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Short Communication

Title Page

Comprehensive evaluation for substrate selectivity of cynomolgus monkey cytochrome P450 2C9, a new efavirenz oxidase

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Abbreviations: CLint, intrinsic clearance ; LC, liquid chromatography; MS/MS, tandem mass spectrometry; P450, cytochrome P450 (EC 1.14.14.1).

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Abstract

Cynomolgus monkeys are widely used as primate models in preclinical studies, because of their evolutionary closeness to humans. In humans, the cytochrome P450 (P450 or CYP) 2C enzymes are important drug-metabolizing enzymes and highly expressed in livers. The CYP2C enzymes including CYP2C9 are also expressed abundantly in cynomolgus monkey liver and metabolize some endogenous and exogenous substances like testosterone, *S*-mephenytoin, and diclofenac. However, comprehensive evaluation regarding substrate specificity of monkey CYP2C9 has not been conducted. In the present study, 89 commercially available drugs were examined to find potential monkey CYP2C9 substrates. Among the compounds screened, 20 drugs were metabolized by monkey CYP2C9 with a relatively high rate. Seventeen of these compounds were substrates or inhibitors of human CYP2C9 or CYP2C19, while 3 drugs were not, indicating that substrate specificity of monkey CYP2C9 resembled those of human CYP2C9 or CYP2C19, with some differences in substrate specificities. Although efavirenz is known as a marker substrate for human CYP2B6, efavirenz was not oxidized by CYP2B6 but by CYP2C9 in monkeys. Liquid chromatography-mass spectrometry analysis revealed that monkey CYP2C9 and human CYP2B6 formed the same mono- and di-oxidized metabolites of efavirenz at 8 and 14 positions. These results suggest that the efavirenz 8-oxidation could be one of the selective markers for cynomolgus monkey CYP2C9 among the major three CYP2C enzymes tested. Therefore, monkey CYP2C9 has the possibility to contribute to limited specific differences in drug oxidative metabolism between cynomolgus monkeys and humans.

Introduction

Cynomolgus monkeys are used in the studies of drug metabolism and toxicity due to their evolutionary closeness to humans as compared with other animal species, however, the differences between monkeys and humans in drug metabolisms are occasionally seen. The cytochrome P450 (P450 or CYP) superfamily consists of a large number of drug-metabolizing enzyme genes. In humans, CYP2C enzymes comprise about 20% of P450 enzymes in the liver and are essential in metabolizing approximately 20% of all prescribed drugs (Shimada et al., 1994; Goldstein, 2001). The CYP2C enzymes, which account for more than 14% of total P450 enzymes, are expressed abundantly in cynomolgus monkey liver, (Uehara et al., 2011). Cynomolgus monkey CYP2C9 exhibits a high amino acid sequence identity to human CYP2C9 (93%) and CYP2C19 (91%) (Uno et al., 2011). Cynomolgus monkey CYP2C9 shows high activity for testosterone and *S*-mephenytoin and low activity for diclofenac, but no activity for paclitaxel or tolbutamide (Mitsuda et al., 2006; Uno et al., 2006). These findings partly showed substrate specificity of cynomolgus monkey CYP2C9, however, a broad evaluation of potential substrates for CYP2C9 has not yet been conducted.

In the present study, we focused on the function of cynomolgus monkey CYP2C9. We report herein that among 89 commercially available drugs examined for potential cynomolgus monkey CYP2C9 substrates, the efavirenz 8-oxidation could be one of the selective markers for cynomolgus monkey CYP2C9 among major CYP2C enzymes including CYP2C9 (formerly 2C43), CYP2C19 (2C75), and CYP2C76 (Uno et al., 2011). Cynomolgus monkey CYP2C9 generally has similar substrate recognition functionality as human CYP2C enzymes, but possibly contributes to limited specific differences in drug oxidative metabolism between cynomolgus monkeys and humans.

Materials and Methods

Efavirenz and 8-hydroxyefavirenz were purchased from Sigma-Aldrich (St. Louis, MO) and Toronto Research Chemicals (Toronto, ON), respectively. The other drugs in Supplemental Table S1 were obtained from as described previously (Hosaka et al., 2015). Cynomolgus monkey P450 recombinant enzymes were expressed in *Escherichia coli* membranes with human NADPH-P450 reductase (Iwata et al., 1998; Daigo et al., 2002). Hereafter, the terms “monkey” or “monkeys” refer to cynomolgus monkeys. Human CYP2B6 was purchased from Corning (Tewksbury, MA). Monkey and human liver microsomes were purchased from BioreclamationIVT (Baltimore, MD). Other reagents used in this study were of the highest quality commercially available.

The substrates were dissolved in final concentrations of 0.5-50 μM in 0.01-0.1% DMSO or 1% methanol. Incubation mixtures contained substrate, 10 or 25 pmol/ml recombinant monkey or human P450s or 0.1 or 0.5 mg/ml monkey or human liver microsomes, 0.25 mM β-NADP$^+$, 2.5 mM glucose 6-phosphate, 0.025 U glucose-6-phosphate dehydrogenase, and 30 mM magnesium chloride in a final volume of 100 μL of 50 mM potassium phosphate buffer, pH 7.4. The mixture was incubated at 37°C for 0-60 min, and then pretreated for LC-MS/MS analysis. Sample preparation and LC-MS/MS analysis were conducted as described previously (Hosaka et al., 2015). All incubations were performed in duplicate.

Residual (%) at 30 minutes after incubation of each substrate were calculated and converted to substrate disappearance (%) as described previously (Hosaka et al., 2015). The kinetic analysis of 8-hydroxyefavirenz formation was done using a nonlinear regression analysis program (KaleidaGraph, Synergy Software, Reading, PA). When substrate inhibition was observed, an equation of $v = V_{\max} \bullet [S]/(K_m + [S] + [S]^2/K_s)$ was used; $[S]$ and K_s were defined as substrate concentration and substrate inhibition constant, respectively.

Results and Discussion

A total of 89 drugs (Supplemental Table S1) were screened for investigating their potential to undergo metabolism by monkey CYP2C9 and other P450s (CYP2C19 and CYP2C76). The substrate depletion assay showed that 20 compounds were metabolized by CYP2C9 at a relatively rapid rate (substrate disappearance > 20%) (Fig. 1A). Most of these compounds were also eliminated by CYP2C19 and/or CYP2C76; however, efavirenz exhibited higher selectivity to CYP2C9 among monkey P450 2C enzymes (Fig. 1B). Because efavirenz has been reported as a substrate for CYP2B6 in humans (Ward et al., 2003; Bumpus et al., 2006), metabolic activities of monkey CYP2B6 and human CYP2B6 toward efavirenz were also evaluated. Efavirenz was slightly metabolized by monkey CYP2B6 and CYP2C76, whose intrinsic clearance (CL_{int}) values (0.15 and 0.14 ml/min per nmol P450) were approximately 10-fold lower than that of monkey CYP2C9 (1.54 ml/min per nmol P450). Monkey CYP2C19 and CYP3A4 showed little activity to efavirenz (CL_{int} value <0.10 ml/min per nmol P450). In human CYP2B6, CL_{int} value was 1.86 ml/min per nmol P450, which was comparable to monkey CYP2C9.

Because efavirenz showed relatively high selectivity to monkey CYP2C9, further investigations on the metabolites of efavirenz were conducted by LC-MS/MS. Fig. 2 shows the extracted ion chromatograms of the efavirenz metabolites generated by monkey CYP2C9 and human CYP2B6. The $[M-H]^-$ ions were detected at *m/z* 330.015 and *m/z* 346.010, which were considered to be mono-oxidized and di-oxidized metabolites of efavirenz (*m/z* 314.020), respectively. Considering their retention times (t_R), it was considered likely that monkey CYP2C9 and human CYP2B6 formed the same metabolite set of efavirenz. Mass spectral pattern of efavirenz and its metabolites were compared. Supplemental Fig. S1 shows that the fragment ions of metabolites at *m/z* 330.015 (peak b in Figs. 2A and 2B) and *m/z* 346.010 (peak c in Figs. 2A and 2B) formed by monkey CYP2C9 and human CYP2B6 were identical, indicating that these P450s generated the same metabolites of efavirenz. The origin of main fragment ions is postulated in Supplemental Fig. S1. Considering that human CYP2B6 has been reported to form 8-hydroxyefavirenz and 8,14-dihydroxyefavirenz (Ward et al., 2003; Bumpus et al., 2006), monkey CYP2C9 also formed the same mono- and di-oxidized metabolites. We conducted further experiments using liver microsomes from monkeys and humans and obtained the same results (data not shown).

The kinetic analysis of efavirenz 8-oxidation revealed that monkey liver microsomes and recombinant monkey CYP2C9 showed similar apparent K_m values, 2.5 μM and 9.9 μM, respectively. Monkey liver microsomes showed Michaelis-Menten kinetics, while recombinant monkey CYP2C9 showed substrate inhibition kinetics, with 3.3 μM of apparent K_s. The reason for this similar affinity but no substrate inhibition by efavirenz in monkey liver microsomes was not clear at present, but these phenomena might be presumably resulted in apparent little or no effects of efavirenz on CYP2C9 in the presence of multiple CYP2C forms and/or a diversity of drug metabolizing enzymes and proteins in monkey liver microsomes through any substrate competitions.

In this study, therefore, 89 marketed compounds, including human CYP2C and non-CYP2C substrates or inhibitors (Rendic, 2002), also found in the Food and Drug Administration Drug-Drug Interaction Draft Guidance, 2006, (http://www.fda.gov/cder/guidance/6695dft.htm) and European Medicines Agency (EMA) guidelines (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf), were screened as potential substrates for monkey CYP2C9.

In the previous study, monkey CYP2C9 showed high oxidation activities for testosterone and *S*-mephenytoin and low activity for diclofenac, but no activity for paclitaxel or tolbutamide (Mitsuda et al., 2006; Uno et al., 2006). In this study, testosterone was identified as a substrate for monkey CYP2C9, whose substrate disappearance was 84.5%. The substrate disappearance of diclofenac (13.2%) was lower than 20%, but was higher than those of paclitaxel (<5%) and tolbutamide (5.2%), which is consistent with the previous reports. *S*-mephenytoin, which is difficult to measure by LC-MS/MS due to poor ionization efficiency, was not evaluated in this study.

According to the review of human P450 metabolism data summarized by Rendic (2002) and other reports (Scott et al., 2013; Obach et al., 2005; Nishiya et al., 2009; Xu and Desta, 2013; Transon et al., 1995; Wen et al., 2001; Rastogi and Jana, 2014; Yamazaki et al., 2000), among the 20 compounds found as monkey CYP2C9 substrates in this study (Fig. 1A), 6 (amitriptyline, diazepam, fluvastatin, sertraline, testosterone, and troglitazone) and 7 (amitriptyline, clomipramine, clopidogrel, diazepam, sertraline, testosterone, and troglitazone) are known as human CYP2C9 and CYP2C19 substrates, respectively. Because monkey CYP2C9 has a high amino acid sequence identity to both human CYP2C9 (93%) and CYP2C19 (91%) (Uno et al., 2011), some human CYP2C19 substrates might possibly be metabolized by monkey CYP2C9. Fifteen (amitriptyline, clopidogrel, diazepam, efavirenz, fluvastatin, gemfibrozil, α-naphthoflavone, nicardipine, nifedipine, nootkatone, pioglitazone, quercetin, sertraline, ticlopidine, and troglitazone) have been reported as the inhibitor of human CYP2C9 and/or CYP2C19, while 9 have been reported as substrate or competitive inhibitors of human CYP2C9 and/or CYP2C19. Although the amino acid sequence of monkey CYP2C9 is highly identical to human CYP2C9 and CYP2C19, a small difference in primary sequence and tertiary structure could result in a slight difference in the substrate recognition property of each P450 enzyme. To our knowledge, 3 compounds identified (7-ethoxyresorufin, pitavastatin lactone, and troleandomycin) have not been reported as substrates or inhibitors of human CYP2C9 or CYP2C19, indicating that monkey CYP2C9 might show different substrate specificity from human P450s in some cases.

Among the evaluated compounds, efavirenz showed high selectivity to monkey CYP2C9 (Fig. 1B). In human, efavirenz is metabolized by CYP2B6 and formed 8-hydroxyefavirenz and 8,14-dihydroxyefavirenz whose molecular weights are 16 and 32 Da larger than the parent compound, respectively (Ward et al., 2003; Bumpus et al., 2006). In contrast to monkey CYP2B6, monkey CYP2C9 formed the same metabolites as human CYP2B6 did (Figs. 2 and Supplemental S1). By the kinetic analysis of efavirenz 8-oxidation, both monkey liver microsome and monkey CYP2C9 showed single μM apparent K_m values (Table. 1). According to the report by Mayumi et al. (2013), CL_{int} values for efavirenz 8-oxidation in cynomolgus monkey and human liver microsomes were 3-4 times higher in the former. On the contrary, CL_{int} values in the recombinant CYP2B6s were about 10 times lower in cynomolgus monkey than human. These findings suggest that CYP2C9 should predominantly metabolize efavirenz in monkeys, playing the role of human CYP2B6 in efavirenz metabolism. Moreover, efavirenz 8-oxidation by recombinant monkey CYP2C9 showed substrate inhibition kinetics (Table 1). Therefore, efavirenz 8-oxidation could be a selective marker reaction of monkey CYP2C9, although more detailed experiment with other monkey P450 isoforms is needed in the future. These differences of substrate specificity in P450 isoforms in drug metabolism might result in species differences in pharmacokinetic profiles and toxicities.

In conclusion, 20 structurally diverse substrates for monkey CYP2C9 were identified among the 89 substrates evaluated. Seventeen of these compounds were substrates or inhibitors of human CYP2C9 or CYP2C19, while 3 drugs were not. These results indicated that monkey CYP2C9 has substrate specificity similar to human CYP2C9 or CYP2C19, but in some cases may show different characteristics. Among the newly identified substrates, efavirenz showed high selectivity to monkey CYP2C9. Efavirenz was mainly metabolized by CYP2B6 in human; however, monkey CYP2B6 showed only marginal activity toward efavirenz. Monkey CYP2C9 and human CYP2B6 generated the same mono- and di-oxidized metabolites of efavirenz at 8 and 14 positions. In addition, this metabolic reaction of efavirenz would possibly be a selective marker reaction of monkey CYP2C9 among major CYP2C enzymes including CYP2C9, CYP2C19, and CYP2C76 (Uno et al., 2011) under the present conditions. Considering these differences in substrate specificity, monkey CYP2C9 may contribute to limited species differences in drug metabolism between monkeys and humans. Accumulation of such information in monkeys will read to profound comprehension for compering drug metabolism in monkeys and humans. Our findings on substrate specificity of monkey CYP2C9 should help to gain a better understanding of drug metabolism in monkeys and a better interpretation of preclinical study data.

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Authorship contribution

Participated in research design: Uno, and Yamazaki.

Conducted experiments: Hosaka, Murayama, and Uehara.

Contributed new reagents or analytic tools: Satsukawa,

Performed data analysis: Hosaka, Shimizu, Iwasaki, Iwano, and Uno.

Wrote or contributed to the writing of the manuscript: Hosaka, Iwano, Uno, and Yamazaki.

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Legends for figures

Fig. 1. Substrate disappearance of compounds metabolized by recombinant monkey P450s.

Each substrate (1 µM) was incubated with recombinant monkey CYP2C9, CYP2C19, or CYP2C76 for 30 min. The substrates whose disappearance exceeded 20% are shown. Substrate disappearance (A) and the ratio relative to monkey CYP2C9 (B) are shown.

Fig. 2. Chromatographic profile of efavirenz after incubation with recombinant monkey CYP2C9 (A) and human CYP2B6 (B).

Efavirenz (1 µM) was incubated with each recombinant monkey P450 for 60 min, and the samples were analyzed by negative full scan mode. The mass chromatograms were obtained after background subtraction with control samples (reaction mixture not containing efavirenz). Peak a, efavirenz; peak b, mono-oxidized metabolite; and peak c, di-oxidized metabolite.

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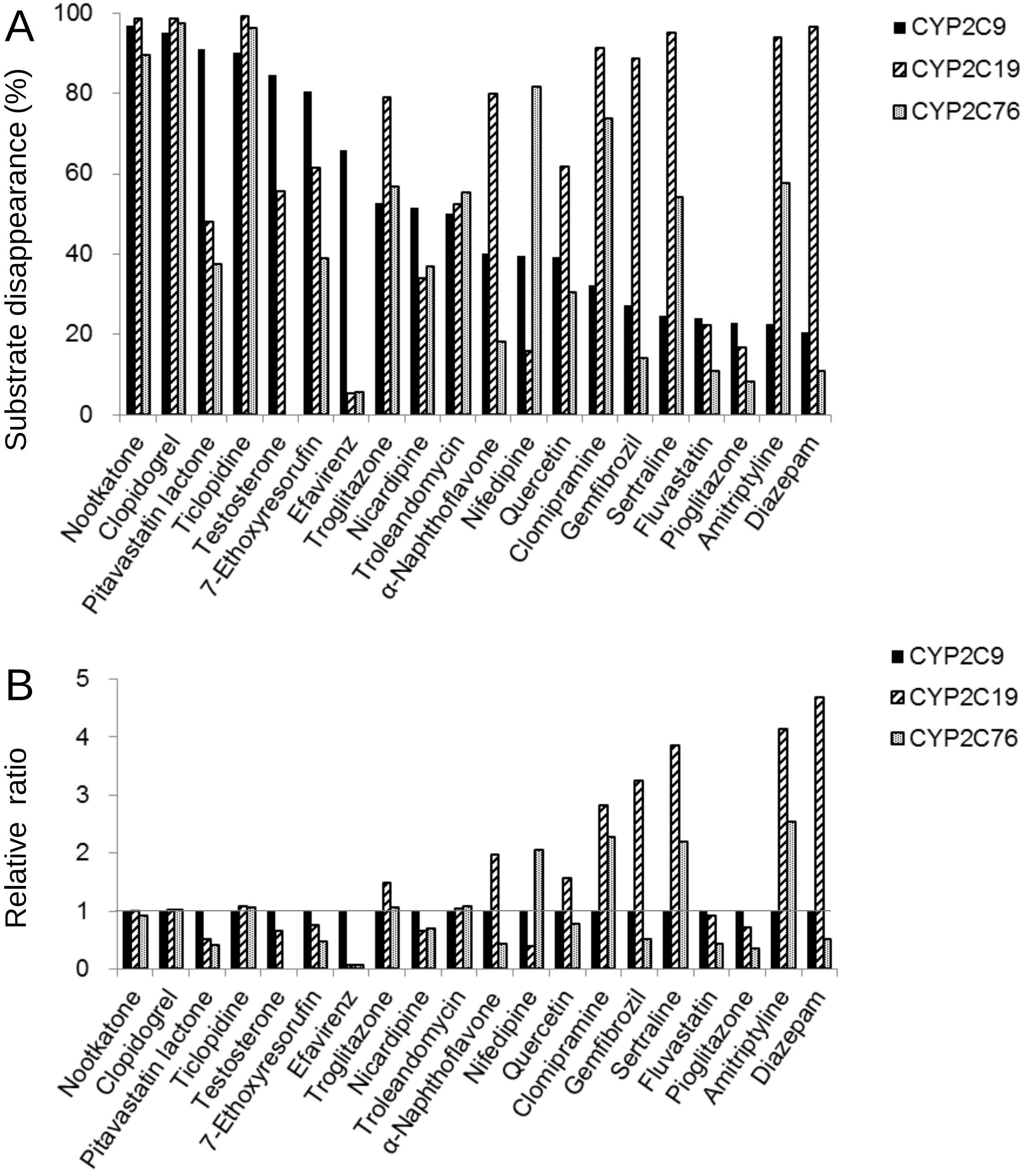
Table 1. Kinetic parameters for efavirenz 8-oxidation

Enzyme	Efavirenz 8-oxidation			
	K_m	V_{max}	V_{max}/K_m	K_s
Monkey liver microsomes	2.5 ± 0.3 [a]	0.23 ± 0.01 [b]	0.09	-
Monkey CYP2C9	9.9 ± 2.2 [a]	5.4 ± 1.0 [b]	0.54	3.3 ± 0.7 [a]
Monkey CYP2C19	-	<0.05	-	-
Monkey CYP2C76	-	<0.05	-	-
Monkey CYP2B6	-	<0.05	-	-

[a] μM, [b] nmol/min/nmol P450. Efavirenz (0.5, 2, 5, 20, and 50 μM) was incubated with monkey liver microsomes (0.1 mg/mL) or recombinant monkey CYP2C or CYP2B6 enzymes (10 pmol/mL) at 37°C for 10 min in the presence of an NADPH-generating system. 8-Hydroxyefavirenz formation was quantified by LC-MS/MS using mefenamic acid as internal standard and showed linearity between 0.1-0.5 mg protein/mL in monkey liver microsomes, 10-50 pmol P450 in monkey CYP2C9, and reaction time range of 10-20 min. Kinetic analysis was done using nonlinear regression analysis employing the Michaelis-Menten equation, $v = V_{max} \cdot [S]/(K_m + [S])$, or $v = V_{max} \cdot [S]/(K_m + [S] + [S]^2/K_s)$ for substrate inhibition.

Fig. 1


A
Substrate disappearance (%)
0
20
40
60
80
100
■ CYP2C9
▨ CYP2C19
▦ CYP2C76
Nootkatone
Clopidogrel
Pitavastatin lactone
Ticlopidine
Testosterone
7-Ethoxyresorufin
Efavirenz
Troglitazone
Nicardipine
Troleandomycin
α-Naphthoflavone
Nifedipine
Quercetin
Clomipramine
Gemfibrozil
Sertraline
Fluvastatin
Pioglitazone
Amitriptyline
Diazepam
B
Relative ratio
0
1
2
3
4
5
■ CYP2C9
▨ CYP2C19
▦ CYP2C76
Nootkatone
Clopidogrel
Pitavastatin lactone
Ticlopidine
Testosterone
7-Ethoxyresorufin
Efavirenz
Troglitazone
Nicardipine
Troleandomycin
α-Naphthoflavone
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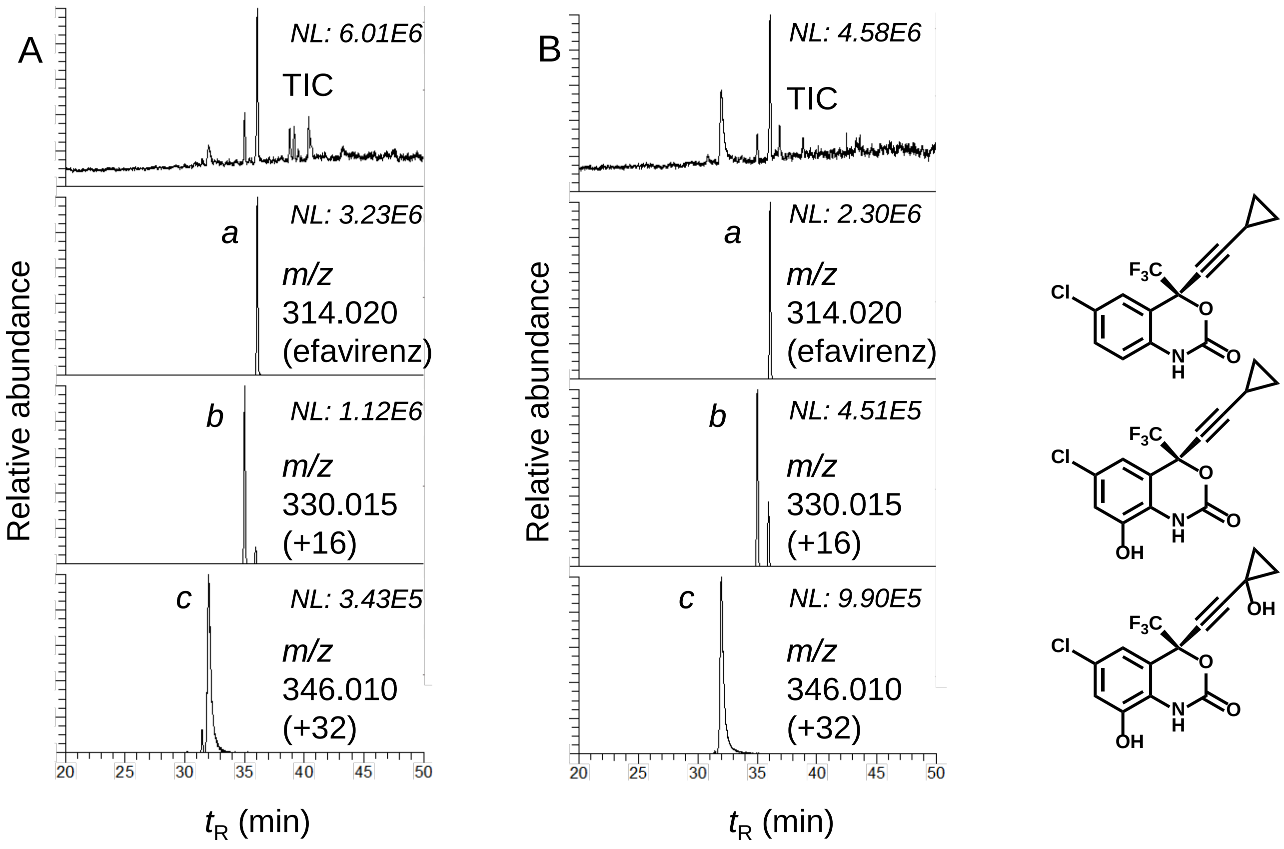
Fluvastatin
Pioglitazone
Amitriptyline
Diazepam

Fig. 2


A
B
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TIC
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TIC
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(efavirenz)
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m/z
314.020
(efavirenz)
b
NL: 1.12E6
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330.015
(+16)
NL: 4.51E5
m/z
330.015
(+16)
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NL: 3.43E5
m/z
346.010
(+32)
NL: 9.90E5
m/z
346.010
(+32)
Relative abundance
20
25
30
35
40
45
50
tR (min)
F3C
Cl
O
N
H
O
OH
OH