JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Berberine and Its Metabolites: Relationship between Physicochemical Properties and Plasma Levels after Administration to Human Subjects

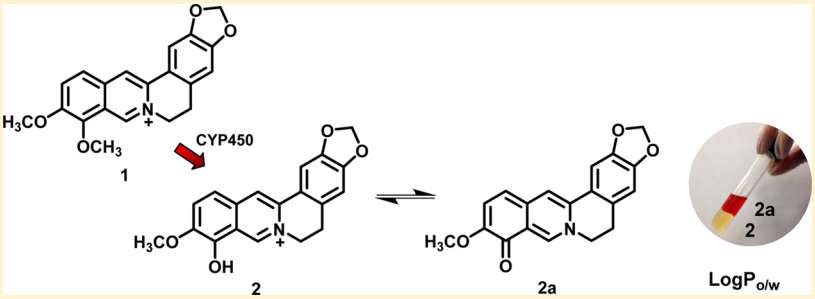
Silvia Spinozzi,[†] Carolina Colliva,[†] Cecilia Camborata,[†] Marinella Roberti,[‡] Cristina Ianni,[‡] Flavia Neri,[§] Claudio Calvarese,[§] Andrea Lisotti,[§] Giuseppe Mazzella,[§] and Aldo Roda*[,†]

[†]Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126 Bologna, Italy

[‡]Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

[§]Department of Medical and Surgical Science, University of Bologna, Via Massarenti 9, 40138 Bologna, Italy

Supporting Information



ABSTRACT: Berberine (**1**) is an alkaloid used widely in the treatment of several diseases. However, its physicochemical properties, pharmacokinetics, and metabolism remain unclear, and conflicting data have been reported. In this study, the main physicochemical properties of **1** and its metabolites were evaluated, including lipophilicity, solubility, pK_a, and albumin binding. A sensitive HPLC-ESIMS/MS method was developed and validated to identify **1** and its main metabolites in human plasma. This method was used to quantify their levels in the plasma of healthy volunteers and hypercholesterolemic patients following a single dose and chronic administration, respectively. In both cases, berberrubine (**2**) was found to be the main metabolite. Surprisingly, **2** is more lipophilic than **1**, which suggests that this compound tautomerizes to a highly conjugated, electroneutral quinoid structure. This was confirmed by NMR studies. These results indicate that the higher plasma concentration of **2** was a consequence of a more efficient intestinal absorption, suggesting that berberrubine is potentially more pharmacologically active than berberine.

In recent years, there has been renewed interest in the potential of purified natural products to provide health and medical benefits and to prevent disease. In some cases, these substances do not require conventional drug approval based on preclinical pharmacokinetic, metabolism, and toxicological studies.[1] Consequently, their biodistribution in the human body, their dose-related metabolism, and target organ activity have been poorly studied. This may hamper lead discovery based on these molecules. Furthermore, understanding the physicochemical properties of these natural compounds is important for the purposes of formulation.

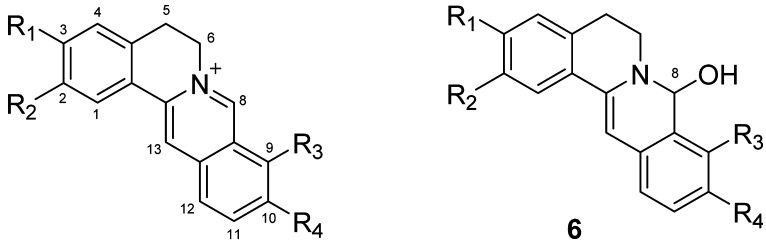
Protoberberine (5,6-dihydrodibenzo[*a*,*g*]quinolizinium) alkaloids found in the bark, rhizomes, roots, and stems of *Berberis vulgaris* L. (Berberidaceae)[2] have been shown to exhibit many different types of biological activities. Among the most well studied of these compounds is berberine (**1**), which has antifungal,[3] anti-inflammatory,[4] antimalarial,[5] anti-HIV,[6] anti-hyperglycemic,[7] immunoregulatory,[8] antitumor,[9] and cholesterol-lowering[10] effects.

Currrently, berberine chloride (**1**) is administered chronically to hypercholesterolemic patients at a relatively high dose of about 1−2 g/day. Toxicity is observed when **1** is administered intravenously to mice at 9 mg/kg body weight.[11] Therefore, the potential side effects of berberine should be carefully evaluated. Despite the widespread use, the physicochemical properties responsible for the biodistribution of this compound are largely unknown or have been measured with inaccurate methodologies. These inaccurate methodologies have produced conflicting results, leading to misinterpretation of the biological profile of berberine chloride (**1**). Its metabolism and the properties and role of the main metabolites so far identified remain unclear.

Previous studies carried out in rats after acute administration of **1** at 200 mg/kg showed that it is metabolized in the liver by CYP450 isoenzymes through oxidative demethylation at

Received: July 26, 2013
Published: March 5, 2014

Chart 1. Structures of Protoberberine and Their 8-Hydroxy Adducts



1: berberine R_1+R_2=OCH_2O, R_3=R_4=OCH_3
2: berberrubine R_1+R_2=OCH_2O, R_3=OH, R_4=OCH_3
3: thalifendine R_1+R_2=OCH_2O, R_3=OCH_3, R_4=OH
4: demethyleneberberine R_1=R_2=OH, R_3=R_4=OCH_3
5: jatrorrhizine R_1=OH, R_2=R_3=R_4=OCH_3

positions 2, 3, 9, and 10 followed by conjugation of these hydroxy group functions with glucuronic acid.[12] In humans, chronic administration of **1** at a dose of 1 g/day has produced the following main primary metabolites: berberrubine (**2**), thalifendine (**3**), demethyleneberberine (**4**), and jatrorrhizine (**5**). Some of these, such as **2**, exhibit pharmacological properties such as potential anticancer activity,[9] up-regulation of low-density lipoprotein receptor, and mRNA expression.[12]

There are conflicting data on the acid/base character of **1**, with two different pK_a values reported, namely, 2.47[13] and 15.7.[14] These values were not experimentally measured in water, but rather in methanol, where the data lacked thermodynamic values. Moreover, the analytical methods used were not suitable for characterizing the purity of **1**. Other reports suggest that protoberberine and its 8-hydroxy adducts (**6**), known as pseudobases (Chart 1), are present together in plants.[15−17] Recently, however, it was discovered that the latter species may arise from the basic conditions used during isolation and purification procedures.[15−17]

In this context, the present study aimed to develop and validate a new HPLC-ESIMS/MS method for the qualitative and quantitative determination of **1** and its metabolites in human plasma, after acute and chronic oral administration to hypercholesterolemic patients. The effect on plasma, cholesterol, and triglycerides was determined before and after treatment. In addition, the main physicochemical properties, including lipophilicity, solubility, pK_a, and albumin binding, were accurately evaluated for **1** and its metabolites. This was achieved by studying proton exchange phenomena and tautomeric equilibria, common in the chemistry of natural compounds, at physiological pH. To conclude the study, the relationship between the plasma levels and physicochemical properties of **1** and its metabolites is discussed. A better understanding is sought for their pharmacokinetic, intestinal absorption, and biodistribution in the target organs, which will be responsible for their therapeutic activity.

RESULTS AND DISCUSSION

Physicochemical Properties of 1 and Its Metabolites.

pK_a and Dissociation Equilibrium. The acid/base equilibrium plays an important role in determining the prevalence of neutral or ionized forms at a given pH, particularly a physiological pH. It affects binding with receptors and the membrane partition process. The structure of protoberberine is characterized by the presence of an iminium cation. This makes determination of the pK_a particularly important because at a pH value higher than the pK_a these compounds could take on the characteristics of neutral molecules. Specifically, the simultaneous presence of the iminium cation and the hydroxy groups in the structure of metabolites involves the presence of multiple species in solution, including protonated and deprotonated forms for each metabolite. Thus, the pK_a values of **1** and its metabolites were determined in silico and compared to those experimentally obtained and reported in the literature (Table 1).[13,14] In silico, it is only possible to predict pK_a values of ionizable

Table 1. Physicochemical Properties of 1 and Metabolites 2, 4, 5, and 2a, Solubility, $LogP_{o/w}$, and $LogD_{o/w}$ in a Gradient of pH (from 4.5 to 8.5), and Binding Association Constant (K_B)

compound	pH[a] gradient	solubility (mM)	$logP_{o/w}$[a]	$logD_{o/w}$	K_B[b] ($\times 10^3$ L mol^{-1}) ± SD
1[c]	8.6	9.6	−1.2	−1.2	
	7.0	9.0	−1.2	−1.2	3.37 ± 0.09
	6.0	7.6	−1.2	−1.2	
	4.5	8.2	−1.2	−1.2	
	in silico		−0.4		
2[c]	8.6	0.5	1.6	4.6	
	7.0	1	1.1	2.5	1.51 ± 0.03
	6.0	2	0.9	1.4	
	4.5	4.2	−0.02	0.01	
	in silico		1.0		
4[c]	8.6	6.5	−0.5	−0.3	
	7.0	3.7	−1.1	−1.1	1.98 ± 0.09
	6.0	0.2	−1.1	−1.1	
	4.5	0.2	−1.1	−1.1	
	in silico		0.1		
5[c]	8.6	12.2	0.1	0.6	
	7.0	12.4	−1.2	−1.2	1.73 ± 0.01
	6.0	1.3	−1.5	−1.5	
	4.5	1.5	−1.5	−1.5	
	in silico		−1.1		
2a	8.6		1.6	4.6	
	7.0		1.5	2.9	n.d.
	6.0		1.3	1.8	
	4.5		0.6	0.7	
	in silico				

[a]Medium: phosphate buffer 0.1 M. [b]Determined at 298 K in 0.1 M Tris-HCl pH = 7.2 (n.d. = not determined). [c]Chloride salt.

dx.doi.org/10.1021/np400607k | J. Nat. Prod. 2014, 77, 766−772

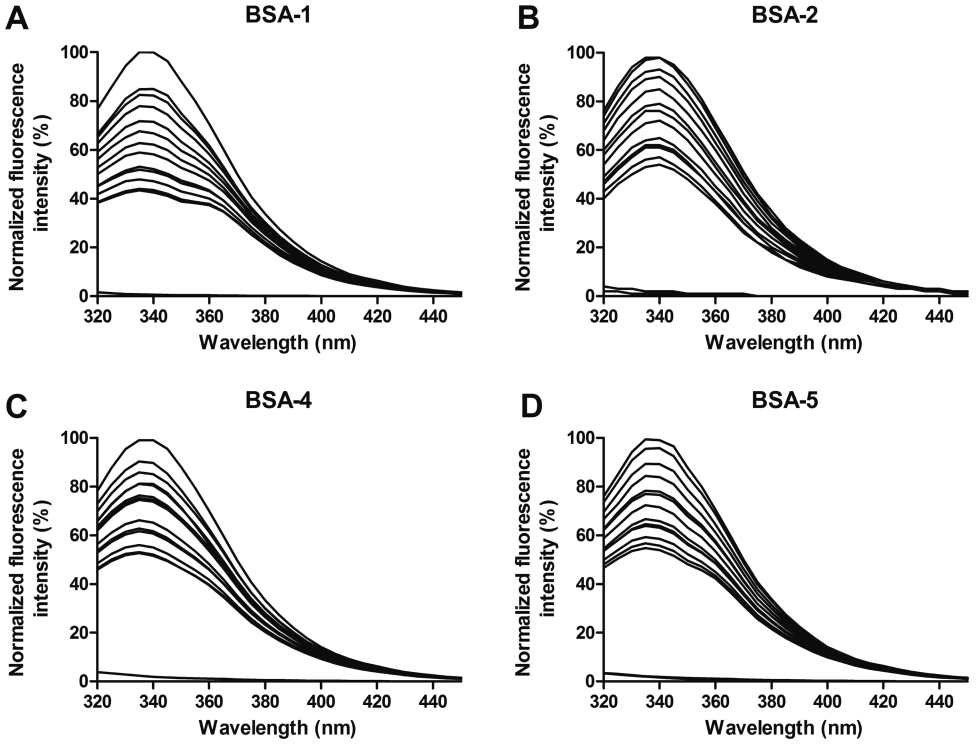
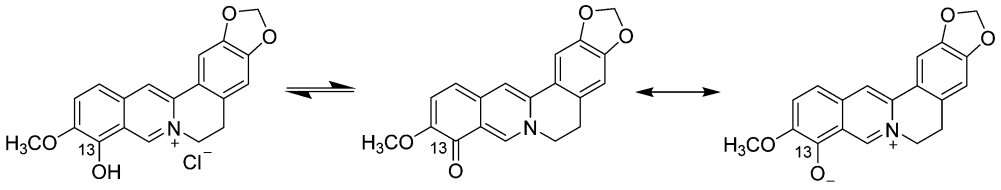


Figure 1. Emission spectra of bovine serum albumin (BSA) (1.0×10^{-5} M) at different concentrations of (A) **1**, (B) **2**, (C) **4**, and (D) **5** (λ_{ext} = 280 nm) from 0.0 to 2.4×10^{-5} M at increments of 0.2×10^{-5} M.

Scheme 1. Three Possible Forms Describing the Metabolite 2



molecules and to generate probable ionized states (and tautomers) as a function of the pH. Consequently, it was not possible to calculate the pK_a of **1** due to the lack of proton donor or acceptor functional groups. For this reason, it was expected that at physiological pH range, the nitrogen of **1** would remain as an iminium cation. Of the two conflicting pK_a data of **1** (15 and 2.47) reported in the literature,[13,14] the more thermodynamic pK_a value should thus be approximately 15 because a 2.47 value describes strong acid behavior. Unlike **1**, its metabolites bear ionizable groups. In particular, **2** and **5** possess one hydroxy group moiety, at position 9 and 3, respectively, while **4** has a hydroxy group function in positions 2 and 3 (Figure 1). The pK_a values of **4** and **5** are quite similar, ≅9.4 and 9.6, respectively. As a consequence, **4** and **5**, similar to **1**, are present at physiological pH as iminium cations and are only partially dissociated. Regarding **2**, an experimental pK_a value of ≅5−6 has been reported in the literature.[18−20] In silico, despite the presence of an ionizable group, the software returned no pK_a value. This is because the quinoid form (**2a**) was identified as the most stable tautomer in water. On the basis of the above results, it has been hypothesized that **2** is able to tautomerize to the highly conjugated, electroneutral, quinoid structure, **2a**[19] (Scheme 1). Indeed, in most of the reported papers, **2** is represented as an enol structure (Scheme 1)[19−21] with a hydroxy group at position C-13. It has, however, also been suggested that either the quinoid structure **2a**[20] or the zwitterionic form[19,20] **2b** may result from the tautomerization process (Scheme 1).[19]

To demonstrate the presence of a keto−enolic equilibrium, the enol **2** and quinoid **2a** forms were synthesized and characterized (see Experimental Section and Supporting Information). ^{1}H NMR spectroscopic analysis was performed on a sample comprising an equimolar mixture of both forms. The spectrum displayed only one average set of signals, demonstrating the existence of fast equilibration between these two tautomeric forms. To further validate this equilibrium, a ^{1}H−^{15}N HMQC correlation analysis was carried out for both structures (see the Supporting Information). Regarding the equilibrium involving quinoid **2a** and zwitterionic **2b** forms, a quinoid-zwitterion resonance may be suggested. This is in agreement with Suau et al.,[20] who proposed an analogous betaine-quinoid resonance for the protoberberine alkaloid 7,8-dehydrocaseamine. It may be envisaged that the aprotic environment would stabilize form **2**, while the presence of water would promote the zwitterion form **2b**. This hypothesis was proved through NMR titration (1D proton and carbon) (see Table S1, Supporting Information). To the best of our

dx.doi.org/10.1021/np400607k | J. Nat. Prod. 2014, 77, 766−772

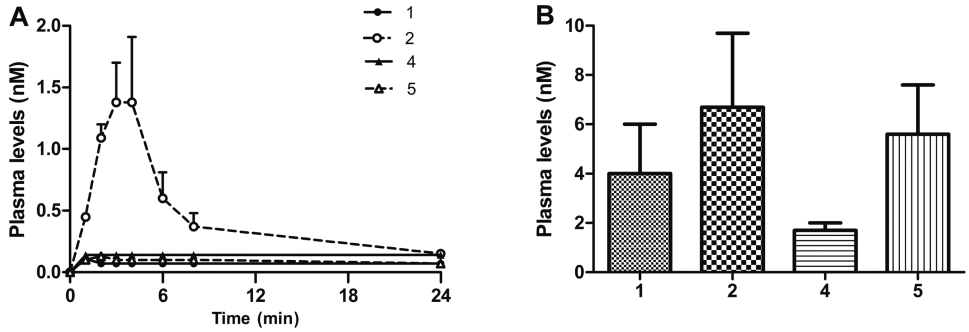


Figure 2. (A) Mean pharmacokinetic profiles of the plasma concentrations of **1**, **2**, **4**, and **5** in healthy volunteers after a single oral administration of chloride **1** of 500 mg. (B) Mean plasma levels of **1**, **2**, **4**, and **5** in 12 hypercholesterolemic subjects after chronic administration of the chloride of **1** at 15 mg/kg body weight day for three months.

knowledge, this is the first time that the tautomerism of **2** has been demonstrated.

Solubility. There have been previous studies on the solubility of **1** in different solvents.[22,23] However, no data on the aqueous solubility of its metabolites have been reported to date. Since **1** has no ionizable groups, it may be expected that its solubility would not be influenced by pH variation. Indeed, this was confirmed by only a slight variation in solubility when the pH was changed, i.e., 9.6 mM at pH 8.6 to 7.6 mM at pH 7.0. In contrast, the solubility of metabolites **4** and **5** increased significantly when pH was increased, with the solubility of **2** decreasing (Table 1). Different pH values were associated with macroscopic changes in the color of **2** in solution, which turned from an intense yellow at acidic pH, due to the prevalence of enolic form **2**, to a red at a basic pH, due to the prevalence of quinoid form **2a**. This bathochromic effect, characteristic of **2**, has been reported in previous reports,[19,20] in which the spectrophotometric and fluorescent properties of protoberberine were studied in detail.[19]

Lipophilicity. The main metabolites of **1** are ionizable molecules. Therefore, the experimental evaluation of 1-octanol/water partition coefficient $LogP_{o/w}$ should be calculated for a single species, unless the distribution coefficient $LogD_{o/w}$ is measured by considering the sum of all the species present in solution at a given pH. The $LogP_{o/w}$ and $LogD_{o/w}$ values of the chloride of **1** and its primary metabolites were thus determined in phosphate buffer 0.1 M at a pH ranging from 4.5 to 8.5 at 25 °C. The results are reported in Table 1. These data show that **1** is not greatly influenced by pH variation, with both $LogP_{o/w}$ and $LogD_{o/w}$ showing negative values. In contrast, the lipophilicity of the metabolites of **1** is much more affected by an acid or basic environment as a result of different ionizations at different pHs. In particular, **2** showed the characteristics of a lipophilic substance ($LogP_{o/w}$ >0), which is in disagreement with its nominal enolic structure. Comparing the coefficient distribution of **2** and its quinoid form **2a**, the lipophilicity increased with increasing pH. In fact, when the single species **2** and **2a** were dissolved in aqueous solution, there was keto−enolic tautomerism, by means of the internal salt (see Scheme 1). The lipophilicity variation of **2**, as a function of pH, may be attributed to the predominance of the enol form (total positive charge) at acid pH and of the quinoid form **2a** at basic pH (total neutral charge), which is much more liposoluble. A different phase color was also observed during the partition of **2** in 1-octanol and aqueous medium: yellow in the aqueous phase and red in 1-octanol. Compound **4** displayed a different lipophilicity at extremes of pH (4.5 and 8.5) with $LogP_{o/w}$ values still below 0, while **5** was more lipophilic at a pH of 8.6, reaching a $LogP_{o/w}$ positive value. These data suggest that the pH of the buffer plays a role in the ionization of these molecules. The biodistribution in human organs of **1** and its metabolites may thus be driven by the extent of their passive intestinal absorption in different districts of the intestinal tract, which are characterized by different pH values.

Albumin Binding. The binding association constant (K_B) with bovine serum albumin (BSA) of **1** and its metabolites was determined by fluorescence quenching of tryptophan, which is present in both binding sites in BSA.[24,25] Figure 1 shows the tryptophan fluorescence quenching effects induced by these compounds. The maximum fluorescence intensity peak, at the value at λ_{em} = 340 nm of BSA, was decreased regularly by increasing the quencher concentration.

The quenching profiles, characterized by the presence of a double peak, were very similar for **1**, **4**, and **5**, whereas a single peak profile was recorded for **2**. This suggests that **1**, **4**, and **5** bind to both binding sites of BSA, while **2** binds to only one. Again, **2** behaved differently from **1** and the other metabolites, although the values of the constant of binding with BSA were quite similar (Table 1).

HPLC-ESIMS/MS Method for in Vivo Study: Pharmacokinetics and Metabolism. A sensitive HPLC-ESIMS/MS method was developed and validated to identify and quantify **1** and its main metabolites in plasma. The synthesized metabolites were used as standard references. The analytical performance of the method is reported in the Experimental Section. This method was used to determine the plasma levels of **1** and its metabolites after a single administration of 500 mg of **1** in 10 healthy subjects and after chronic administration of 15 mg/kg body weight/day in 12 hypercholesterolemic subjects for three months. In addition, the hypolipidemic effect was evaluated by measuring serum biomarkers including total serum cholesterol, triglycerides, LDL, HDL, and bile acids before and after treatment (Table S2, Supporting Information). Figure 2A shows the mean plasma concentrations of **1** and its primary metabolites at different times after oral administration, as obtained in the pharmacokinetic study. The maximum plasma levels were very low for **1**, **4**, and **5**, i.e., 0.07 ± 0.01, 0.14 ± 0.01, and 0.13 ± 0.02 nM, respectively. Their pharmacokinetic profiles were very similar. With **1** and **4**, a plateau was reached one hour after administration. This increased to 2 h for **5**. These plateaus persisted for up to 24 h after ingestion. In contrast, the maximum plasma concentration of **2** was almost 10 times higher, i.e., 1.4 ± 0.3 nM, 4 h after the administration of **1**, with a slow decrease until the residual concentration of

dx.doi.org/10.1021/np400607k | J. Nat. Prod. 2014, 77, 766−772

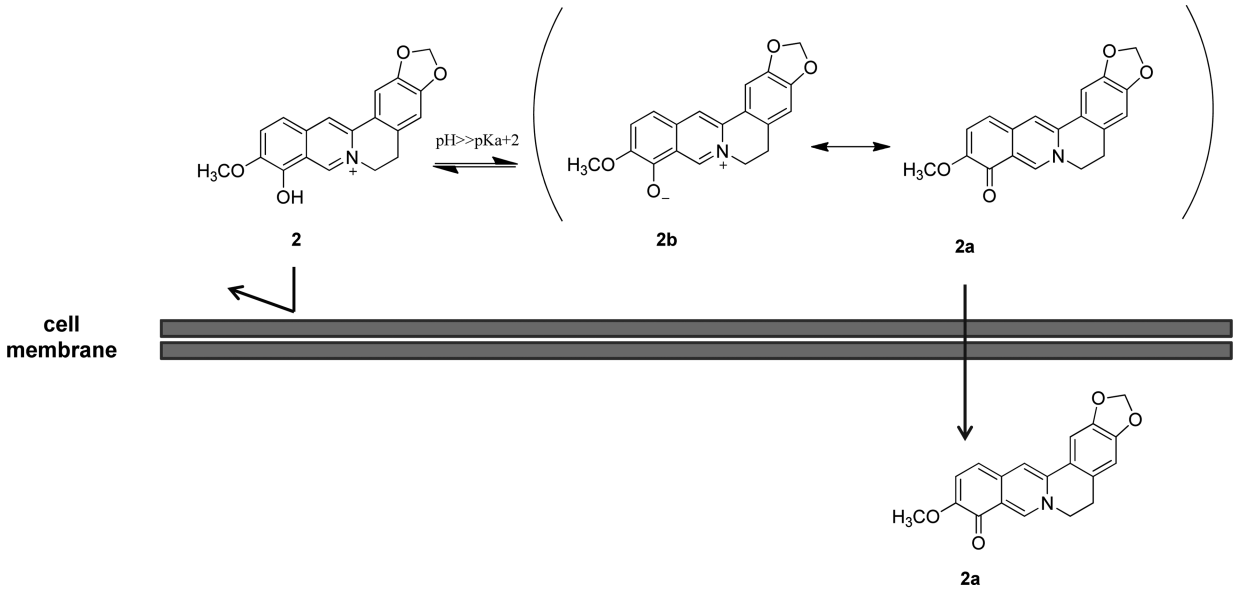


Figure 3. pH-mediated passive diffusion of **2** through the cell membrane.

0.15 ± 0.02 nM after 24 h. The plasma levels of **1** and its metabolites after chronic administration were considerably higher than those after acute administration. This is because the daily dose of 15 mg/kg body weight after chronic administration is almost twice the single dose of 500 mg used in acute administration. A possible bioaccumulation during chronic administration could also be responsible for these values. The maximum steady-state concentrations were 4.0 ± 2.0, 6.7 ± 3.0, 1.7 ± 0.3, and 5.6 ± 2.0 nM for **1**, **2**, **4**, and **5**, respectively (Figure 2B). A high intersubject variability in plasma levels was observed compared to the acute single-dose pharmacokinetic study. Once again, **2** was the main compound present in the plasma.

Relationship between Plasma Levels and Physicochemical Properties. The physicochemical properties of **1** and its metabolites, in particular pK_a and lipophilicity, play a major role in determining plasma levels after oral administration. These different plasma levels are the result of different intestinal absorptions. The pK_a values obtained in this study showed that **1**, **4**, and **5** are permanently charged at the physiological pH range. In contrast, **2** is in keto−enol tautomeric equilibrium with its quinoid form **2a** (Scheme 1). In vivo, the dynamic equilibrium between **2** and **2a** is continuously modulated, with the former being prevalent at acidic pH values and the latter being prevalent at basic pH values. In solution, these two species thus interconvert rapidly into each other. The membrane in the systemic compartment could promote their separation. This is because **2** in its quinoid form, as a neutral molecule, could cross membranes better than the enol form (Figure 3). As a consequence, **2a** probably undergoes efficient intestinal absorption by passive diffusion, which results in the higher plasma concentration of **2** after oral administration, compared to **1** and the other metabolites. The positive correlation between the lipophilicity and the plasma levels of **2** suggests that **1** has an unusual metabolism. It produces the metabolite **2**, which, in its quinoid form, becomes even more lipophilic than the administered drug, as shown by their Log$P_{o/w}$ values (Table 1). Compound **2** thus accumulates in the systemic compartment more than **1**. For this reason, it could be more pharmacologically active than **1**, although this hypothesis should be demonstrated by the direct administration of **2**.

In terms of therapeutic activity during chronic administration, there were significant reductions in total and LDL cholesterol ($p < 0.05$ obtained by the paired two-tailed Student's t test) (Table S2, Supporting Information). This is in agreement with previously published data.[29,30] The total triglyceride and cholesterol-HDL levels were not significantly different ($p > 0.05$).

In conclusion, the overall data suggest the importance of accurate preclinical studies of natural products that are administered at relatively high daily doses to a new population of subjects. Indeed, the biodistribution, metabolism, and accumulation in target organs, such as the excretory pathway, could be very different for a natural compound administered pure at higher doses than when being given in a plant extract. Unexpected metabolites could be formed in patients with liver disease or other related pathologies. These could accumulate in a particular biological fluid or organ, resulting in potential toxicity or other undesired effects.

It is proposed that the protocol used herein to assess **1** be adopted for studying any new isolated natural products intended for chronic administration to patients. In this way, toxicity or side effects could be predicted by preclinical studies of the new chemical entity. Before administration in humans, researchers should use accurate analytical approaches, such as modern spectrometry-based techniques combined with HPLC, to assess the purity of the pharmaceutical compound and to perform any stability tests. In particular, these HPLC-ESIMS/MS techniques should be used to study metabolism, with the main metabolites accurately identified in the different bodily fluids after oral administration.

EXPERIMENTAL SECTION

General Experimental Procedures. Berberine chloride (**1**), as a standard for HPLC (purity ≥97%), (*R*,*S*)-noscapine, and Tris-HCl salt were purchased from Sigma (St. Louis, MO, USA). Jatrorrhizine chloride (**5**) was purchased from AlloraChem srl (Rimini, Italy). All other reagents were of HPLC-grade: methanol and acetonitrile were purchased from Carlo Erba Reagents (Milan, Italy) and LiChrosolv. HPLC-grade water was prepared using the Millipore Milli-Q Synthesis

dx.doi.org/10.1021/np400607k | J. Nat. Prod. 2014, 77, 766−772

A10 system (Molsheim, France). Reaction progress was monitored by TLC on precoated silica gel plates (Kieselgel 60 F254, Merck) and visualized by UV254 light. Flash column chromatography was performed on silica gel (particle size 40−63 μm, Merck). Unless otherwise stated, all reagents were obtained from Sigma-Aldrich and used without further purification.

Synthesis of Compounds 2, 2a, and 4. Compound **2** was prepared starting from **1**, by pyrolysis in solvent-free conditions under a vacuum[26] or under microwave irradiation[27] (Scheme S1, Supporting Information). In contrast to the microwave-assisted synthetic procedure described by Das et al.,[27] which used a commercial microwave oven, in the present study the reaction was performed with a scientific single-mode microwave apparatus, which allowed reproducible conditions to be established. Compound **4** was semisynthesized through the hydrolysis reaction of the acetal ring of compound **1**, in the presence of sulfuric acid and phloroglucin[26] (Scheme S2, Supporting Information).

HPLC-ESIMS/MS. Liquid chromatography was performed using a 2690 Alliance system (Waters, Milford, MA, USA) equipped with a built-in 120-position cooled autosampler. Analytical separation was conducted on a Phenomenex Luna C_{18} (5 μm, 150 mm × 2.0 mm i.d.) column. The mobile phases were 10 mM formic acid in water adjusted to pH 4.0 with ammonia (solvent A) and a solution of acetonitrile−methanol (95:5 v/v) (solvent B). Separation was achieved at a 0.15 mL min^{-1} flow rate under gradient elution conditions (5 min at 95% A and 10 min at 40% A), followed by a column purge (5 min at 20% A) and column re-equilibration (10 min at 0% A). The injected sample volume was 10 μL. The analytical column was maintained at 30 °C. In the optimized analytical conditions, the mean retention times were for **1** 10.1 ± 0.1 min, **2** 10.6 ± 0.1 min, **4** 9.4 ± 0.1 min, and **5** 9.7 ± 0.1 min (n = 20). The column effluent was introduced into the ESI source, operating in the positive-ionization mode, connected to a triple-quadruple mass spectrometer (Quattro-LC, Micromass) operating in the multiple reaction monitoring acquisition mode [m/z 336 → 320 (**1**), m/z 322 → 307 (**2**), m/z 324 → 280 (**4**), m/z 338 → 323 (**5**), and m/z 414 → 220 (*R,S*)-noscapine]. Nitrogen was used as nebulizer gas at 75 L/h flow rate and as desolvation gas at 850 L/h. Ion source block and desolvation temperatures were set to 130 and 250 °C, respectively. Capillary voltage was 3.0 kV. The cone voltage was 45 kV. MassLynx software version 4.0 was used for data acquisition and processing.

Quantification. A seven-point calibration curve was prepared daily and injected in duplicate. Calibration samples were obtained in the 1.5−20 nM concentration range prepared in plasma for plasma matrix samples and in mobile phase for physicochemical property samples using (*R,S*)-noscapine as internal standard (IS, 2.5 nM). Linear calibration curve parameters were obtained from the plot of the analyte peak area/internal standard peak area versus analyte concentration using a least-squares regression analysis (weight = $1/x^2$). Correlation coefficients were ≥0.996 for all analytes. The HPLC-ESIMS/MS method was validated according to the current guidelines[28] and has good sensitivity (LOQ 1.5 nM), selectivity, precision (CV% intra- and interassay <9%), and accuracy (bias % inter- and intra-assay <7%) for all analytes.

Plasma Sample Preparation. Analytes were extracted from plasma using an Oasis HLB (hydrophilic−lipophilic balance 200 mg, 6 mL) SPE column. The optimized extraction procedure utilized: conditioning with 2 mL of MeOH and 2 mL of H_2O Milli-Q; loading with 780 μL of plasma (+10 μL of IS, 40 ng/mL) diluted with 2 mL of ammonium formate (10 mM pH 7.0), washing with 1 mL of formic acid (2%, v/v) and 2 mL of ammonium formate (10 mM pH 7.0), elution with 2 mL of MeOH, followed by 1 mL of MeOH containing 1% (v/v) CH_3COOH and 1 mL of MeOH containing 2% (v/v) NH_4OH, vacuum drying, and reconstitution with 400 μL of mobile phase. The recovery percentage was ≥98% for all analytes.

Determination of pK_a Values in Silico. The pK_a values were determined in silico using Epik module version 2.2 from Schrödinger Suite 2010, with water as the solvent. The Maestro interface of Schrödinger Suite 2010 (Maestro, version 9.1, Schrödinger, LLC, New York, NY, 2010) was used to build all molecules.

Determination of Solubility in Physiological pH Range. Excess amounts of the chlorides, **1**, **2**, **4**, and **5** were added to Na phosphate buffer 0.1 M from pH 4.5 to 9 in a sealed vial. The samples were left for 1 week under continuous stirring at 25 °C. Then, each solution was filtrated with RC membrane 0.45 μm syringe filters and appropriately diluted with mobile phase before injection in HPLC-ESIMS/MS.

Determination of Lipophilicity by 1-Octanol/Water Partition Coefficient and Distribution. The measurements of the 1-octanol/water partition coefficients were carried out starting from an initial concentration of 0.1 mM for all analytes in Na phosphate buffer 0.1 M from pH 4.5 to 9.[29] Then, 2 mL of this standard solution was added to 2 mL of 1-octanol presaturated with Na phosphate buffer, and the samples were left to equilibrate for 1 week under continuous stirring at 25 °C. After centrifugation, the two phases were carefully separated. The aqueous solution was collected and appropriately diluted with mobile phase before injection in HPLC-ESIMS/MS. The general equation used for the calculation of $LogD_{o/w}$ is reported in the Supporting Information (eq S1).

Determination of Albumin Binding Association Constants by Fluorescence Quenching. The measurements were performed using bovine serum albumin (1.0×10^{-5} mol L^{-1}) in Tris-HCl buffer solution (0.05 mol L^{-1} Tris, 0.15 mol L^{-1} NaCl, pH 7.4). The emission spectra (excitation at 295 nm and emission wavelengths of 300−500 nm) were recorded with increasing concentrations of quencher (**1**, **2**, **4**, **5**) from 0 to 2.4×10^{-5} mol L^{-1} at increments of 0.2×10^{-5} mol L^{-1} at room temperature. The experimental results published on the quenching mechanism of fluorescence of human serum albumin (HSA) by **1** indicate that it is a static quenching procedure[30] and that the binding association can be determined by plotting a modified Stern−Volmer equation (see eq S2, Supporting Information).

Human Feeding Study. Berberine was administered to human volunteers according to a clinical study protocol, approved on April 21, 2009, by S. Orsola-Malpighi Hospital Review Board, No. 7-2209-U-SPER. The complete study protocol is described in the Supporting Information. To evaluate the metabolic effects of berberine chloride (**1**) on cholesterol and biliary metabolisms, 12 consecutive euglycemic subjects were enrolled, affected by mild to moderate hypercholesterolemia (c-LDL >130 mg/mL and <190 mg/mL). All patients received 15 mg/kg daily of berberine chloride (**1**) for three months. The age range of the patients was between 18 and 70 years old. No patient received any medication affecting biliary, glucose, or cholesterol metabolism.

■ ASSOCIATED CONTENT

Supporting Information

General synthetic procedures and spectroscopic characterization of compounds **2**, **2a**, **2b**, and **4**. General equations used for the determination of $LogD_{o/w}$ and K_B. In vivo study protocol and comparison between basal vs after treatment (V0 vs V3) of cholesterol (total, LDL, and HDL), total triglycerides, total serum bile acid, fasting glucose, and fasting insulin. This information is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +39 051 343398. Fax: +39 051 343398. E-mail aldo.roda@unibo.it.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the University of Bologna for financial support. We acknowledge Prof. A. Mazzanti and Dr. S. Grilli for fruitful discussions on the spectroscopic characterization of the

synthesized metabolites. Ms. G. Fox is thanked for editing and proofreading the manuscript.

REFERENCES

(1) Zeisel, S. H. *Science* **1999**, *285*, 1853−1855.
(2) Bentley, K. W. *Nat. Prod. Rep.* **1997**, *14*, 387−411.
(3) Sarma, B. K.; Pandey, V. B.; Mishra, G. D.; Singh, U. P. *Folia Microbiol.* **1999**, *44*, 164−166.
(4) Kuo, C. L.; Chi, C. W.; Liu, T. Y. *Cancer Lett.* **2004**, *203*, 127−137.
(5) Iwasa, K.; Kim, H. S.; Wataja, Y.; Lee, D. U. *Eur. J. Med. Chem.* **1998**, *33*, 65−69.
(6) Gudima, S. O.; Memelova, L. V.; Borodulin, V. B.; Pokholok, D. K.; Mednikov, B. M.; Tolkachev, O. N.; Kochetkov, S. N. *Mol. Biol. (Moskow)* **1994**, *28*, 1308−1314.
(7) Ni, Y. X. *Zhong Xi Yi Jie He Za Zhi* **1988**, *8*, 711−713 and 707.
(8) Kim, T. S.; Kang, B. Y.; Cho, D.; Kim, S. H. *Immunology* **2003**, *109*, 407−414.
(9) Ikekawa, T.; Ikeda, Y. *J. Pharmacobiodyn.* **1982**, *5*, 469−474.
(10) Kong, W.; Wei, J.; Abidi, P.; Lin, M.; Inaba, S.; Li, C.; Wang, Y.; Wang, Z.; Si, S.; Pan, H.; Wang, S.; Wu, J.; Wang, Y.; Li, Z.; Li, J.; Jiang, J. D. *Nat. Med.* **2004**, *10*, 1344−1351.
(11) Kheir, M. M.; Wang, Y.; Hua, L.; Hu, J.; Li, L.; Lei, F.; Du, L. *Food Chem. Toxicol.* **2010**, *48*, 1105−1110.
(12) Li, Y.; Ren, G.; Wang, Y. X.; Kong, W. J.; Yang, P.; Wang, Y. M.; Li, Y. H.; Yi, H.; Li, Z. R.; Song, D. Q.; Jiang, J. D. *J. Transl. Med.* **2011**, *9* (62), 1−10.
(13) Roja, G.; Bhangale, A. S.; Juvekar, A. R.; Eapen, S.; D'Souza, S. F. *Biotechnol. Prog.* **2005**, *21*, 1688−1691.
(14) Simanek, V.; Preininger, V.; Lasovsky, J. *Collect. Czech. Chem. Commun.* **1976**, *41*, 1050−1055.
(15) Marek, R.; Seckarova, P.; Hulova, D.; Marek, J.; Dostal, J.; Sklenář, V. *J. Nat. Prod.* **2003**, *66*, 481−486.
(16) Shamma, M.; Rahimizadeh, M. *J. Nat. Prod.* **1986**, *49*, 398−405.
(17) Grycova, L.; Dostal, J.; Marek, R. *Phytochemistry* **2007**, *68*, 150−175.
(18) Jeon, Y. W.; Jung, J. W.; Kang, M.; Chung, I. K.; Lee, W. *Bull. Korean Chem. Soc.* **2002**, *23*, 391−394.
(19) Pavelka, S.; Smékal, E. *Collect. Czech. Chem. Commun.* **1976**, *41*, 3157−3169.
(20) Pavelka, S.; Kovar, J. *Collect. Czech. Chem. Commun.* **1976**, *41*, 3654−3669.
(21) Suau, R.; Silva, M. V.; Valpuesta, M. *Tetrahedron* **1991**, *47*, 5841−5846.
(22) Lu, Y. C.; Lin, Q.; Luo, G. S.; Dai, Y. Y. *J. Chem. Eng. Data* **2006**, *51*, 642−644.
(23) Battu, S. K.; Repka, M. A.; Maddineni, S.; Chittiboyina, A. G.; Avery, M. A.; Soumyajit, M. *AAPS PharmSciTech* **2010**, *11*, 1466−1475.
(24) Hu, Y. J.; Liu, Y.; Xiao, X. H. *Biomacromolecules* **2009**, *10*, 517−521.
(25) Samanta, A.; Paul, B. K.; Guchhait, N. *J. Photochem. Photobiol. B* **2010**, *101*, 304−312.
(26) Li, Y. H.; Li, Y.; Yang, P.; Kong, W. J.; You, X. F.; Ren, G.; Deng, H. B.; Wang, Y. M.; Wang, Y. X.; Jiang, J. D.; Song, D. Q. *Bioorg. Med. Chem.* **2010**, *18*, 6422−6428.
(27) Das, B.; Srinivas, V. N. S. *Synth. Commun.* **2002**, *32*, 3027−3029.
(28) Food and Drug Administration. *Guidance for Industry: Bioanalytical Method Validation*; U.S. Department of Health and Human Services, FDA, Center for Drug Evaluation and Research: MD, 2001; pp 2−22.
(29) Roda, A.; Minutello, A.; Angellotti, M. A.; Fini, A. *J. Lipid Res.* **1990**, *31*, 1433−1443.
(30) Hu, Y.; Ehli, E. A.; Kittelsrud, J.; Ronan, P. J.; Munger, K.; Downey, T.; Bohlen, K.; Callahan, L.; Munson, V.; Jahnke, M.; Marshall, L. L.; Nelson, K.; Huizenga, P.; Hansen, R.; Soundy, T. J.; Davies, G. E. *Phytomedicine* **2012**, *19*, 861−867.