nicotine metabolites in a bile sample (20 μl) from a male Sprague–Dawley rat administered 10 μCi of racemic nicotine ([2′-^{14}C]pyrrolidine). See text for details of solvent program. Peaks: 1 = cotinine; 2 = 3-hydroxycotinine glucuronide; 3 = nicotine N-glucuronide.

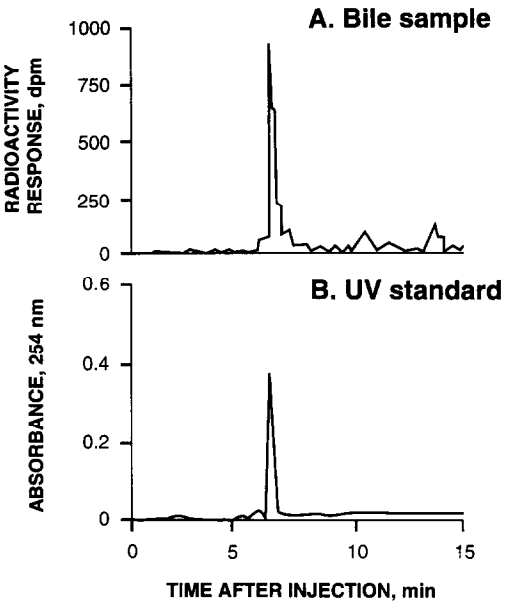

Fig. 3. (A) Radiochromatography of metabolite peak 2 following incubation with β-glucuronidase as described in the text. (B) Chromatogram of UV standard for 3-hydroxycotinine. The solvent program is described elsewhere [1].

the resulting aglycone eluted with the authentic standard of 3-hydroxycotinine on both the present system and an HPLC system described previously [1] (Fig. 3). Based on these results, we have identified peak 2 as 3-hydroxycotinine glucuronide. Although peak 2 in Fig. 2 is broad, it is symmetrical. It is possible that the breadth is due to different ionic forms of 3-hydroxycotinine glucuronide. Also, the presence of different chemical forms (*i.e.* N-, N′- and O-glucuronide) cannot be ruled out, although only 3-hydroxycotinine O-glucuronide has been detected in urine from human smokers [16].

Peak 3 co-eluted with the nicotine N-glucuronide standard. After incubation with β-glucuronidase, peak 3 revealed on HPLC a metabolite peak corresponding to nicotine. Therefore, peak 3 appears to be nicotine N-glucuronide.

With the use of the present separation technique, direct measurement of the individual glucuronides of nicotine and its metabolites should allow more thorough investigation of pathways of nicotine metabolism than previously possible. Future investigations of the biliary excretion of

these conjugates could contribute to a better understanding of the processes affecting nicotine disposition, such as enterohepatic circulation.

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