Effect of Berberine on In Vitro Metabolism of Sulfonylureas: A herb-drug interactions study

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Abstract:	Rationale: Patients with type 2 diabetes may co-ingest herbal and prescription medicine to control their blood sugar levels. Competitive binding of drug and herb may mutually affect their metabolism. This can alter the level of drug and its kinetics in the body, potentially causing toxicities or loss of efficacy. Understanding how metabolism of sulfonylureas like glyburide and gliclazide can be affected by the presence of berberine and vice versa can provide valuable information on the possible risk of toxicities caused by co-ingestion of drugs. Methods: Berberine and sulfonylureas (glyburide and gliclazide) were co-incubated with rat liver microsomes in the presence of NADPH regenerating system. The metabolites of berberine and sulfonylureas were analysed using liquid chromatography with high resolution mass spectrometry in the positive ion mode. The role of individual isozymes in the metabolism of berberine, glyburide and gliclazide was investigated by using specific inhibitors. Results: In vitro metabolism of berberine lead to the formation of demethyleneberberine (B1a) and B1b through demethylenation. Berberrubine (B2a) and its isomer (B2b) was formed through demethylation. The isozymes CYP3A and CYP2D were found to be involved in the metabolism of berberine. In vitro metabolism of glyburide and gliclazide lead to the formation of hydroxylated metabolites. The isozymes CYP3A and CYP2C were found to be involved in the metabolism of glyburide. Gliclazide was metabolised by CYP2C. In vitro co-incubation of glyburide or gliclazide with berberine showed that each drugs metabolism was compromised as both share a common isozymes. A strong negative linear correlation of glyburide or gliclazide metabolites levels and the concentration of berberine confirmed the effect of berberine on the metabolism of sulfonylureas. Conclusions: The metabolism of sulfonylureas and berberine was affected when these compounds were co-incubated with each other. This may be attributable to competitive binding of the herb and drug to the catalytic sites of same isozymes.

Effect of Berberine on *In Vitro* Metabolism of Sulfonylureas: A herb-drug interactions study

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***Keywords*:** Berberine; glyburide; gliclazide; isomers; interactions; isozymes

Abstract

Rationale: Patients with type 2 diabetes may co-ingest herbal and prescription medicine to control their blood sugar levels. Competitive binding of drug and herb may mutually affect their metabolism. This can alter the level of drug and its kinetics in the body, potentially causing toxicities or loss of efficacy. Understanding how metabolism of sulfonylureas like glyburide and gliclazide can be affected by the presence of berberine and vice versa can provide valuable information on the possible risk of toxicities caused by co-ingestion of drugs.

Methods: Berberine and sulfonylureas (glyburide and gliclazide) were co-incubated with rat liver microsomes in the presence of NADPH regenerating system. The metabolites of berberine and sulfonylureas were analysed using liquid chromatography with high resolution mass spectrometry in the positive ion mode. The role of individual isozymes in the metabolism of berberine, glyburide and gliclazide was investigated by using specific inhibitors.

Results: *In vitro* metabolism of berberine lead to the formation of demethyleneberberine (B1a) and B1b through demethylenation. Berberrubine (B2a) and its isomer (B2b) was formed through demethylation. The isozymes CYP3A and CYP2D were found to be involved in the metabolism of berberine. *In vitro* metabolism of glyburide and gliclazide lead to the formation of hydroxylated metabolites. The isozymes CYP3A and CYP2C were found to be involved in the metabolism of glyburide. Gliclazide was metabolised by CYP2C. *In vitro* co-incubation of glyburide or gliclazide with berberine showed that each drugs metabolism was compromised as both share a common isozymes. A strong negative linear correlation of glyburide or gliclazide metabolites levels and the concentration of berberine confirmed the effect of berberine on the metabolism of sulfonylureas.

Conclusions: The metabolism of sulfonylureas and berberine was affected when these compounds were co-incubated with each other. This may be attributable to competitive binding of the herb and drug to the catalytic sites of same isozymes.

1. Introduction

Many patients use herbal medicines alongside prescription medicines to treat their medical conditions. The co-administration of these compounds can cause competitive metabolism as cytochrome P450 (CYP) is the main family of isozymes responsible for the biotransformation of compounds [1]. One therapeutic area where both herb and prescription medicine may be co-ingested is in the treatment of type 2 diabetes. Sulfonylureas (glibenclamide (glyburide) and gliclazide) are used as the frontline pharmacotherapy to treat adult type 2 diabetes[2-4]. Sulfonylureas stimulate pancreatic β-cells to increase insulin secretion, which causes the depolarisation of β-cell membrane through inhibition of K_{ATP} channels. This leads to the opening of Ca^{2+} channel voltage gate which in turn results in the influx of Ca^{2+} inside the cells. Exocytosis is achieved as insulin-containing secretory granules are released in circulation [5]. The efficacy of sulfonylureas is determined from β cell function [6]. Sulfonylureas are mainly eliminated through metabolic transformation. Studies that have been reported on metabolism of glyburide have confirmed the production of 10 hydroxylated metabolites catalysed by CYP3A4/5/7, CYP2C9, CYP2C8 and CYP2C19 (Fig. 1A) [7-11] Gliclazide has been shown to be extensively metabolised by the activity of CYP2C9 and CYP2C19 to produce a range of hydroxylated metabolites (Fig. 1B) [3,12-14]. Berberine is an active alkaloid component (Fig. 1C) found in certain plants such as *Phellodendron cortex* (Huang Bai), *Coptis chinensis* (Huang Lian), *Hydrastis canadensis, Berberis aquifolium, Berberis vulgaris and Berberis aristata* [15,16]. Berberine has shown to have anti-diabetic effects [16,17].

The hypoglycaemic effect of berberine is attributed to: activation of AMPK (AMP-activated protein kinase)[18], β cell regeneration [19]and increase of muscle glucose uptake [20]. Berberine inhibits dipeptidyl peptidase 4 (DPP-4) to regulate the secretions of glucagon-like peptide (GLP-1) [21, 22]. In addition, berberine inhibits the aerobic respiration, and induces the glycolysis through glucose uptake [18,23-25]. Berberine is metabolised by CYP2D6, CYP1A2, CYP2E1, CYP2C9, CYP2C19 and CYP3A4 to produce demethylated and demethylenated metabolites [26]. Competitive metabolism may mutually affect the metabolism of both compounds. This can change the level of the drug and its kinetics in the body to cause toxicity or loss of efficacy [1]. To date, there is no study that has been reported on how the competitive metabolism of

berberine and the sulfonylureas (glyburide and gliclazide) would affect the metabolism of both compounds as they share the same isozymes. The aim of this paper was to investigate the *in vitro* interaction of berberine-sulfonylureas by considering that binding of one drug to the catalytic site of isozyme could compromise the metabolism of the other drug.

2 Materials and Methods

2.1 Materials

Male and female rat (Sprague-Dawley) liver microsomes, glucose-6-phosphate dehydrogenase (G-6-PD) from leuconostoc mesenteroides - recombinant, expressed in *E. coli*, ammonium sulphate suspension, D-glucose 6-phosphate sodium salt, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt hydrate (β-NADPH), magnesium chloride, berberine chloride hydrate, glyburide, glipizide, gliclazide, ketoconazole, tienilic acid, quinidine and cimetidine were obtained from Sigma-Aldrich (Seldown Lane, Poole, UK). Acetonitrile of HPLC grade, formic acid and methanol were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). Mini-Uni 0.45 µm prep syringeless filter were supplied by GE Healthcare Life Sciences (Little Chalfont, Bukinghamshire, UK). ACE3 C18-AR Column and ACE3 C18 guard column were purchased from Hichrom Limited (Lutterworth, Leicestershire, UK). Sodium hydroxide and potassium phosphate monobasic of analytical reagent grade were obtained from BDH and Griffin (Ealing,Middlesex, UK) respectively. Berberrubine chloride and demethyleneberberine were custom manufactured by AvaChem Scientific (San Antonio, Texas, USA). GraphPad Prism 7.04® and Minitab 17® were purchased from GraphPad Software (San Diego, CA, USA) and Minitab Ltd. (Progress Way, Coventry, UK) respectively.

2.2 Preparation of drugs

Stock solutions of berberine, cimetidine, gliclazide, glyburide, ketoconazole, quinidine, and tienilic acid were prepared at a concentration up to 2 mg/mL in methanol. Working solutions at a concentration of 0.6 mg/mL were prepared by diluting the stock solution with water. A single solution of cimetidine was prepared at a concentration of 1 mg/mL in deionised water.

2.3 LC–IT-TOF MS instrumentation and conditions

Samples were analysed using a Shimadzu LC-IT-TOF MS. (Milton Keynes, Bukinghamshire, UK). A Shimadzu Prominence HPLC system composed of a degassing unit (model number L202543), pumps (model number L201044), autosampler (model number L201743), column oven (model number L202043), detector (model numberL201343) and system controller (model number L202344) hyphenated to an ion-trap time of flight mass spectrometer (model number C20234470341) (IT-TOF MS) was used. LC-MS solutions software V3® was used to acquire data and set the instrument parameters. Separation of sample was carried out using an ACE3 C18 150 x 2.1 mm, 3 µm column with a ACE3 C18 cartridge guard column. The mobile phase consisting of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B) was used at a flow rate of 0.21 mL/min. A linear gradient elution profile: 0-10 min, 25 to 90 % B; 10-15 min, 90 % B; 15 to 15.10 min, 25 % B. The column and autosampler temperature were maintained at 40 and 4°C respectively. The sample injection volume was 10 µL. The mass spectrometer was equipped with an electrospray ionisation source (ESI) and was operated in dual positive and negative ion acquisition mode. MS and MS/MS data was acquired in the range *m/z* 100 to 590 with an ion accumulation time of 30 msec. Analysis was carried out using nitrogen as nebuliser gas at a flow rate of 1.5 L/min and curved desorption line (CDL) and heat block temperature of 200°C. The detector and interface voltage was set at 1.60 and 4.5 kV respectively. Argon was used as collision gas at 75 psi. A mixture of acetonitrile, isopropanol and water (33.3:33.3:33.3, v/v) containing 0.1% formic acid was used for the injection needle rinse.

2.4 *In vitro* metabolism conditions of selected drugs

2.4.1 Incubation conditions

A co-factors solution composed of 5 mM magnesium chloride, 2 mM NADP, disodium salt and 5 mM D-glucose 6-phosphate sodium salt was prepared in 0.1 M potassium phosphate buffer, pH 7.4. NADPH regenerating system was prepared by adding glucose-6-phosphate dehydrogenase (1 U/mL) to the co-factors solution. A small volume (50 µL) of cofactors solution together with 50 µL of G-6-PD and 40 µL of 0.1 M KH_2PO_4 buffer, pH 7.4 was transferred to a 1.5 mL Eppendorf tube. An aliquot (50 µL) of microsomes (0.5 mg/mL) was then added. The incubation mixture was pre-incubated for 5 min in a water bath at 37°C and subsequently berberine or sulfonylurea (glyburide or gliclazide) was added at a final

concentration of 20 µM. The final volume of incubation mixture was 0.2 mL. A small volume (20 µL) of reaction termination solvent consisting of a mixture of water, acetonitrile and formic acid (92:5:3 %, v/v) was added after 1 h. The samples were subsequently kept on ice for 10 min. Ice cold methanol (60 %, v/v) was then added and the mixture was placed on ice for 10 min. Samples were centrifuged at 16,000 g for 5 min and supernatant was collected and filtered using Mini-Uni prep syringeless filters (0.45µm) to remove precipitated proteins before loading 10 µL of sample onto the HPLC column.

2.5 Role of CYP isozymes in the metabolism of sulfonylureas and berberine

In vitro phase I metabolism profile of berberine, glyburide and gliclazide was established by incubating 20 µM of these compounds with 0.5 mg/mL male rat liver microsomes and the NADPH regenerating system. To investigate which isozymes are involved in the metabolisms of berberine, glyburide and gliclazide, specific isozyme inhibitors were used. Ketoconazole, quinidine, cimetidine and tienilic acid are specific inhibitors for isozymes CYP3A, CYP2D, CYP1A2 and CYP2C respectively. To the *in vitro* incubation mixture containing 20 µM berberine or glyburide or gliclazide and NADPH regenerating system, ketoconazole, quinidine, cimetidine or tienilic acid were added at various concentrations to estimate the involvement of isozymes of CYP3A, CYP2D, CYP1A2 and CYP2C respectively. The mixture was incubated as described above. The concentration of the inhibitors used was validated in preliminary experiments to obtain the IC50 values.

2.6 Berberine-sulfonylureas interactions

The interactions of glyburide and gliclazide with berberine were studied by incubating the sulfonylureas (20 µM) with various concentrations of berberine (0.1 to 80 µM) in the presence of 0.5 mg/mL microsomes and the NADPH regenerating system. Control samples were prepared with berberine and sulfonylurea alone in the same concentration range with 0.5 mg/mL microsomes and the NADPH regenerating system. All samples were prepared in triplicate.

2.7 Measurement of berberine and sulfonylureas metabolites

Demethyleneberberine and berberrubine were prepared over a concentration range 0.31 to 20 µg/mL and 0.11 to 28 µg/mL respectively. The internal standard, glipizide, was spiked into all the standards at a final concentration of 5.6 µg/mL. These were used to generate standard

curves. The level of glyburide metabolites (4-trans-hydroxycycohexyl glyburide, 4-cis-hydroxycyclohexyl glyburide and 3-trans-hydroxycycohexyl glyburide) and gliclazide metabolites 6-α- hydroxygliclazide and methylhydroxygliclazide were semi-quantitated due to unavailability of the authentic standards. The change in HPLC-peak area of metabolite relative to the control was calculated. Although the metabolites such as 3-cis-hydroxycyclohexyl glyburide, 6-β- hydroxygliclazide, 7- β-hydroxygliclazide, 7-α-hydroxygliclazide were detected and not quantitated due to insufficient separation (Fig. 2,3).

2.8 Data treatment

The statistical significance of the data was established by using GraphPad Prism 7.04® and Minitab 17®. The normal distribution of data was verified by probability plots. All data are represented as mean ± SD. Two samples t-test and Pearson correlation tests were used to determine the statistical significance at 95% confidence interval with p values (0.05). The p values were used with following symbols; * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ and $P > 0.05$ was not significant.

3 Results and Discussion

3.1 *In vitro* phase I metabolism profile of berberine and sulfonylureas

Glyburide and gliclazide metabolites eluted in the increasing order of hydrophobicity using reverse phase chromatography with a linear gradient elution profile from 25 to 90 % of acetonitrile (containing 0.1 % formic acid) over 15 min. Sulfonylureas metabolites were identified from both the MS and MS/MS spectra.

3.2 *In vitro* phase I metabolism profile of glyburide

Glyburide and its metabolites eluted in the following order G1a<G1b<G1c<G1d<G (Fig. 2 and Table 1). Metabolites of glyburide were identified from both the MS and MS/MS spectra. The elution order of the hydroxylated metabolites of glyburide has been reported using reverse phase chromatography [9,27]. These authors determined the isomeric nature of the metabolites using synthesised standards. In the present study, using similar separation conditions the elution profile for glyburide and its metabolites is likely to be similar. Glyburide eluted at 10.0 min and showed $[M+H]^+$ ion at *m/z* 494.151 ($C_{23}H_{29}ClN_3O_5S$). Fragmentation of this precursor ion yielded the product ions at *m/z* 395.048, 369.065, 169.005 as shown in Fig. 2. The mass spectrum obtained can be explained by fragmentation of glyburide precursor ion. The ion *m/z* 395.048 is formed via the loss of $C_6H_{13}N$ (99 Da), *m/z* 369.065 results from the loss of $C_7H_{11}NO$ (125 Da), *m/z* 169.005 via the loss of $C_{15}H_{23}N_3O_3S$ (325 Da). Identical fragments have been reported by other authors[28, 29].

MS and MS/MS Spectra and fragmentation pathway of glyburide metabolites following incubation of glyburide with rat male liver microsomes and the NADPH regenerating system are shown in Fig. S-1. 4-trans-hydroxycyclohexyl glyburide (G1a) (*m/z* 510.143) and its isomers G1b (4-cis-hydroxycyclohexyl glyburide), G1c (3-cis-hydroxycyclohexyl glyburide) and G1d (3-trans-hydroxycyclohexyl glyburide) eluted at 7.5, 7.8, 7.9 and 9.1 min and showed $[M+H]^+$ ion respectively. The increase in mass of glyburide by 16 Da to form the isomeric metabolites can be attributed to hydroxylation of the compound [8-10]. The MS and MS/MS spectra and retention time was used to identify the hydroxylated metabolites of glyburide. From identical MS and MS/MS spectra of G1a, G1b, G1c and G1d it was evident that these were isomers. Fragmentation of this precursor ion yielded the product ions at *m/z* 492.133, 410.040, 385.060, 369.064 as shown in Fig. S-1. The mass spectrum obtained can be explained by fragmentation of glyburide metabolites precursor ion i.e. *m/z* 492.133 formed via the loss of H_2O (18 Da), *m/z* 411.040 formed via the loss of $C_6H_{13}N$ (99 Da), *m/z* 385.060 via the loss of $C_7H_{11}NO$ (125 Da), *m/z* 369.064 via the loss of $C_7H_{11}NO_2$ (141 Da). Precursor ion was hydroxylated at the terminal region of cyclohexyl group [29,30]. The product ions *m/z* 411.040 and 385.060 have been reported by [28]. Structure of product ions *m/z* 385.060 and *m/z* 369.064 proposed are identical to those reported by [27].

3.3 *In vitro* phase I metabolism profile of gliclazide

Gliclazide and its metabolites eluted in the following order Gz1a<Gz1b<Gz1c<Gz1d <Gz1e<Gz (Fig. 3 and Table 1). The elution order of the hydroxylated metabolites of gliclazide has been reported using reverse phase chromatography[31-33]. The authors determined the elution order of the metabolites using synthesised standards. In the present study, using similar reverse phase conditions a similar elution profile for gliclazide and its metabolites was obtained. Gliclazide eluted at 9.3 min as $[M+H]^+$ ion at *m/z* 324.135 ($C_{15}H_{22}N_3O_3S$). Fragmentation of this precursor ion yielded the product ions at *m/z* 281.128, 168.112, 151.086, 127.122, 110.022 as shown in Fig.3. The mass spectrum obtained can be explained by the mass changes of gliclazide precursor ion i.e. *m/z* 281.128 via the loss of C_3H_7 (43 Da) , *m/z* 168.112 via the loss of $C_7H_8O_2S$ (156 Da), *m/z* 151.086 via the loss of $C_7H_{11}NO_2S$ (173 Da), *m/z* 127.122 via the loss of $C_8H_7NO_3S$ (197 Da), *m/z* 110.020 via the loss of $C_8H_{10}N_2O_3S$ (214 Da). Identical fragments were reported by [34-36].

6-α-hydroxygliclazide (Gz1a) (*m/z* 340.129) as a metabolite of gliclazide with its isomers 6-β-hydroxygliclazide (Gz1b), 7-α-hydroxygliclazide (Gz1c), 7-β-hydroxygliclazide (Gz1d) and methylhydroxygliclazide (Gz1e) eluted at 5.5 , 5.9, 6.0 , 6.4 and 6.8 min and showed $[M+H]^+$ ion at *m/z* 340.131 respectively. These metabolites are mostly likely produced through hydroxylation of gliclazide (*m/z* 324.135) [14,33]. The MS/MS spectrum and retention time was used to identify the hydroxylated metabolites of gliclazide and from identical MS and MS/MS spectra it was evident that Gz1a, Gz1b, Gz1c, Gz1d, Gz1e were isomers. Fragmentation of this precursor ion yielded the product ions at *m/z* 322.119, 110.095, 143.119, 168.113 as shown in Fig. S-2. The mass spectrum obtained can be explained by fragmentation of gliclazide metabolites i.e. *m/z* 322.119 formed via the loss of H_2O (18 Da), *m/z* 110.095 via the loss of $C_8H_{10}N_2O_4S$ (230 Da), *m/z* 143.119 via the loss of $C_8H_7NO_3S$ (197 Da), *m/z* 168.113 via the loss of $C_7H_8O_3S$ (172 Da).

3.4 *In vitro* phase I metabolism profile of berberine

Berberine metabolites eluted in the following order B1a<B1b<B2a<B2b<B. MS and MS/MS spectra was used to identify the analytes (Fig. 4 and Table 1). Berberine eluted at 6.8 min and showed [M+] at *m/z* 336.123 ($C_{20}H_{18}NO_4$). Fragmentation of this precursor ion yielded the product ions at *m/z* 321.100, 305.102, 292.097, 275.084 as shown in Fig 4. The mass spectrum obtained can be explained by the mass changes from the berberine precursor ion i.e. *m/z*

321.100 via the loss of CH_3 (15 Da), *m/z* 305.102 via the loss of CH_3O (31 Da), *m/z* 292.097 via the loss of C_2H_4O (44 Da) and *m/z* 275.084 via the loss of $C_2H_5O_2$ (61 Da). Identical fragments were reported by [16].

Demethyleneberberine ($C_{19}H_{18}NO_4$) (B1a) *m/z* 324.122 and B1b were produced following *in vitro* incubation of berberine with rat male liver microsomes and the NADPH regenerating system. B1a and B1b eluted at 4.9 and 5.8 min respectively and showed [M]+ ion at *m/z* 324.122. The MS and MS/MS spectra of B1a and B1b were identical. B1a was produced through demethylenation and this was confirmed using authentic standard. B1b showed similar empirical formula based on accurate mass from B1a and eluted after 0.9 min. This could be due to a different or additional biotransformation. To date B1b has not been reported in the literature. The catechol of B1a could be methylated by catechol-O-methyl transferase and demethylated at the other end of the molecule to give B1b (Fig. 7). The most abundant product ions of demethyleneberberine was observed at *m/z* 309.109 via the loss of CH_3 (15 Da) and *m/z* 281.101 via the loss of C_2H_3O (43 Da) (Fig. S-3).

Berberrubine ($C_{19}H_{16}NO_4$) (B2a) *m/z* 322.107 and its isomer thalifendine (B2b) was produced following *in vitro* incubation of berberine with rat male liver microsomes and the NADPH regenerating system through demethylation (Fig. S-4). Berberrubine showed [M]+ ion at *m/z* 322.107. This was apparent from 14 Da lower mass (*m/z* 322.107) than the parent compound (*m/z* 336.124) and from MS and MS/MS spectra of authentic standards[16,37]. B2a and B2b eluted at 5.9 and 6.2 min respectively. MS and MS/MS spectra and retention time of berberrubine was similar to the authentic standard. The identical MS and MS/MS spectra of berberrubine confirmed that B2a and B2b were isomers and showed similar fragmentation pattern. The most abundant product ion of berberrubine was observed at *m/z* 307.087 via the loss of CH_3 (15 Da) (Fig. S-4).

3.5 Role of CYP isozymes involved in the metabolism of sulfonylureas

The role of CYPs in producing hydroxylated metabolites of glyburide and gliclazide was investigated using ketoconazole and tienilic acid as CYP3A and CYP2C inhibitors respectively [37-39]. The IC50 values of ketoconazole and tienilic acid to inhibit the production of glyburide metabolites are shown in S-Table 1. The hydroxylation metabolism of glyburide mediated by CYP3A and CYP2C is depicted in S-Fig. 6. From these results it has been seen that CYP3A and CYP2C play a key role in the metabolism of glyburide. These findings are consistent with

previous studies that have been conducted using human liver microsomes and in pregnant women by [9,11]. The IC50 values of tienilic acid to inhibit the production of gliclazide metabolites are listed in S-Table 1. The metabolism of gliclazide mediated by CYP2C to produce the hydroxylated metabolites is shown in S-Fig.7. The results obtained demonstrate that CYP2C is involved in the metabolism of gliclazide.

3.6 Role of CYP isozymes in metabolism of berberine

Cimetidine has been shown to be a reversible competitive inhibitor of CYP1A2 [41]. The role of CYP1A2 in the metabolism of berberine was verified by correlation analysis of the production of berberine metabolites and the concentrations of cimetidine present in the *in vitro* incubation mixture. A weak positive correlation was noticed with no statistical significance (p- value > 0.05) (see Fig. S-5). The increasing concentration of cimetidine did not show any significant decrease in the formation of berberine metabolites. The role of CYP1A2 in the metabolism of berberine was only mentioned by [37], no similar findings were observed in the present study.
As a highly selective competitive inhibitor of CYP2D6 [39,40], quinidine was found to inhibit the metabolism of berberine (S-Table 1). The isozyme CYP3A was also found to play a role in the metabolism of berberine. The presence of ketoconazole an inhibitor of CYP3A, in the incubation mixture resulted in inhibition of the production of B1a, B1b, B2a, B2b (S-Table 1). A study conducted by [37] using mice and human liver microsomes identified that CYP3A4 is involved in the metabolism of berberine. This is consistent with our findings. The metabolism of berberine mediated by CYP2D and CYP3A is shown in S-Fig.8.

3.7 Interactions of berberine and sulfonylureas

3.7.1 Measurement of berberine metabolites

Separate standard curves were derived for demethyleneberberine and berberrubine in the berberine vs glyburide and –gliclazide interactions study. The correlation coefficient values were >0.99.

3.7.2 Interactions of berberine and glyburide

Berberine and glyburide share the common isozyme CYP3A4 in their metabolism [11,37]. The potential interaction in the metabolism of berberine and glyburide was investigated by co-incubating berberine with glyburide with rat liver microsomes. It was found that the metabolism of berberine was affected by the presence of glyburide. Lower levels of demethyleneberberine and berberrubine were observed when berberine was co-incubated with 20 µM glyburide confirming statistical significance, $p < 0.05$ for 2 and 4 µM respectively of berberine. There was no statistical significance for the difference in metabolism of berberine at 6, 8 and 10 µM in the presence of glyburide at 20 µM (Fig. 5).

The effect of berberine on the production of glyburide metabolites was verified using the correlation analysis of glyburide metabolites production and the concentration of berberine present in the *in vitro* metabolism incubation mixture (S-Table 2). A strong negative linear relationship with a correlation coefficient of ($r = -0.96$) was observed between the production of glyburide metabolites, including both 4-trans-hydroxycyclohexyl glyburide and 4-cis-hydroxycyclohexyl glyburide, and the concentrations of berberine (2-10 µM). However, there was no significant statistical difference in the formation of 3-trans-hydroxycyclohexyl glyburide in presence and absence of berberine.

The results from interaction study, suggest that the competitive binding of glyburide and berberine to isozyme CYP3A may be responsible for the lower biotransformation of both compounds when they are co-incubated with microsomes.

3.7.3 Interactions of berberine and gliclazide

The isozyme CYP2C9 has been shown to mediate the metabolism both of berberine and gliclazide [17, 26, 37]. In the current work potential interactions between berberine and gliclazide were investigated by co-incubating the two compounds with rat liver microsomes. The interactions were investigated with berberine in the range 2-80 µM in the presence of gliclazide at 20 µM. It was found that the metabolism of berberine at 10 µM was affected by the presence of 20 µM gliclazide. A lower level of demethyleneberberine was formed when berberine was co-incubated with gliclazide. This was found to be statistically significant with a $p < 0.05$. B1b was also formed at lower level in presence of 20 µM gliclazide and berberine at concentration of 2, 40 and 80 µM with statistical significance of $p < 0.05$. Berberrubine isomer was formed at lower level following the incubation of berberine at concentration of 2, 6, 10, 20 and 40 µM in presence of 20 µM gliclazide confirming statistical significance, $p < 0.05$ (Fig. 6).

The effect of berberine on the production of gliclazide metabolites was verified using the correlation analysis (S-Table 2). The strong negative linear relationship between formation of gliclazide metabolites and the concentration of berberine (2-80 μM) was observed with a correlation coefficient of r = -0.80. There was a significant decrease in the formation of (6-α-hydroxygliclazide) (Gz1c) and methylhydroxygliclazide (Gz1e), indicating that berberine inhibited the metabolism of gliclazide. This may be attributable to berberine and gliclazide competing for binding to the same CYP2C isozyme.

The studies conducted by [17,26,37] on the role of isozymes involved in the metabolism of berberine have confirmed the role of CYP2C. Although berberine affected the metabolism of gliclazide, this sulfonylurea did not significantly affect the metabolism of berberine . This may be because several isozymes (CYP3A4, CYP2D6, CYP2C19, CYP2E1) are involved in the metabolism of berberine [37]. This may help to explain why gliclazide did not significantly affect the metabolism of berberine.

3.8 Conclusions

In vitro metabolic profiles of berberine and sulfonylureas (glyburide, gliclazide) were established using liquid chromatography with high resolution mass spectrometry in the positive ion mode. This was utilised to study the potential interactions of berberine and sulfonylureas. Glyburide and berberine both share a common CYP3A isozyme and due to this their metabolism was mutually affected when both compounds were co-incubated with rat microsomes. Co-incubation of berberine and gliclazide with microsomes resulted in inhibition of metabolism of gliclazide. This may be attributable to the two compounds competing for the same isozyme CYP2C, which is the only isozyme that is involved in the metabolism of gliclazide The molecular mechanisms of berberine and sulfonylurea interactions could be attributable to the compounds sharing the same isozymes. The herbs containing berberine and other active components may give different results as a complex mixture. Further *in vitro* and *vivo* interaction studies are necessary to gain better understanding of the impact of drug-herb interactions on their metabolism.

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Conflicts of interest: The authors declare that there are no conflicts of interest.

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Ethical approval: Protocols for the *in vitro* studies were approved by the Natural Sciences Ethics sub-Committee at the Middlesex University (London, United Kingdom) [reference: 1883].

Table 1 LC MS/MS retention times and precursor and product ions of berberine and sulfonylureas.

Name	t_R (min)	Exact mass	Measured mass	Error (ppm)	Formula
Berberine (B)	6.8	336.123	336.124	2.03	$C_{20}H_{18}NO_4$
Demethyleneberberine (B1a)	4.9	324.123	324.122	1.49	$C_{19}H_{18}NO_4$
B1b	5.9	324.123	324.122	1.49	$C_{19}H_{18}NO_4$
Berberrubine (B2a)	5.9	322.107	322.107	0.41	$C_{19}H_{16}NO_4$
Berberrubine's isomer (B2b)	6.2	322.107	322.107	0.41	$C_{19}H_{16}NO_4$
Glyburide (G)	10.0	494.151	494.151	0.09	$C_{23}H_{29}ClN_3O_5S$
4-trans-hydroxycyclohexyl glyburide (G1a)	7.5	510.146	510.146	0.90	$C_{23}H_{29}ClN_3O_5S$
4-cis-hydroxycyclohexyl glyburide (G1b)	7.8	510.146	510.146	0.90	$C_{23}H_{29}ClN_3O_5S$
3-cis-hydroxycyclohexyl glyburide (G1c)	7.9	510.146	510.146	0.90	$C_{23}H_{29}ClN_3O_5S$
3-trans-hydroxycyclohexyl glyburide (G1d)	9.1	510.146	510.146	0.90	$C_{23}H_{29}ClN_3O_5S$
Gliclazide (G)	9.2	324.138	324.137	0.88	$C_{15}H_{22}N_3O_3S$
6-β- hydroxygliclazide(Gz1a)	5.5	340.133	340.132	2.35	$C_{15}H_{22}N_3O_4S$
7- β- hydroxygliclazide (Gz1b)	5.9	340.133	340.132	2.35	$C_{15}H_{22}N_3O_4S$
6-α- hydroxygliclazide (Gz1c)	6.1	340.133	340.132	2.35	$C_{15}H_{22}N_3O_4S$
7-α- hydroxygliclazide (Gz1d)	6.4	340.133	340.132	2.35	$C_{15}H_{22}N_3O_4S$
Methylhydroxygliclazide(Gz1e)	6.8	340.133	340.132	2.35	$C_{15}H_{22}N_3O_4S$

Fig.1

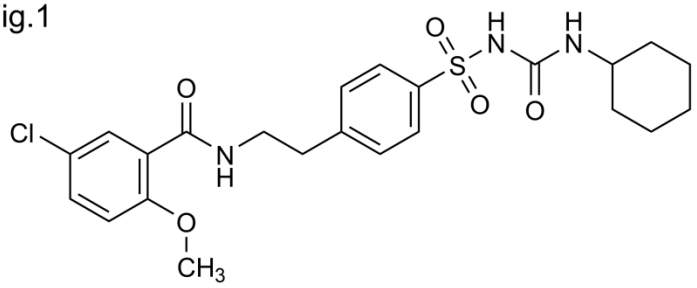

Fig.1(A) Glyburide, M.W. 494.004 g/mol

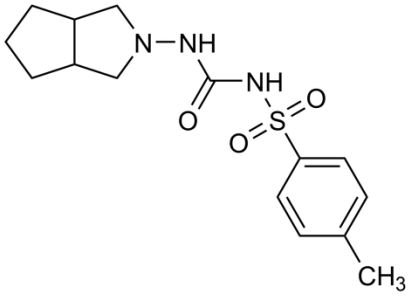

Fig. 1(B) Gliclazide,M.W. 323.4 g/mol

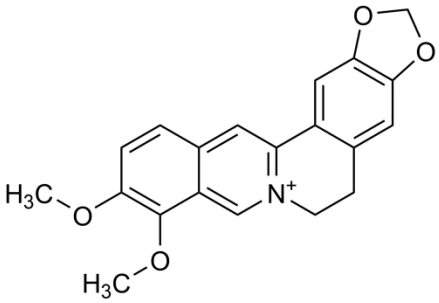

Fig. 1(C) Berberine,M.W. 336.3612 g/mol

Fig.1 Chemical structures of berberine and sulfonylureas.

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Fig.2

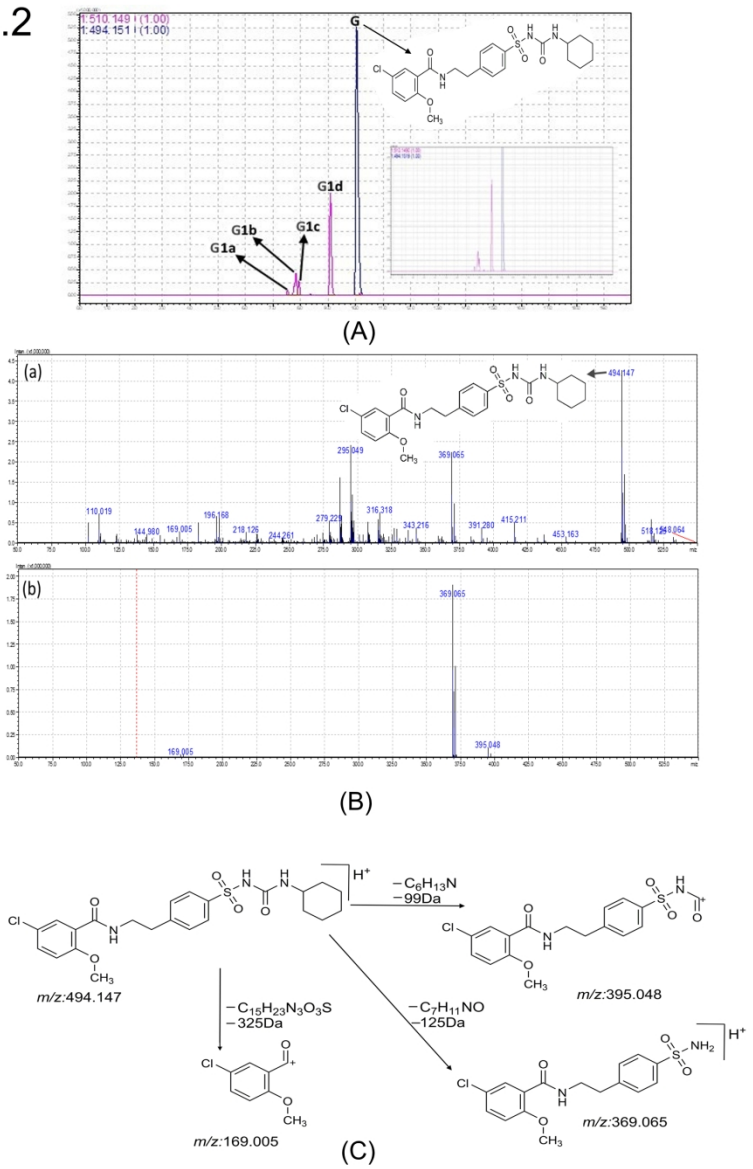

Fig. 2 Metabolic profile of glyburide. Extracted ion chromatogram (EIC) of glyburide (20 μM) and its metabolites following incubation with rat male liver microsomes (G= glyburide, G1a,G1b, G1c, G1d= metabolites) (A); MS and MS/MS spectra of glyburide (m/z 494.151) acquired using positive ion mode (B); structure of product ions (C).

571x807mm (96 x 96 DPI)

Fig.3

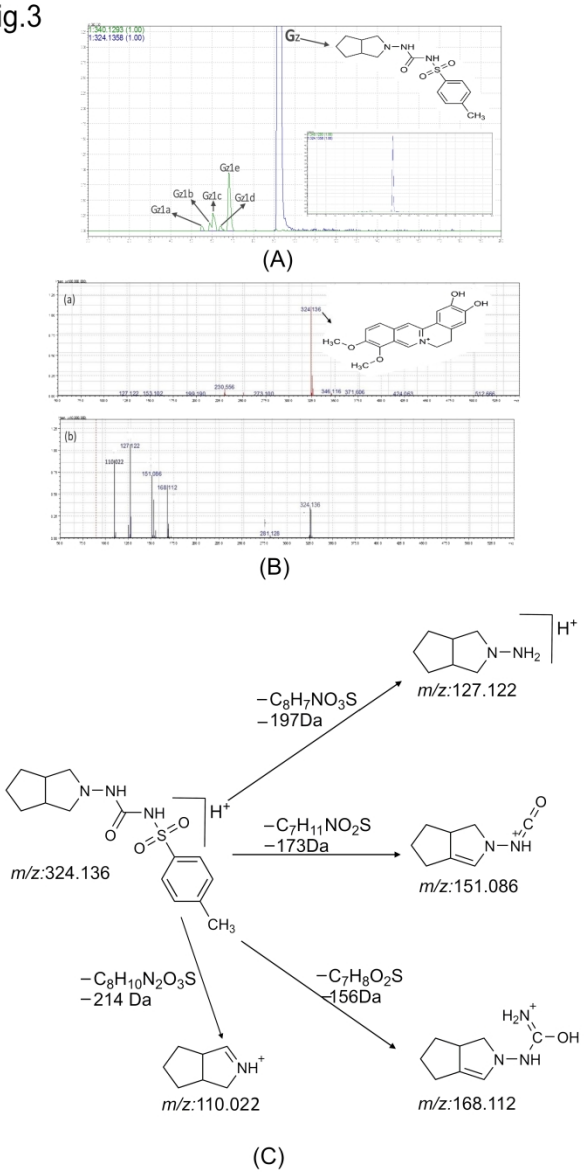

Fig. 3 Metabolic profile of gliclazide. Extracted ion Chromatogram of (EIC) gliclazide (20 μM) and its metabolites following incubation with rat male liver microsomes (Gz= gliclazide, Gz1a, Gz1b, Gz1c, Gz1d, Gz1e = metabolites) (A); MS and MS/MS spectra of gliclazide (m/z 324.135) acquired using positive ion mode (B); structure of product ions (C).

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Fig.4

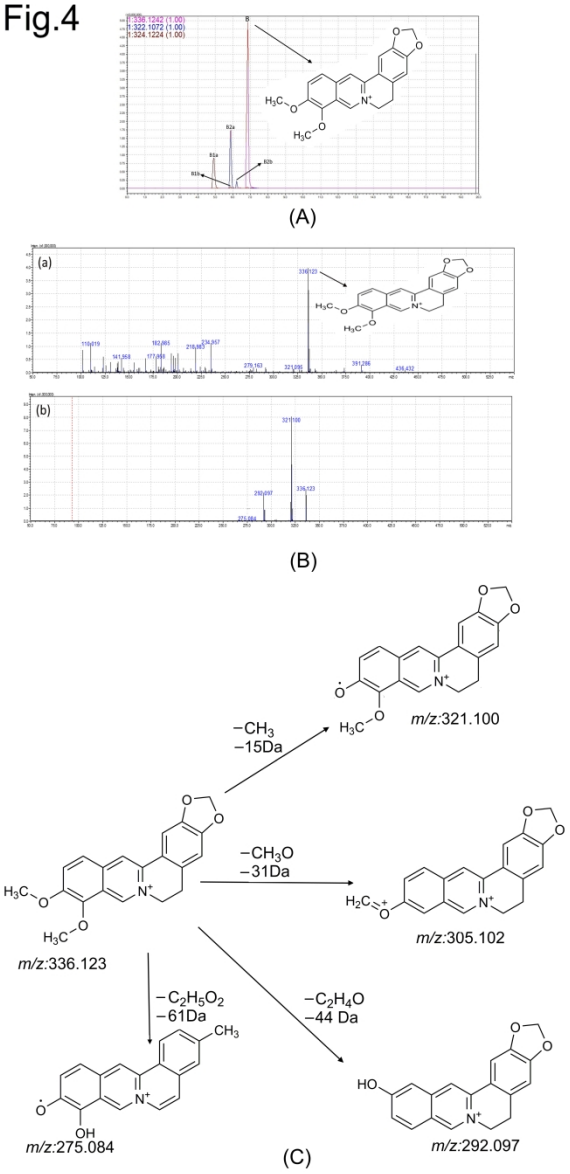

Fig. 4 Metabolic profile of berberine. Extracted ion chromatogram (EIC) of berberine (20 μM) and its metabolites following incubation with rat male liver microsomes (B= berberine, B1a, B1b, B2a, B2b = metabolites). (A); MS and MS/MS spectra of berberine (m/z 336.124) acquired using positive ion mode (B); structure of product ions (C).

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Fig. 5

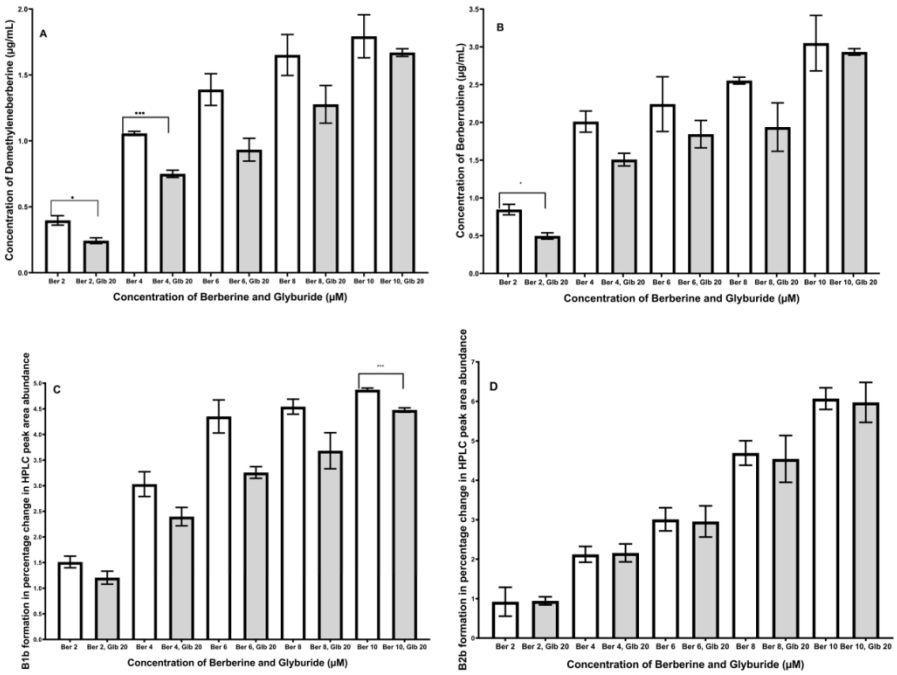

Fig. 5 Plots of berberine metabolites as a function of concentration of berberine following co-incubation of berberine (Ber) (2- 10 µM) with glyburide (Glb) 20 µM. The control represents the normal metabolism of berberine in the absence of glyburide at a fixed concentration. Each data point in the plots represents the mean value of three replicates; (A) Demethyleneberberine (B1a); (B) Berberrubine (B2a); (C) B1b and (D) B2b represent relative percentage change.

373x300mm (96 x 96 DPI)

Fig. 6

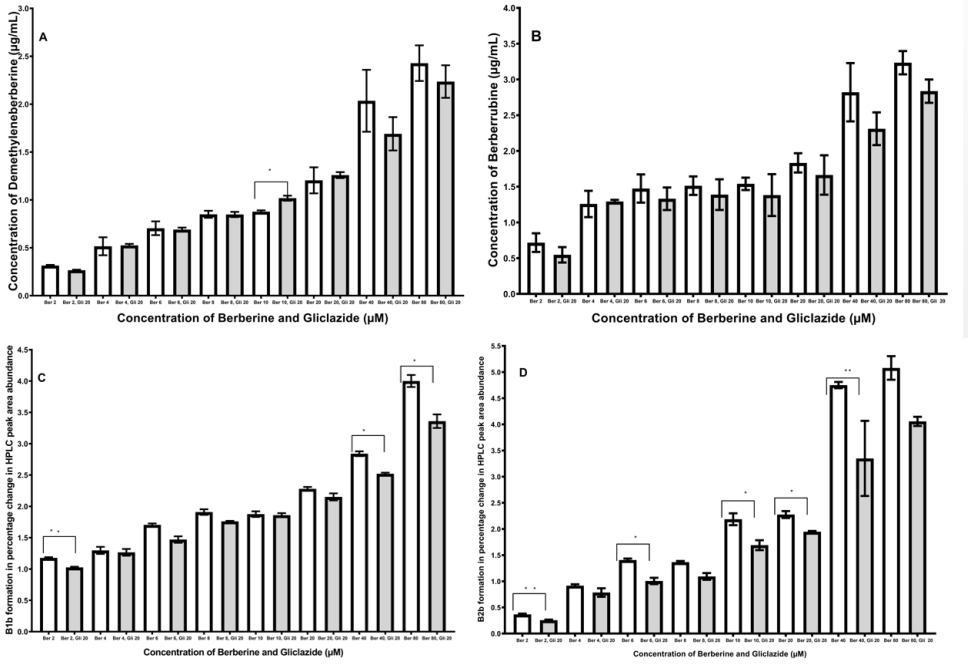

Fig. 6 Plots of berberine metabolites as a function of concentration of berberine following co-incubation of berberine (Ber) (2 to 80 µM) with gliclazide (Gli) at a fixed concentration of 20 µM. The control represents the normal metabolism of berberine in the absence of gliclazide at the same concentration range. Each data point in the bar chart represents the mean value of three replicates; (A) Demethyleneberberine (B1a); (B) Berberrubine (B2a); (C) B1b and (D) B2b represent relative percentage change.

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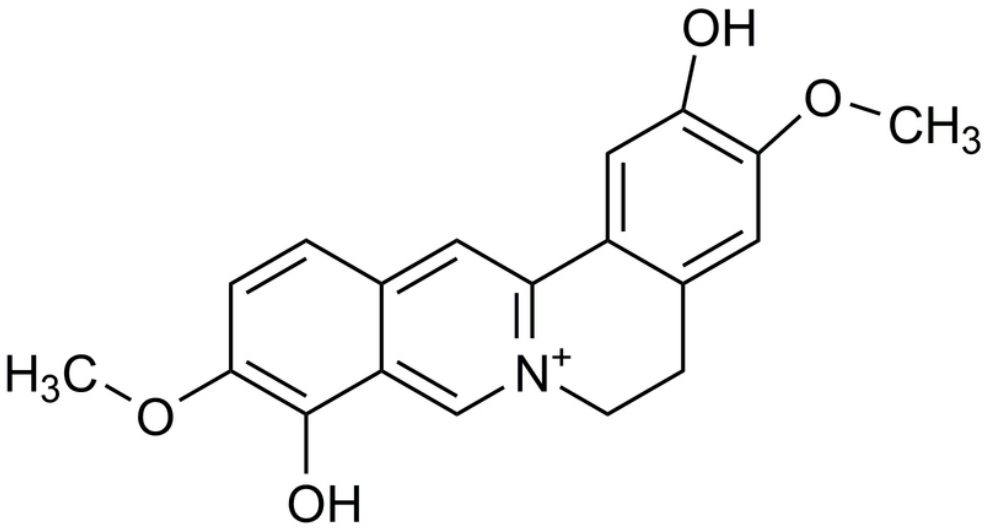

Fig. 7 Proposed structure of B1b.

68x37mm (300 x 300 DPI)