

Introduction

Diabetes is a major health concern for Canadian aboriginal populations, such as the Cree of Eeyou Istchee (CEI)¹ who inhabit the James Bay area in northern Quebec (Figure 1A). Western antidiabetic drugs are used to treat this disease. However, the Cree also possess knowledge of traditional healing methods and often use medicinal plants together with the conventional pharmaceuticals. The Canadian Institutes for Health Research Team in Aboriginal Antidiabetic Medicines (CIHR-TAAM), has demonstrated that many of these Cree medicinal plants have effectiveness in diabetes assays². One prominent Cree medicinal plant is the tamarack larch tree (*Larix laricina*), known in Cree as Watnagan (Figure 1B). It has been reported as a traditional medicine across Canada to treat many conditions, including jaundice, asthma, tuberculosis³, and is ranked as one of the most important medicines for the treatment of diabetic symptoms⁴. One component of the tree's bark of particular interest is the compound rhaponticin, a glycoside stilbene compound (Figure 3). It is the parent compound of its aglycone, rhapontigenin, which is believed to be the active form of the molecule⁵.

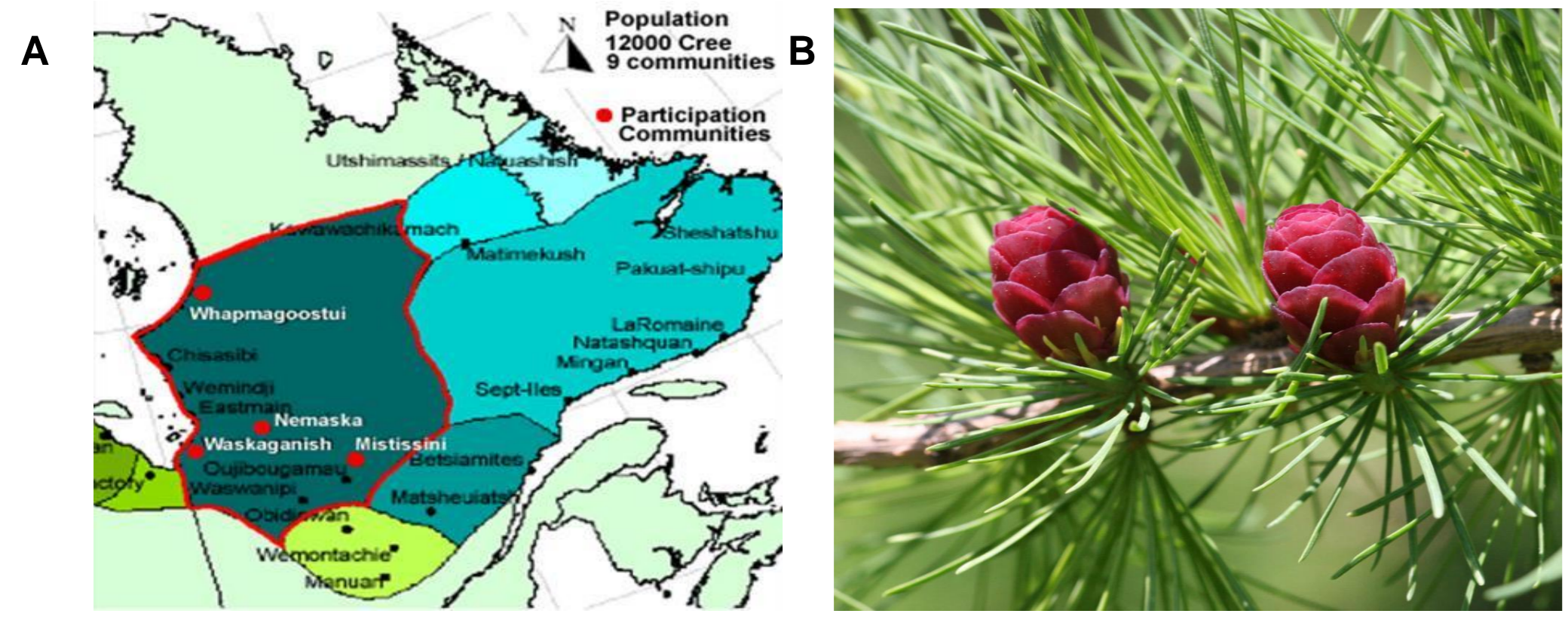


Figure 1. (A) Territory occupied by the CEI (outlined in red). (B) Small red cones of the tamarack larch (*Larix laricina*).

Gliclazide and repaglinide (Figure 2) are drugs used in the treatment of diabetes mellitus type II. Gliclazide is metabolized primarily by CYP 2C9 and CYP 2C19 into seven known metabolites^{6,7}. Repaglinide is metabolized primarily by CYP 3A4 and CYP 2C8⁸. Thus, if CYP activity was affected it could result in safety issues related to drug concentration.

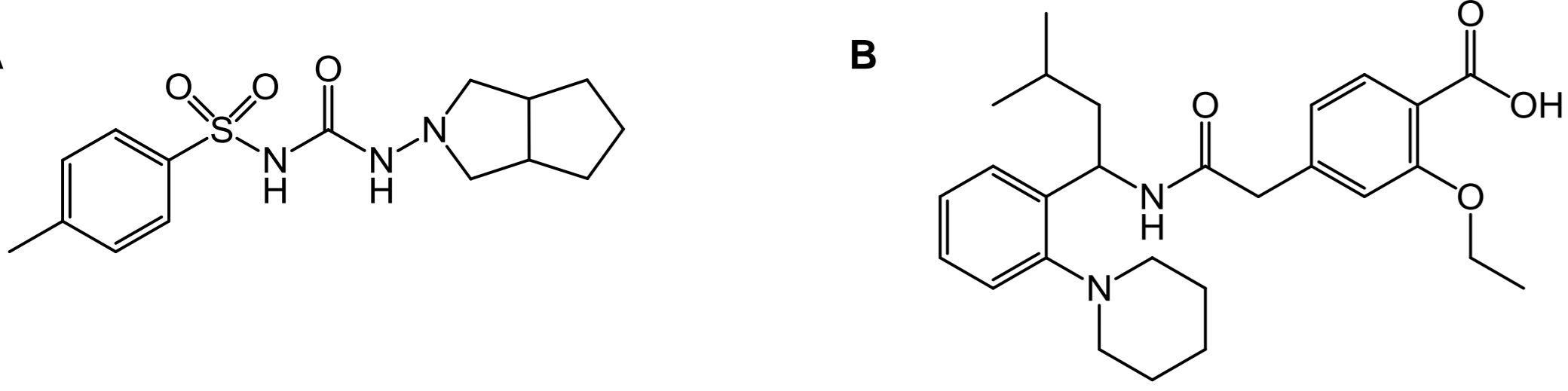


Figure 2. Structures of (A) gliclazide and (B) repaglinide.

Rationale and Objectives

In order to assess the effect of *Larix* phytochemicals on diabetic drug metabolism, the objectives were:

1. Investigate the inhibition of rhaponticin and its active derivative, rhapontigenin, on CYP isozyme activity using a standardized assay.
2. Characterize gliclazide metabolism using HPLC and MS.
3. Evaluate the effects rhaponticin has on gliclazide and repaglinide metabolism

Materials and Methods

Sample Preparation
Rhapontigenin was produced from rhaponticin by biotransformation using β -glucosidase (Figure 3). The conversion to the aglycone was confirmed by HPLC and ¹H NMR. Gliclazide, rhaponticin and rhapontigenin were made into stock solutions in 100% methanol at concentrations of 1 mg/mL.

CYP inhibition assay
In 96-well plates the test compound (10 μ L) was incubated with CYP enzyme (10 nM), Tris or PBS buffer (0.19 μ M), NADPH (0.6 mM), and a corresponding fluorometric substrate (1 μ M) at a final volume of 200 μ L for 20 or 60 min. The initial and final fluorescence was measured using a Cytofluor 4000 Fluorescence Measurement System.

Gliclazide metabolism assay
A reaction mixture was prepared containing either CYP2C9, CYP2C19 or HLM (30 μ L), NADPH (2.42 mM), MgCl₂ (6 mM) and Tris buffer (70 μ M, pH 7.4) at a final volume of 500 μ L. Gliclazide was added at a concentration of 10 μ g/mL. The reaction was incubated at 37°C for 90 min. The mixture was analyzed for metabolites using a HPLC-DAD method (Agilent 1100 Series). Metabolite structures were characterized by a HPLC-ESI-MS/MS system (Agilent 1200 Series, 3200QTRAP).

Effects of Cree compounds on metabolism
Rhaponticin and rhapontigenin (0.01 or 0.02 mg/mL) were co-incubated with a repaglinide HLM metabolism or gliclazide CYP 2C19 metabolism reaction mixture at 37°C for 90 min. A HPLC-DAD method (Agilent 1100 Series) was used to analyze the metabolites. The areas under the peaks were determined and expressed as a ratio relative to a control.

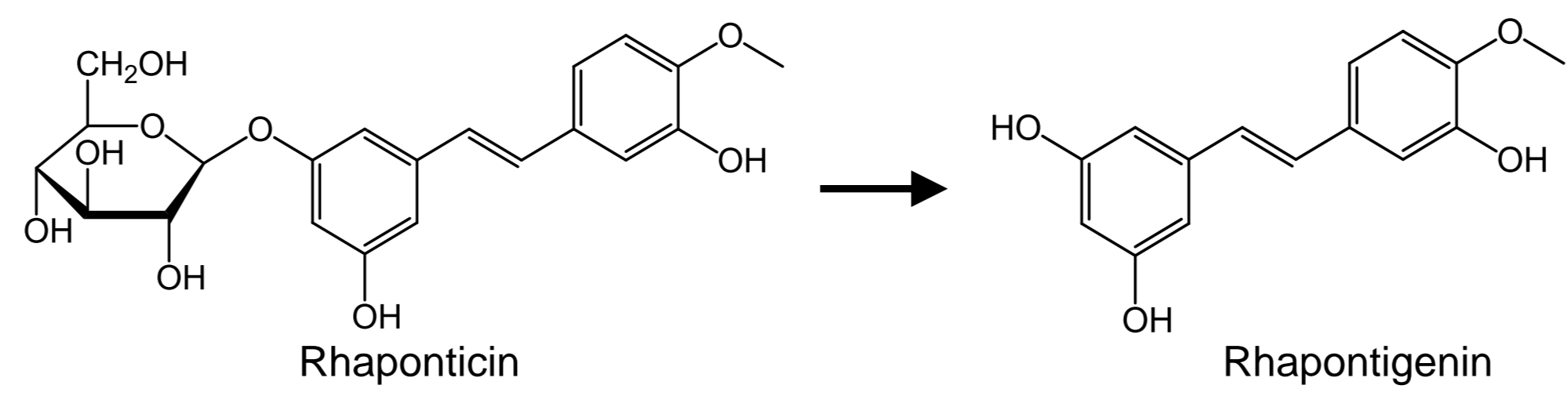


Figure 3. Biotransformation of rhaponticin into rhapontigenin

Results

Gliclazide and rhaponticin showed no significant inhibition of any of the CYP isozymes (Figure 4A). Meanwhile, it was determined that rhapontigenin had IC₅₀ values of 2.7 μ M (0.7 μ g/ml), 7 μ M (1.8 μ g/ml) and 30 μ M (7.7 μ g/ml) for CYP 2C9, 2C19 and 3A4, respectively (Figure 4B). Gliclazide metabolism by 2C19 resulted in the appearance of 5 major metabolites (Figure 6 & 7). While rhaponticin and its aglycone did not have any substantive effects on repaglinide metabolism by HLM (Figure 5) the aglycone demonstrated significant inhibition of gliclazide metabolism by CYP 2C19 (Table 1).

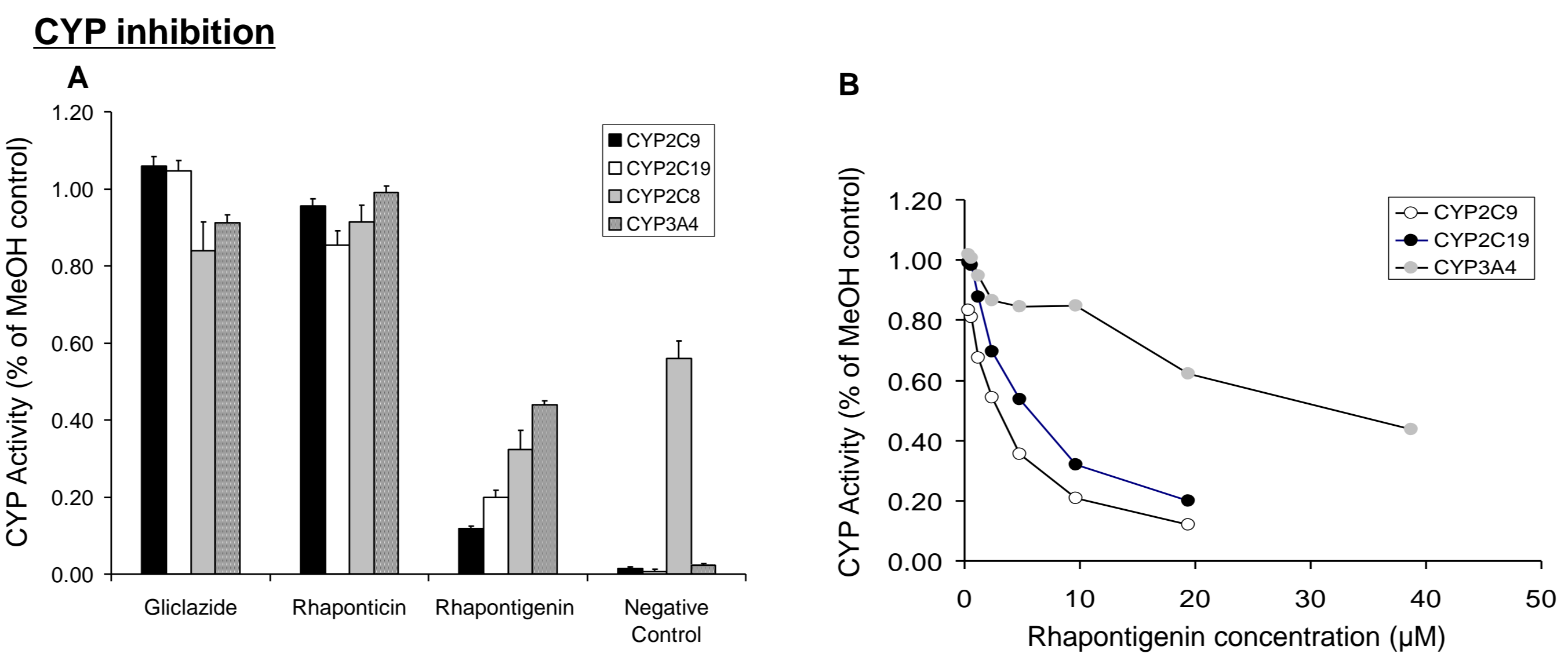


Figure 4. Inhibition effects of various compounds on CYP enzymes using microtitre fluorometric assays. (A) The % CYP enzyme activity (+SEM) was calculated for gliclazide, rhaponticin and rhapontigenin relative to the methanol vehicle control for CYP2C9, 2C19, 3A4 (n=6) and 2C8 (n=3). (B) Inhibition of CYP2C9, 2C19 and 3A4 by varying concentrations of rhapontigenin.

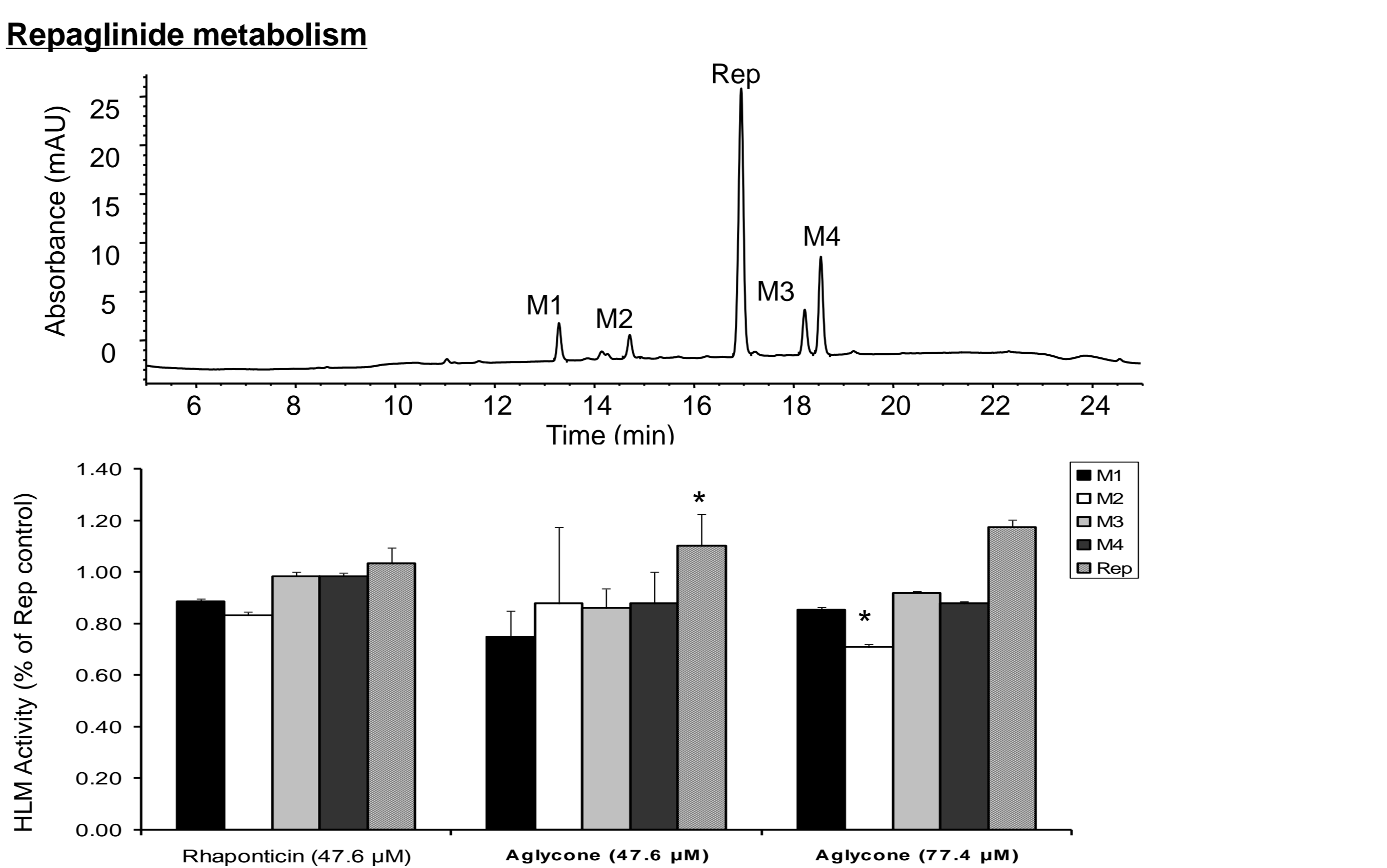


Figure 5. Effects of rhaponticin and rhapontigenin on repaglinide metabolism. (A) HPLC trace of repaglinide and metabolites, M1-M4, after metabolism by HLM. (B) The % activities (+SEM, n=3) expressed as ratios relative to a repaglinide control of the area underneath the elution peaks. An asterisk (*) denotes a significant change in activity compared to the repaglinide control; p<0.05.

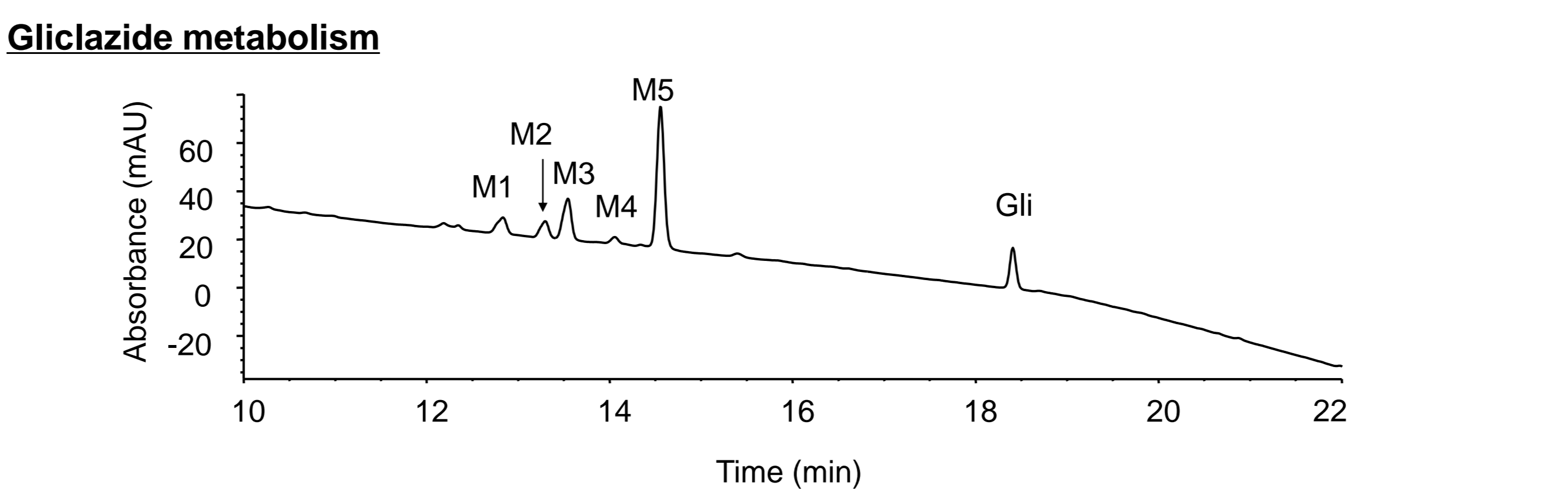


Figure 6. HPLC trace of gliclazide (Gli) and metabolites (M1-M5) by CYP 2C19 metabolism.

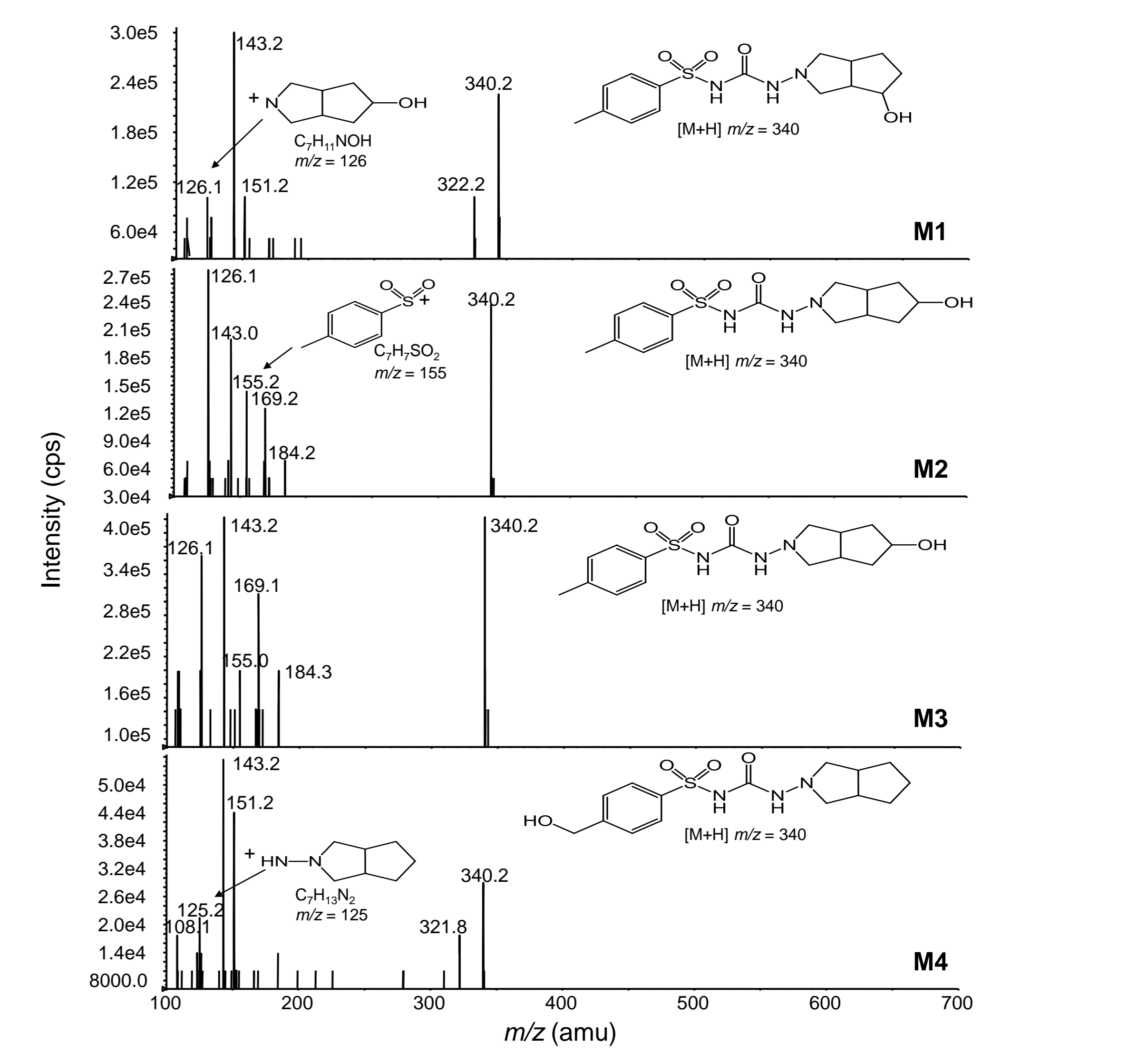


Figure 7. The mass spectral analysis of gliclazide metabolites. Enhanced product ions of metabolites M1-M4.

Effects of rhaponticin and rhapontigenin on gliclazide metabolism

Table 1. Effects of rhaponticin and rhapontigenin on gliclazide metabolism. The % activities (\pm SEM, n=3) expressed as ratios relative to a gliclazide control of the area underneath the elution peaks

Compound	% of CYP2C19 activity (\pm SEM)
Rhaponticin	1.21 (0.13)
Rhapontigenin	1.9 (0.25)

Discussion and Conclusions

- ◆ Rhapontigenin is the active metabolite of rhaponticin; it inhibits CYP 2C9 and 2C19 activity and, to a lesser degree, CYP 3A4 and 2C8.
- ◆ Rhapontigenin inhibition of CYP enzymes suggests a risk of potential drug interactions during the hydrolysis process of metabolism.
- ◆ Rhaponticin and its aglycone do not show any substantive inhibition effects on repaglinide metabolism.
- ◆ Rhaponticin and rhapontigenin do appear to interfere with gliclazide metabolism by CYP 2C19.
- ◆ CYP isozyme inhibition by rhapontigenin might be an issue for the CEI who take tamarack larch remedies together with, not only gliclazide, but any drug metabolized by the CYP 2C family.

Future Studies

Clinical tests may be necessary to elucidate the *in vivo* effects rhaponticin has on the pharmacokinetics and pharmacodynamics of gliclazide.

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