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High-performance liquid chromatography method for determination of N-glucuronidation of 4-aminobiphenyl by mouse, rat, and human liver microsomes

Mustafa Al-Zoughool *, Glenn Talaska

Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, OH 45267-0056, USA

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Abstract

A simple and sensitive method for determination of the N-glucuronidation activity of mouse, rat, and human liver microsomes toward the carcinogenic arylamine 4-aminobiphenyl (4-ABP) using high-performance liquid chromatography with ultraviolet detection has been developed. The method uses chemically synthesized 4-ABP-*N*-glucuronide (4-ABP-G) as a standard for method validation. Validation was done with respect to specificity, linearity, precision, accuracy, and lower limits of detection. The method was specific since there were no interference peaks from the reaction matrix. The calibration curve for 4-ABP-G was linear from 50 to 5000 pmol/200 μl with $R^2 = 0.999$. The newly developed method has good precision and accuracy. The intra- and interday precisions were less than 5 and 10%, respectively, and the highest values for intra- and interday accuracies were −4.6 and −12%, respectively. The lower limit of detection was 10 pmol/200 μl. The developed method was used to determine the glucuronidation activity of mouse, rat, and human liver microsomes. Human liver microsomes were the most active in 4-ABP glucuronidation (344.1 pmol/min/mg) followed by rats (30.6 pmol/min/mg) and then mice (12.3 pmol/min/mg). Human UGT1A4 supersomes were much more active than UGT1A9 (184.4 mol/min/mg versus 25.2 mol/min/mg). These results are consistent with those of earlier studies that used the radioactive [C^{14}]UDPGA.

Keywords: 4-Aminobiphenyl; N-glucuronidation; HPLC; Liver microsomes

An occupational and/or environmental exposure to arylamines is known to cause human urinary bladder cancer [1]. Occupational exposures occur among production workers in rubber, coal, and textile industries, while cigarette smoke and fossil fuels represent the major environmental sources [2,3]. 4-Aminobiphenyl (4-ABP),[1] a representative arylamine, is a potent human bladder carcinogen [4] and its DNA adducts have been found in exposed individuals [5]. Bladder cancer is the fourth most common cancer type (by incidence) in men 60 years of age and older and ranks 11th among all cancer deaths in both sexes [6]. Arylamines must be activated by metabolic enzymes to exert their mutagenic and carcinogenic effects. Phase I and phase II metabolic enzymes operate on arylamines to yield activated and detoxified products. The quantitative and qualitative differences in the activities of these enzymes determine the potency of the specific arylamines and dictate the target organ. 4-ABP is metabolized in the liver by oxidation, N-acetylation, and/or N- or O-glucuronidation [7]. All are competing pathways. Glucuronidated 4-ABP metabolites are excreted via the bladder where they can be hydrolyzed to mutagenic compounds by the acidic environment of the bladder lumen [8]. N-glucuronidation is catalyzed by

* Corresponding author. Fax: +1 513558 0518.
E-mail address: alzougmh@email.uc.edu (M. Al-Zoughool).

[1] *Abbreviations used:* 4-ABP, 4-aminobiphenyl; UGT, UDP-glucuronosyltransferase; ESI electrospray ionization; HLM, human liver microsomes.

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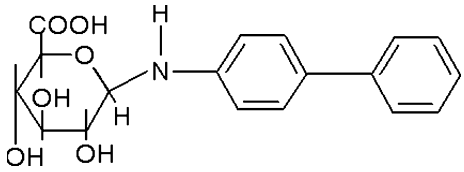

Fig. 1. Structure of the 4-aminobiphenyl-*N*-glucuronide.

UDP-glucuronosyltransferases (UGTs), which are a superfamily of microsomal enzymes that are located in the luminal side of the endoplasmic reticulum in such a way that their activity is controlled by membrane composition [9].

A variety of UGT assays have been developed to measure UGT enzyme activity toward a broad spectrum of endo- and xenobiotics [10–13]. Some of these methods used the *N*-glucuronide as a standard [14,11], which is expensive and available only for limited drugs and chemicals. Others employed the radioactive [C^{14}]UDPGA [10], which is also expensive and difficult to handle. Radiometric HPLC assays also have a high background noise due to the scintillant, which necessitates the use of high amounts of the radiolabel. Because arylamine *N*-glucuronide standards are unavailable commercially, the HPLC methods developed to measure *N*-glucuronides of 4-ABP had to use [C^{14}]UDPGA [15] to increase the sensitivity since N-glucuronidation reaction usually has very low activity [10]. We have developed a sensitive and simple method to directly determine the N-glucuronidation activity of mice, rat, and human liver microsomes toward 4-ABP using HPLC-UV. The newly developed method uses a chemically synthesized 4-ABP-*N*-glucuronide (4-ABP-G) (Fig. 1) as a standard to determine the amount of the glucuronides formed. The method was validated with respect to sensitivity, specificity, linearity, precision, accuracy, and limits of detection. The method was employed to characterize the kinetics of 4-ABP N-glucuronidation in mouse liver microsomes for which no data are currently available. The method was also used to determine the N-glucuronidation activity of rat and human liver microsomes.

Materials and methods

4-Aminobiphenyl, UDPGA, alamethicin, and D-glucuronic acid were all obtained form Sigma–Aldrich (St Louis, MO). Pooled human liver microsomes were obtained from Gentest (Woburn, MA). They were a homogenous mixture of liver specimens from a total of 54 male and female donors between 20 and 70 years of age. Recombinant human UGT1A4 and UGT1A9 supersomes expressed from human UGT1A4 and UGT1A9 cDNA in baculovirus-infected insect cells were purchased from Gentest. Male and female C57BL/6J strain mice were obtained from Jackson Labs (Bar Harbor, ME). Average age was 12 weeks and average weight was 40 g. Long Evans male and female rats were from Jackson Labs. They were 12–14 weeks of age (average weight 690 g).

Chemical synthesis of 4-ABP-G

The *N*-glucuronide was synthesized by modification of methods of Lilienblum and Bock [16] and Babu, S. et al [17]. Briefly, 10 mM solution of 4-ABP in methanol was mixed with 20 mM D-glucuronic acid in 0.1 M ammonium acetate buffer (pH 7.4). The solution was kept in dark at 4 °C with continuous stirring for 36 h. The resulting *N*-glucuronides were purified by collection of their peak following HPLC injection.

Identification of the 4-ABP-G

The infrared (IR) spectrometry was done using Nicolet NEGNA 760. Recording IR was done by 64 scans using KBr disk. Scanning was from 4000 to 400 cm^{-1}.

LC-MS-MS analysis

Sample was analyzed by electrospray ionization (ESI) using Micromass Q-TOF II mass spectrometer (Waters). The sample was dissolved in 50% water/acetonitrile, 0.1% formic acid as an ESI buffer and then directly infused into the mass spectrometer using a syringe at 4 μl/min. The capillary needle voltage was 3 kV and the source temperature was 150 °C. The nebulizer gas (nitrogen) flow was set at 250 L/h.

Determination of the quantity of the 4-ABP-G

The synthesized 4-ABP-G is completely hydrolyzed by incubation for 30 min in 0.1 M acetate buffer (pH 5.5). The released 4-ABP was determined using a standard curve for 4-ABP. Moles of 4-ABP-G were back-calculated from the moles of 4-ABP released from the hydrolyzed 4-ABP-G (assuming 1:1 molar ratio).

Preparation of microsomes

Microsomes were prepared by centrifuging pooled rat or mouse liver homogenates at 10,000 rpm for 20 min followed by ultracentrifugation at 40,000 rpm for 1 h. Microsomal pellets were resuspended in a solution of 0.05 KH_2PO_4 buffer (pH 7.4) containing 20% glycerol, stored in aliquots at −70 °C and used within 2 months. Protein concentration was determined using bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

UGT reactions

Pooled liver microsomes (from mice, rats, or humans) or human UGT1A4 and UGT1A9 supersomes