Measuring Swainsonine in Serum of Cancer Patients: Phase I Clinical Trial

Jose A. Baptista,1 Paul Goss,2 Mai Nghiem,3 Jiri J. Krepsinsky,1 Michael Baker,2 and James W. Dennis1,3,4

Swainsonine, an indolizidine alkaloid and competitive inhibitor of Golgi α-mannosidase II (EC 3.2.1.114), reduces tumor growth and stimulates immune function in mice. On the basis of these observations, a phase I clinical trial was initiated to determine whether swainsonine could be administered safely to cancer patients. We describe a method for extraction, acetylation, and quantification of swainsonine in human serum samples. Methyl α-D-mannopyranoside and methyl β-D-galactopyranoside were added to serum samples as internal standards and, after sequential extraction of lipids and proteins with chloroform and acetonitrile, respectively, samples were acetylated with acetic anhydride and 4-dimethylaminopyridine and separated by gas–liquid chromatography. The identity of swainsonine and the internal standards after their extraction from serum and acetylation was confirmed by gas chromatography/mass spectrometry. Swainsonine was recovered at an efficiency of 90%, relative to internal standards, and calibration graphs were rectilinear from 3 to 18 mg/L with a detection limit of ≤0.1 mg/L. The CV for multiple samples was ≤6.7%. In patients receiving swainsonine (50–550 μg/kg per day) continuously for 5 days by intravenous infusion, serum concentrations of the drug reached 3–11.8 mg/L, 100 to 400 times greater than the 50% inhibitory concentration for Golgi α-mannosidase II and lysosomal α-mannosidases. Accurate measurements of swainsonine in biological fluids with this method should facilitate further clinical studies with the drug.

Indexing Terms: enzyme inhibitor/digosaccharides/gas chromatography–mass spectrometry/drug monitoring

The indolizidine alkaloid swainsonine was first extracted from Australian Swainsona canescens (1), subsequently from North American plants of the genera Astragalus and Oxycotis (2), and also from the fungus Rhizoctonia leguminocola (3). Swainsonine is a potent inhibitor of Golgi α-mannosidase II (EC 3.2.1.114), an enzyme required in the catabolism or processing of N-linked oligosaccharides on glycoproteins. As such, swainsonine blocks expression of the β(1→6)-branched complex-type N-linked oligosaccharides, which have been shown to increase after malignant transformation in human and rodent cells (4, 5). In several experimen-
tal tumor models, the β(1→6)-branched oligosaccharides are apparently required for invasion in vitro and for efficient tumor cell metastasis in vivo (6, 7). Consistent with these observations, murine tumor cells cultured in the presence of swainsonine showed reduced potential for organ colonization, and solid tumor growth was also inhibited in mice treated with the drug (8, 9). In particular, the growth rate of human tumors, including MeWo melanoma and HT29m colon carcinoma tumor cell lines growing as xenografts in athymic nude mice, was reduced by treating the mice with swainsonine (10, 11). Swainsonine also appears to stimulate and activate the immune system (reviewed in 12). In particular, the alkaloid alleviates both chemically induced and tumor-associated immune suppression in mouse (13), increases natural killer cell activity in mice (14) and lymphokine-activated killer cell activities of human peripheral lymphocytes (15), and increases lymphocyte and bone marrow cell proliferation (16).

A phase I clinical trial was undertaken to determine whether swainsonine in the dosage range of 50 to 550 μg/kg per day could be administered safely to humans by intravenous infusion over 5 days. The subjects were terminally ill cancer patients with either leukemia or breast, colon, lung, pancreatic, or head and neck cancers. The clinical results of the phase I study will be presented elsewhere (17). Here we document a method for the extraction, acetylation, and quantification of swainsonine in human serum samples.

Materials and Methods

Materials

Solvents were HPLC grade: chloroform from Fisher Scientific, Toronto, Canada, and acetonitrile (low water content) from J. T. Baker, Toronto, Canada. Dry acetonitrile was obtained from Aldrich, Milwaukee, WI, and acetic anhydride and sodium acetate (Analar) were from BDH, Toronto, Canada. 4-Dimethylaminopyridine (4-DMAP) and the internal standards methyl α-D-mannopyranoside (me-Man) and methyl β-D-galactopyranoside (me-Gal) were obtained from Sigma Chemical Co., St. Louis, MO.5 The basic alumina solid-phase cartridges were purchased from Alltech Associates, Deerfield, IL. Chemically synthesized and pyrogen-free swainsonine was purchased from Toronto Research Chemicals, Toronto, Canada, and purified by recrystallization to constant melting point; purity was confirmed by proton nuclear magnetic resonance proton spectroscopic analy-

426 CLINICAL CHEMISTRY, Vol. 40, No. 3, 1994

5 Nonstandard abbreviations: me-Man, methyl α-D-mannopyranoside; me-Gal, methyl β-D-galactopyranoside; 4-DMAP, 4-dimethylaminopyridine; GC, gas–liquid chromatography; and MS, mass spectrometry.
Acetylation of Swainsonine from Serum

We prepared the internal standards me-Man and me-Gal in H2O at 200 mg/L, and added 45 µL (9 µg) of each standard to 1.5 mL of serum in Kimax tubes (13 mm × 16 cm). We added 2 mL of cold chloroform, gently mixed the samples to avoid foaming, and centrifuged them at 1000g for 10 min at 4°C. The supernates were quantitatively transferred into clean Kimax tubes with a Pasteur pipette, followed by addition of 2 mL of cold acetonitrile and vigorous vortex-mixing. Each mixture was centrifuged at 1000g, the supernate saved, and the protein pellet washed twice with 2 mL of cold acetonitrile. The combined supernates were evaporated under a stream of nitrogen at 60°C. The residue was further dried in a desiccator over P2O5 at room temperature for 12 h.

Acetylation of Swainsonine

To the dry samples we added 1 mL of dry acetonitrile, 15 mg of 4-DMAP, and 15 mg of sodium acetate. After the samples were cooled to 0°C, 0.5 mL of acetic anhydride was added, and the samples were left overnight at room temperature. The acetylated reaction products were then partitioned between 1 mL of chloroform and 1 mL of distilled water with gentle shaking. Polar contaminants were dissolved in the aqueous layer, whereas the acetylated saccharides and swainsonine dissolved in the lower chloroform layer. The chloroform layer was washed with 1 mL of water, and traces of water and acetic anhydride were removed by passing the samples over 500-mg cartridges of basic alumina. The solvent was evaporated at 60°C under a stream of nitrogen, the residue dissolved in 30 µL of chloroform, and 1-µL aliquots were injected into the gas–liquid chromatograph.

Gas–Liquid Chromatography (GC)

For GC analysis of each derivatized sample we used a Varian (San Fernando, CA) Model 3400 gas chromatograph, equipped with flame ionization detector and capillary column [15 m × 0.53 mm (i.d.), Megabore] coated with a 1.2-µm thin layer of DB225. The column temperature was kept at 180°C for 22 min after the injection, then raised to 230°C at 1°C/min; the latter temperature was maintained for 30 min. The injector port temperature was 240°C, and the detector block 280°C. Samples of 1 µL were injected by direct flash vaporization, and prepurified dry helium (Canox) was used as the carrier gas at a flow rate of 6 mL/min. Quantitative analyses were done with detector range of 10–10, using a Hewlett-Packard (Palo Alto, CA) integrator, Model 3396B, with attenuation set at 1.

For the GC/mass spectrometry (MS) experiments we used a Varian Saturn II GC/MS PC system, utilizing the SPI programmed temperature injector with a packed sleeve for biological samples. Samples were run in chemical ionization mode, with isobutane as reagent gas.

Results and Discussion

Extraction and Acetylation of Swainsonine

Swainsonine was first purified by Colegate et al. (1) from dry leaves of Swainsona canescens mixed with ethyl acetate, followed by drying and extracting the residue with water. The water-soluble material was further purified by column chromatography on Dowex 50W×8 and CM-Sepharose CL–6B. Modifications of the procedure have been described and used to quantify swainsonine in other plants and fungus (18, 19). To optimize a procedure for quantifying swainsonine in human serum, we tested several methods of extraction and derivatization and adopted the following procedure. The major obstacles in quantifying swainsonine were the considerable quantities of protein and lipids in serum. Therefore, we first extracted serum samples with chloroform to remove lipids and other hydrophobic material. Then we added acetonitrile to the aqueous phase containing swainsonine, to a concentration of 660 mL/L, and pelleted the precipitated protein by centrifugation. The protein pellet was washed twice with acetonitrile and the washes were pooled. This procedure appears to release swainsonine adhering to serum macromolecules, because the recovery of swainsonine added to serum samples was 90% (see below). Acetonitrile extraction of serum was more efficient than methanol extraction, producing fewer contaminating peaks on the GC chromatograms.

GC analysis for swainsonine can be performed after any of several methods of derivatization (18, 19). Molyneux et al. (18) produced trimethylsilyl ethers of swainsonine, but these derivatives hydrolyze easily if the anhydrous conditions are not maintained scrupulously. To overcome this difficulty, we acetylated swainsonine with 4-DMAP and acetic anhydride, an efficient reaction yielding a product of relatively high volatility and stability.

To quantify swainsonine in serum by GC, one must add one or more internal standards to the samples at the beginning of the analytical procedure. This procedure allows for subsequent losses, assuming the losses are the same for the unknown and standards. The latter assumption is valid if the standards are chemically similar to the class of compounds being analyzed and if they show no reactivity with the sample. After testing various saccharides, we decided to use me-Man and me-Gal as internal standards because, acetylated, they emerge from the GC column well-separated from acetylated swainsonine and serum contaminants (Fig. 1). Elution times for acetylated swainsonine, me-Man, and me-Gal were 8.8, 14.7, and 19.1 min, respectively.

We ran GC/MS experiments with chemical ionization to analyze the structures of acetylated swainsonine and standards. The mass fragmentation patterns for the standards me-Gal and me-Man were confirmed by comparing the data with the National Institute of Standards and Technology Mass Spectral Library (Varian) (Fig. 2). Swainsonine, both the pure material and that extracted from serum, produced a very strong molecular ion with good resolution (Fig. 2). Chemical ionization MS pro-
duced a better signal-to-noise ratio than did electron ionization MS.

Calibration curves were prepared by adding swainsonine and the internal standards to serum from untreated normal subjects. Swainsonine was added to serum at 3.0, 6.0, 9.0, 13.5, and 18.0 mg/L; me-Man and me-Gal were added to all samples at 9 mg/L each. Serum samples were then extracted, acetylated, and separated by GC as described above. Peak areas (A) for swainsonine and internal standards (IS) expressed as a ratio (i.e., A_s/A_IS) were plotted as a function of the concentrations of the added swainsonine and internal standard expressed as a ratio (i.e., x/I.IS). Measurements of swainsonine in the serum of our phase I subjects ranged from 0.2 to 11.5 mg/L, and the sensitivity of the method as described was ~0.1 mg/L (i.e., 10-fold the signal-to-noise ratio). If necessary, sensitivity can be increased by at least 10-fold by using a narrow-bore GC column and injecting more of the sample.

To assess recovery of swainsonine relative to the internal standards for multiple runs, we added 9 µg of each compound to four independent serum samples and processed for GC. Relative to me-Man and me-Gal, recovery of swainsonine was 90% and 89%, respectively, and the CV was 2.7%. Concentrations of swainsonine measured in 1.5-mL samples of patients' serum fell within the range of the calibration curve and could therefore be read directly from the graph shown in Fig. 3.

Quantification of Swainsonine

Nineteen patients were infused continuously by saline drip containing swainsonine for 5 days, under a protocol approved by the University of Toronto and the Health Protection Branch, Ottawa, Canada. Blood samples were collected into tubes containing heparin at 0, 1, 8, 24, 48, 72, 96, and 120 h after beginning treatment.
Swainsonine is both water- and lipid-soluble and would therefore be expected to diffuse efficiently into tissues. In tissue culture, swainsonine has been shown to attain concentrations inside the cells similar to that in the culture medium within minutes (20).

The relation between dosage and serum drug concentrations was non-linear, suggesting multiple mechanisms of drug clearance. Patients received the drug in a daily volume of 1 L of isotonic saline; therefore, at 120 h, the serum concentration was approximately equal to that of infusate for the lowest dosage (i.e., 50 μg/kg per day) and was only 30% of that obtained for the infusate concentration at 450 μg/kg per day. The plot for dosage vs (steady-state serum drug concentration × clearance) was second-order, suggesting a slow clearance mechanism at low dosages (i.e., 0.5 mL/h per kg) and a more rapid clearance rate in patients given higher doses, 150 to 550 μg/kg per day (i.e., 3.8 mL/h per kg) (17). The mechanisms underlying the dose-dependent clearance rates for swainsonine may be either a drug-induced clearance mechanism that enhances the apparent rate at higher dosages, or saturation of a swainsonine-binding molecule in serum, which would decrease the rate at low dosages. The former may involve conjugation in the liver or kidneys and requires further analysis of swainsonine metabolites to address this question. The method we have described for extracting and quantifying swainsonine in human serum should facilitate further clinical studies with the drug.

This work was supported by research grants from the National Cancer Institute (NCI) of Canada and the Medical Research Council (MRC) of Canada to M.B. and J.W.D., and an MRC maintenance grant to the Carbohydrate Research Centre (MT 6499), University of Toronto. J.W.D. is a Terry Fox Research Scientist of the NCI of Canada.

References


