Ningbo College of Health Sciences[1], Ningbo; South China University of Technology[2], Guangzhou; Anhui Medical University[3], Hefei; Medical School of Ningbo University[4], Ningbo, PR China

Metabolism of fenofibrate in beagle dogs: New metabolites identified and metabolic pathways revealed

Julin Yang[1,*], Wenxia Luo[2,*], Li Chen[3], Renke Dai[2], Aiming Liu[4]

Received November 3, 2012, accepted November 30, 2012

Dr. Aiming Liu, Medical School of Ningbo University, 818, Fenghua Road, Ningbo 315211 PR China
**These authors contributed equally to this work.*
liuaiming@nbu.edu.cn

Pharmazie 68: 414–420 (2013) *doi: 10.1691/ph.2013.2860*

Fenofibrate is a prototypical agonist of peroxisome proliferator-activated receptor alpha (PPARα) which is well known to be associated with species related carcinogenesis. Important species differences have been reported in its metabolism and elimination pattern. Its new metabolites have been revealed in *Cynomolgus* monkeys and Sprague-Dawley rats. However in beagle dogs, several polar metabolites of fenofibrate have not been identified yet. In this study, beagle dogs were orally dosed with fenofibrate mixed with feeds. Urine and plasma samples were collected and subject to LC-MS/MS by comparison with authentic compounds and confirmed using an API 4000 Q-TRAP system. *In vitro* cultured primary hepatocytes were used to reveal metabolic pathways and confirm the data *in vivo*. Seven new metabolites of fenofibrate in dogs were identified, and their metabolic pathways were revealed. Fenofibrate in beagle dogs was found to be more prone to be metabolized into other secondary metabolites than fenofibric acid, compared with that in rats.

1. Introduction

Fenofibrate is one of the fibrate drugs which are agonists of peroxisome proliferator-activated receptor α (PPARα), a transcription factor that regulates lipid, glucose, and amino acid homeostasis *via* a number of routes (Qi et al. 2000; Peters et al. 2005). It is believed to be metabolized in several stages, and firstly hydrolyzed by esterases into a pharmacologically active metabolite, fenofibric acid (FA) (Caldwell 1989; Guay 1999). By activating PPARα, FA exerts favorable therapeutic effects on dyslipidemia (Elisaf 2002; Farnier 2008). Although considered safe and effective in clinical practice, chronic administration of fibrates in rodent causes species related hepatocarcinogenesis. Fortunately, both nonhuman primates and human remain refractory to this toxicity (Reddy et al. 1980; Rao and Reddy 1987; Ward et al. 1998; Klaunig et al. 2003; Hoivik et al. 2004). Myotoxicity, unlike hepatocarcinogenesis, has been reported in clinic with low incidence, but can lead to severe reactions including acute kidney failure (Blane 1987; Clouatre et al. 1999; Ritter and Nabulsi 2001; Ghosh et al. 2004). The intriguing findings that some metabolites of PPARα agonists could cause genotoxicity and complicated drug-drug interactions suggest that in-depth understanding of fenofibrate metabolism may help explain the toxic actions (Sallustio et al. 1997; Backman et al. 2002; Prueksaritanont et al. 2002; Shitara et al. 2004).

The metabolism of fenofibrate has been studied in humans, rats, guinea pigs, dogs and rat hepatocytes in the 1990 s (Weil et al. 1990; Cornu-Chagnon et al. 1995), and important species differences have been reported (Caldwell 1989). Metabolites FA and reduced fenofibric acid (RFA) were observed in all the above mentioned species. However, fenofibric acid ester glucuronide (FAEG) and reduced fenofibric acid glucuronide (RFAEG), were reported in rats, guinea pigs and human but not in dogs. Unknown polar metabolite(s) were detected in rats, guinea pigs, and dogs, but were not investigated further (Weil et al. 1988). Compound A (4-chloro-4′-hydroxybenzophenone) was detected in human and compound X (2-[4-(4-chloro-benzoyl)-phenoxy]-2-methyl-propionic acid methyl ester) was found to be an *in vitro* metabolite in rat hepatocytes (Cornu-Chagnon et al. 1995). Recently, fenofibrate metabolites in *Cynomolgus* monkeys and Sprague-Dawley rats were studied using UPLC-QTOF-MS-based metabolomics coupled with LC-MS/MS (Liu et al. 2009a,b). Besides the above metabolites, reduced compound A (AR), compound B (4-chloro-4'-isopropoxybenzophenone), fenofibric acid taurine (FAT) and reduced fenofibric acid taurine (RFAT) were reported as new *in vivo* metabolites in both species. Among them, FAT and RFAT indicate a novel metabolism pathway of fenofibrate (Liu et al. 2009a,b).

As to the abundance of different metabolites and the elimination routes, species related differences have also been observed. Both FA and RFA are considered as main metabolites in rats and dogs. In contrast, the FAEG is the predominant metabolite in humans. In rats and dogs, about 70–80% of fenofibrate and its metabolites are eliminated in the feces, whereas in humans the main elimination route is renal excretion (Caldwell 1989). To better understand the mechanism underlying therapeutic effects and toxic actions of fenofibrate in different species, it is important to know its metabolism profile in commonly used animal models, including beagle dogs.

In this study, urine and plasma samples of dogs were collected following oral administration of fenofibrate. Metabolites in these samples were identified using LC-MS/MS and confirmed with Q-TRAP (hybrid Triple Quadrupole/Linear Ion trap mass

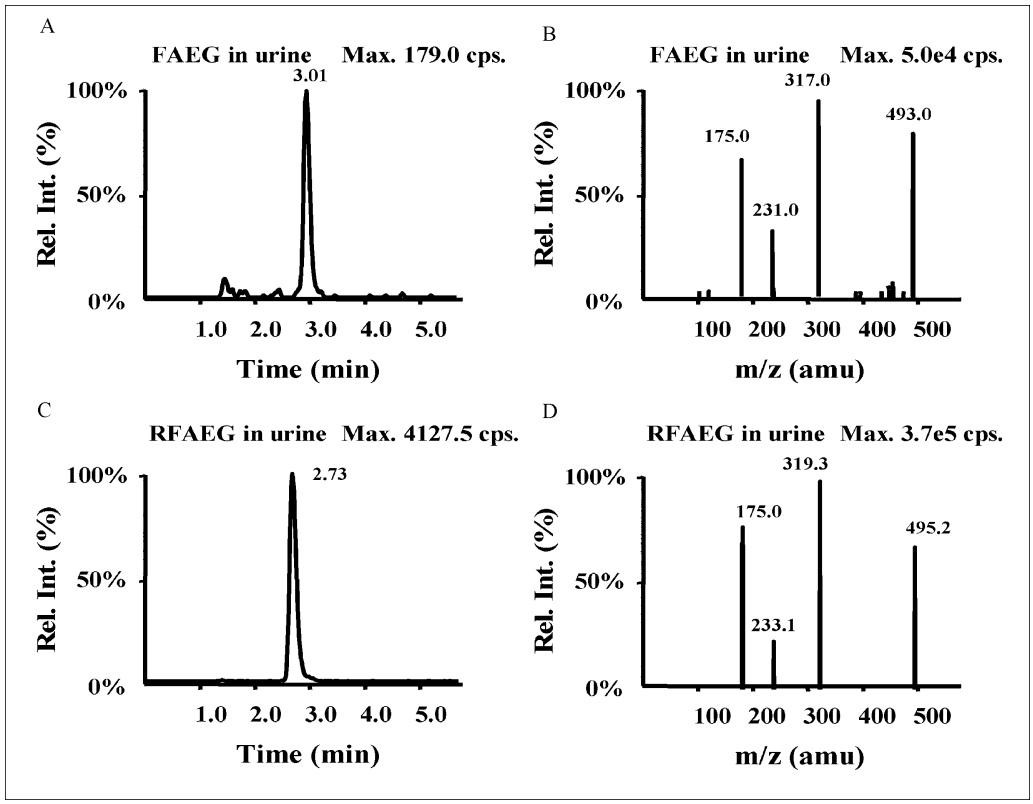

Fig. 1: Chromatographs and mass spectra of FAEG and RFAEG in dog urine samples. Their retention times were 3.01 min (A) and 2.73 min (B), respectively. The main fragment ions by full-scan in negative mode for FAEG (C) and RFAEG (D) indicated 318.0659 is for FA, 320.0815 for RFA, and 176.0321 for common ligand glucuronic acid

spectrometer) by comparison with authentic compounds. *In vitro* metabolism in dog hepatocytes was studied to confirm these metabolites and reveal their metabolic pathways. Additionally, the *in vivo* and *in vitro* formation of RFA in beagle dogs and Sprague-Dawley rats was compared and a distinguished difference was indicated.

2. Investigations and results

2.1. Identification of metabolites in dogs

FA and RFA, not FAEG and RFAEG, have been reported to be metabolites in dog urine and plasma. In this study, FAEG and RFAEG were identified in both dog urine and plasma samples by LC-MS/MS, even though they were not reported in a previous study. Their MS/MS spectra by Q-TRAP are shown in Fig. 1. The fragments of FAEG, FA (493.0 → 317.0) and glucuronic acid (493.0 → 175.0) were detected in EPI mode. Similarly, RFA (495.2 → 319.3) and glucuronic acid (495.2 → 175.0), fragments of RFAEG were also detected (Fig. 1B and D).

FAT and RFAT, the taurine-conjunction metabolites of fenofibrate, have been identified in both *Cynomolgus* monkey and Sprague-Dawley rats by our group. In this study, synthetic compounds were used to identify them with respect to their retention times and MS/MS spectrum. Both retention time and MS/MS profile of FAT were the same between authentic compounds and urine samples (Fig. 2). And this was also true for RFAT (data not shown), indicating FAT and RFAT were both metabolites of fenofibrate in dogs.

It is noteworthy that compound X could be detected in urine, but not in plasma by the 4000 Q-TRAP system, indicating its more abundance in urine. The same MS/MS profile with that of authentic compound confirmed it as a metabolite in urine samples (Fig. 3). For compound B, it was identified in the plasma samples with both LC-MS/MS and 4000 Q-TRAP. But in the urine samples, it was only detected by 4000 Q-TRAP, indicating that it was less in urine than in plasma (Fig. 4). However, compound A could not be detected as an *in vivo* metabolite by 4000 Q-TRAP in plasma or urine samples. While, when compound B, the precursor of compound A, was incubated in hepatocytes for 24 h, compound A was produced (Fig. 5).

2.2. Metabolism pathway of fenofibrate in dog hepatocytes

Fenofibrate, FA, RFA, FAT, RFAT, compounds AR, B, X, and A were added to dog hepatocyte cultures to investigate their metabolic pathways. Production of metabolites was determined using MRM mode by LC-MS/MS. Combining the *in vivo* and *in vitro* results, the metabolism pathway of fenofibrate in dogs was proposed in Fig. 6.

2.3. Metabolism of FA in dogs

As a pharmacologically active metabolite of fenofibrate, FA is also believed to be a main metabolite. In dog plasma and urine samples of the present study, it was interesting to find that RFA was more abundant than FA, as well as FAEG, RFAEG, FAT, RFAT and metabolite B. Take RFA for example, the ratio of RFA/FA in 5-hour plasma sample following oral administration of fenofibrate in dogs was about 46 (46 ± 16, $n = 5$) times than that in rats (Fig. 7A). In experiments with dog or rat hepatocytes incubated with three concentrations of FA, RFA was measured along the incubation time. When incubated with 10 μM FA, the formation of RFA in dog hepatocytes was about 3–7 times over that in rat hepatocytes, during the 70-minute metabolism (Fig. 7B). Similar results were observed when low concentrations of FA (0.4 and 2 μM) were employed. These *in vivo* and *in vitro* data indicated that in beagle dogs fenofibrate is more prone to be metabolized into other metabolites than FA, in comparison with rats.

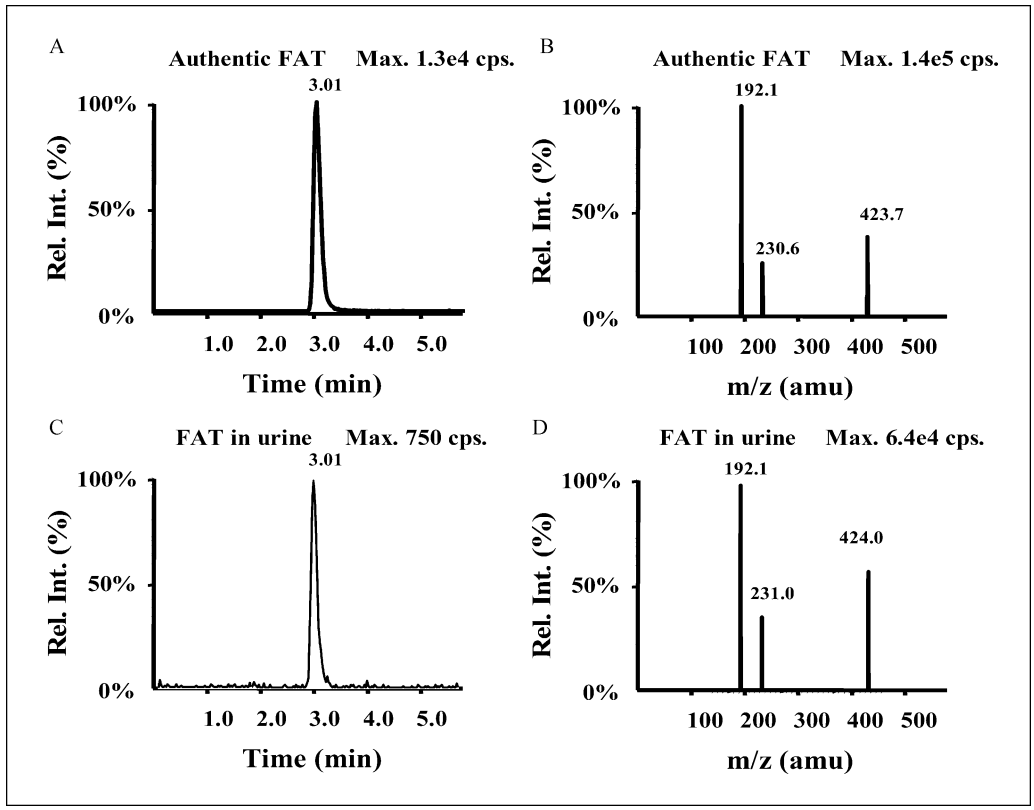

Fig. 2: Chromatographs and mass spectra of synthetic FAT and metabolite FAT in urine samples. Both the retention time and fragmentation profile of synthetic FAT (A and B) was identical with metabolite FAT in urine samples (C and D)

3. Discussion

Metabolism of fenofibrate has been studied in human, dogs, rats, and guinea pigs. Several metabolites were recently reported in *Cynomolgus* monkeys and Sprague-Dawley rats (Liu et al. 2009a,b). Compound A, a metabolite in monkeys, not in rats, was also found in dogs in this study. Compound AR, a previously identified metabolite in rat and monkey, was not detected in dog plasma, urine, or primary hepatocytes. Thanks to the sen-

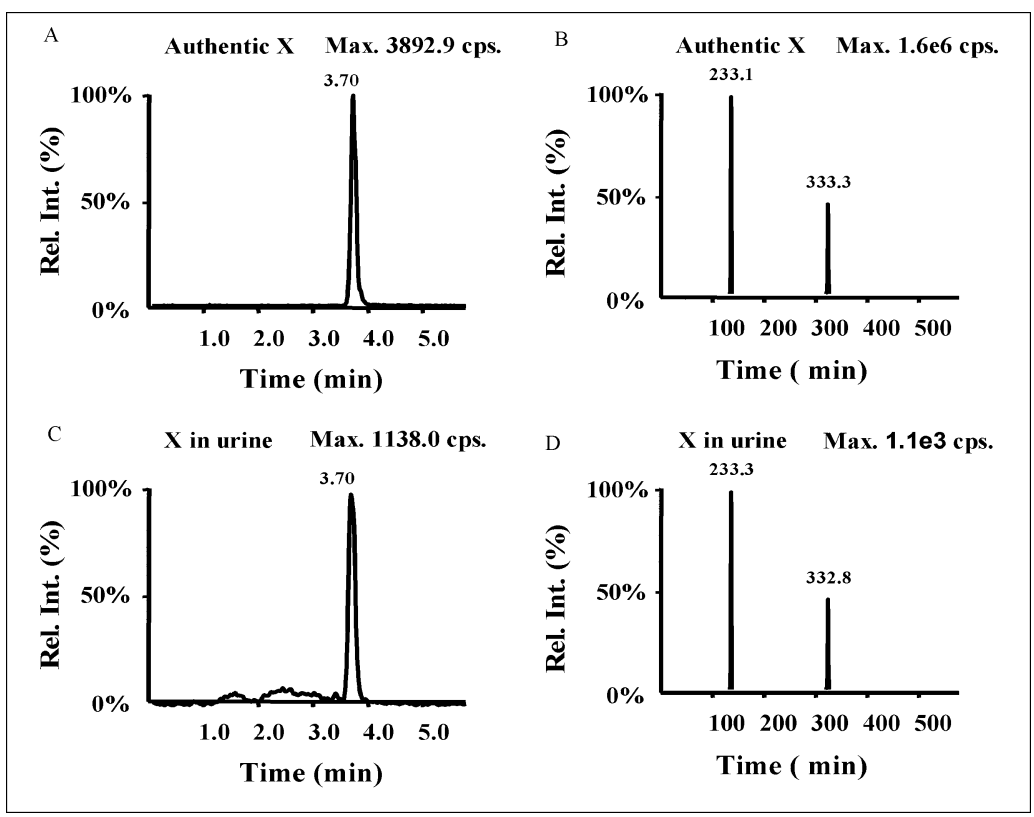

Fig. 3: Chromatographs and mass spectra of synthetic and metabolite X. The retention times and fragmentation profile for authentic compounds (A and B) and metabolite in urine (C and D) were identical

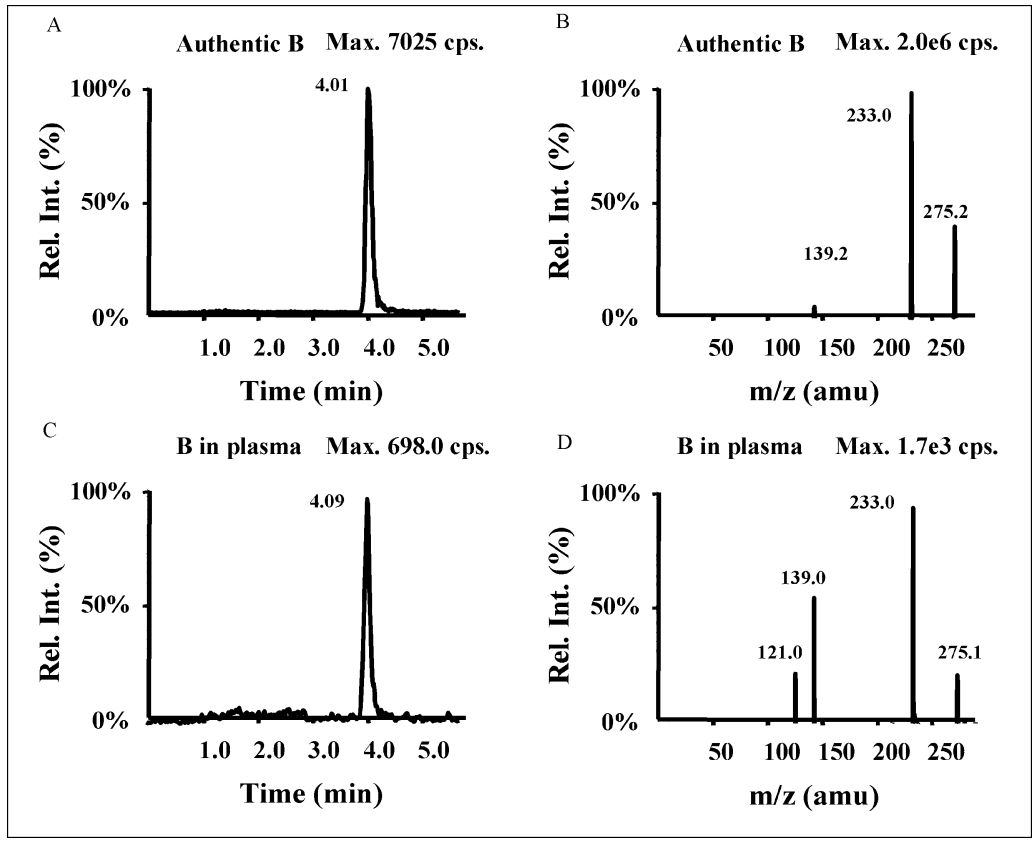

Fig. 4: Chromatographs and mass spectra of synthetic B and metabolite B in urine samples. Both the retention time and fragmentation profile of synthetic B (A and B) was identical with metabolite B in urine samples (C and D)

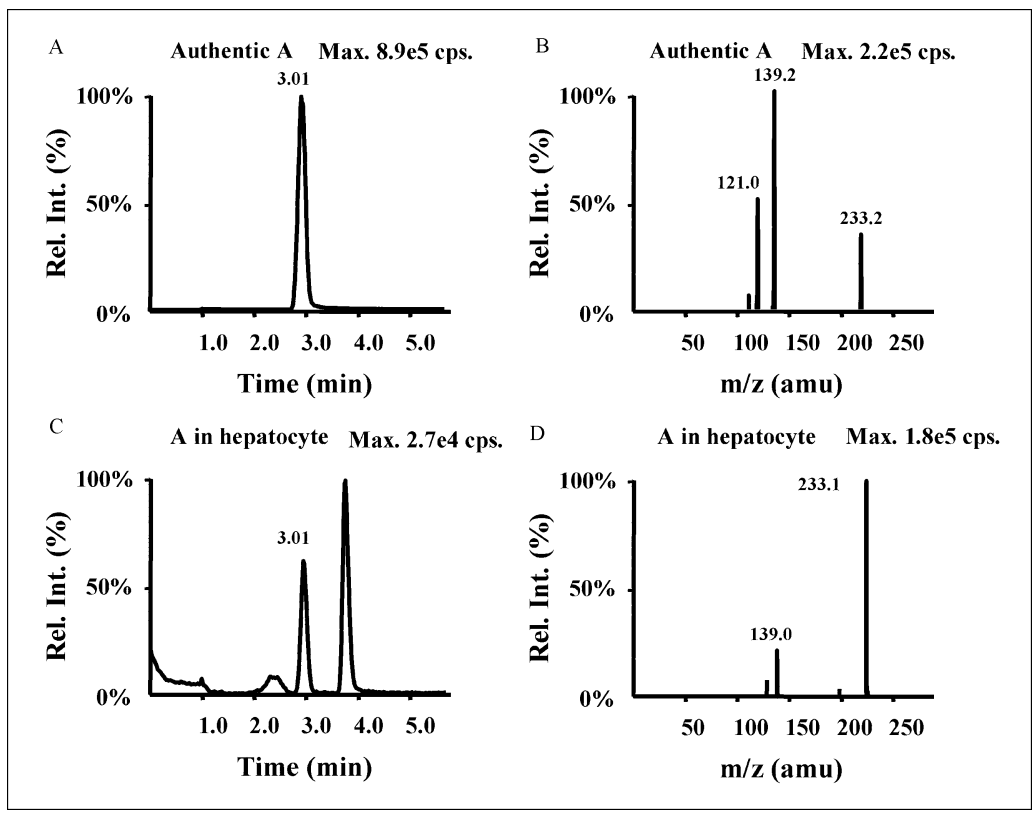

Fig. 5: Chromatographs and mass spectra of synthetic A and metabolite A in urine samples. Both the retention time and fragmentation profiles of synthetic A (A and B) was identical with the metabolite A in urine samples (C and D)

Table: Summary of fenofibrate metabolites in different species

Metabolites	Human	Monkey	Guinea Pig	Rat		Dog	
				In vivo	Hepatocyte	*In vivo*	Hepatocyte
FA	√	√	√	√	√	√*	√
RFA	√	√	√	√	√	√*	√
FAEG	√	√	√	√	√	√	√
RFAEG	√	√	√	√	√	√	√
FAT		√		√	√	√	√
RFAT		√		√	√	√	√
B		√		√	√	√	√
X		√		√	√	√	√
AR		√			√		
A	√						√

[a]Metabolites of fenofibrate in human, guinea pig, and dog (*) were obtained from the indicated references (Weil et al. 1988; Weil et al. 1990; Cornu-Chagnon et al. 1995). [b]The data of monkey, rat and rat hepatocytes were from our previous studies (Liu et al. 2009a; Liu et al. 2009b).

sitivity of 4000 Q-TRAP system which can detect low abundant metabolites, identification of compound B and compound X was possible. Conclusively, there were seven fenofibrate metabolites in dogs newly identified in the present study. The phase II metabolism pathways, glucuronidation and taurine conjugation in dogs were first confirmed in beagle dogs. Comparison of fenofibrate metabolism profile in commonly used species is summarized in the Table.

LC-MS/MS has been widely used in drug discovery and development (Xu et al. 2005). In this study, we used a hybrid Triple Quadrupole/Linear Ion trap mass spectrometer, the API 4000 Q-TRAP system, which enables full-scan MS, MS/MS, and MS3 with superior selectivity (Wen et al. 2008). This system is considered ideal for biomarker discovery and metabolite identification. With its high-sensitive triple quadrupole, quantitation in multiple reactions monitoring (MRM) can also be performed. In the present study, metabolite X in plasma and B in urine could only be detected by 4000 Q-TRAP system, demonstrating superiority in term of sensitivity. Additionally, the MS/MS spectra detected by Q-TRAP were more dependable than those acquired by LC-MS/MS in independent runs. So, advance in detection technology increase quality of the data in this study, and helps to find more metabolites of low concentration.

Nine of the 29 drugs withdrawn from market between 1974 and 1993 were reported as carboxylic acid-containing drugs (Bakke et al. 1995; Skonberg et al. 2008). As pharmacologically active metabolite of fenofibrate, FA is also a carboxylic-acid compound. It is true that more FA can be produced in *Cynomolgus* monkeys and Sprague-Dawley rats (previous studies, data not shown). Although important species differences in the metabolism and elimination patterns of fenofibrate have been reported, in rats and dogs, fenofibric acid and reduced fenofibric acid both are considered principal metabolites (Caldwell 1989). However in this study, as an interesting phenomenon it was observed that RFA, as well as other metabolites, were produced much more than FA in dog plasma and urine (46-fold for RFA was an example, Fig. 7A). By *in vitro* experiments, dog hepatocytes produced RFA faster (about 3–7 times) than rat hepatocytes, when incubated with FA in the concentration range 0.4 to 10 μM (Fig. 7B). This indicated that transformation of FA into RFA was quite different between dogs and rats. While no safety issues have ever been reported for FA or RFA, the relevance of this difference to clinic remains unclear. Additionally, the amino acid sequence of dog PPARα was found to be 97% identical to that of humans and the action of PPARα in dogs was considered similar to that in humans (Nagasawa et al. 2004). Treatment of obese dogs with fenofibrate markedly lowered serum TG and TC, similar to the effects observed in humans (Serisier et al. 2006). So this difference observed probably will not compromise its pharmacological activity.

In summary, fenofibrate metabolism in beagle dogs was investigated using *in vivo* and *in vitro* approaches. Seven metabolites

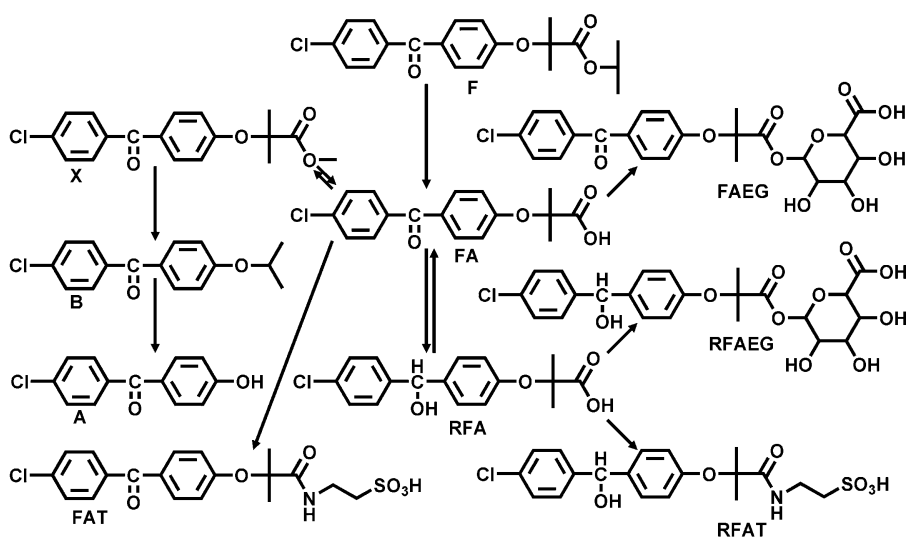

Fig. 6: Proposed metabolic pathways of fenofibrate in beagle dogs. The *in vitro* metabolism using beagle dog hepatocytes was utilized to suggest metabolic pathways. Conclusion was drawn by combining data from hepatocytes from three dogs

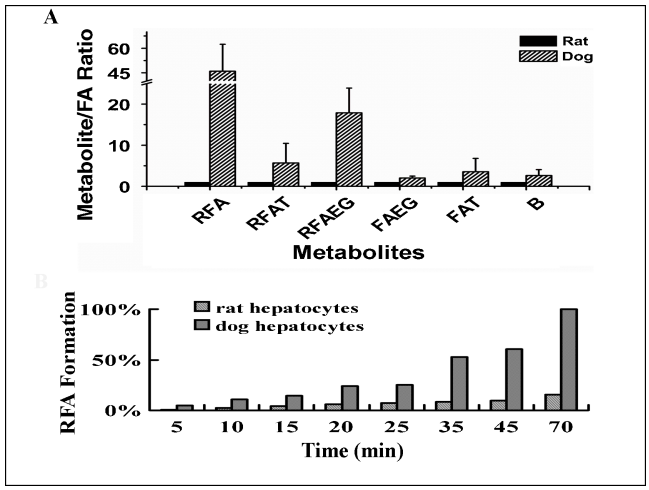

Fig. 7: *In vivo* and *in vitro* production of RFA in dogs, in comparison with that in rats. When normalized to that in rats, relative abundance of metabolites versus that of FA in dogs showed FA was more prone to be metabolized into secondary metabolites (A). As an example to study biotransformation from FA to RFA, production of RFA in dog hepatocytes was 3–7 times faster than that in rat hepatocytes, when incubated with 10 μM FA (B)

of fenofibrate were identified in dogs, as well as their metabolic pathways. RFAEG, FAEG, RFAT and FAT were new phase II conjugative metabolites of fenofibrate in beagle dogs. Different from the knowledge that FA is a main metabolite of fenofibrate, in beagle dogs, FA is more prone to be metabolized into secondary metabolites, leading to relatively lower systemic exposure to FA in dogs than that in rats.

4. Experimental

4.1. Chemicals and reagents

Fenofibrate, FA and compound A were purchased from Shangqiu Chemry Chemicals Co. Ltd. RFA, FAT, RFAT, compound AR, compound B, and compound X were synthesized and characterized by NMR as described in previous studies (Liu et al. 2009a,b). Bezafibrate, acetonitrile and methanol (HPLC grade) were purchased from Sigma Aldrich (St. Louis, USA). Purified water was produced by a Millipore Elix system (Millipore, USA). All other chemicals were analytical or HPLC grade from commercial resource.

4.2. Animal treatment and sampling

Beagle dogs (1 year old, weighing 9–13 kg) and Sprague-Dawley rats (8 weeks old, weighing 190–210 g) were originally provided by the Experimental Animal Center, Southern Medical University. They were maintained under specific pathogen-free conditions in the Animal Center of Guangzhou Institute of Biomedicine and Health (GIBH), Chinese Academy of Sciences (CAS). Animals were housed in cages under a standard 12 h light/dark cycle with free access to purified water and standard commercial diet. Environmental controls for the animal rooms were set to maintain 20–25 °C with a humidity of 40–70%. Before experiments, the animals were allowed to acclimatize for at least 7 days. The animal protocols for experiments were reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of GIBH, CAS.

The dogs (n = 6, three male and three female) were orally dosed with 850 mg·kg^{-1} fenofibrate mixed with food. Urine and plasma samples before fenofibrate dosing were collected and used as blank matrix. 0.5–36 h after dosing, urine was step-wise collected and stored at –80 °C until metabolite identification. Blood samples were collected in heparin-coated tubes 5 h after administration. These samples were centrifuged at 1500 g for 10 min, following which plasma was isolated and transferred to –80 °C for metabolite confirmation.

4.3. Identification of metabolites using LC-MS/MS and 4000 Q-TRAP

Identification of these metabolites in dogs was performed on an an API3000 mass spectrometer coupled with a Shimadzu 10A LC pump (Shimadzu, Japan) and MPS3C auto-sampler (Gerstel, Germany), controlled by Analyst 1.4.2 workstation. The metabolites identified by the above LC-MS/MS were confirmed using enhanced product ion (EPI) mode on API4000 Q-TRAP mass spectrometer (Applied Biosystems, USA) coupled with a Shimadzu 20AD XR LC pump and 20A XR SIL auto-sampler (Shimadzu, Japan). Both detecting systems used Capcell Pak C18 columns (5 μm, 2.0 mm ID × 50 mm; Shiseido, Japan) for chromatographic separation.

The MS/MS spectra of authentic metabolite compounds were determined by an API3000 mass spectrometer (Applied Biosystems, USA). When relative quantification is performed, ion-pairs were selected by multiple reactions monitoring (MRM) analysis of those compounds. Transition mass-to-charge ratios (*m/z*) 317.0/230.9 for FA, 319.2/233.1 for RFA, 426.1/192.3 for RFAT, and 424.0/192.0 for FAT were used for identification in negative MRM mode. Transitions *m/z* 233.1/138.9 for compound A, *m/z* 275.3/233.1 for compound B and 333.3/233.1 for compound X were used in positive MRM mode.

After being thawed at room temperature, both the dog urine samples and plasma samples were diluted with 2 volumes of iced methanol. After vortexing for 2 min, all the samples were centrifuged at 16,000 g 4 °C for 30 min to remove precipitated proteins. The supernatant was subject to lyophilization to concentrate metabolites, following which the residue was reconstituted with water-methanol solution (1:2, v/v). Again, after centrifuging at 16,000 g 4 °C for 30 min, the supernatant was transferred and aliquot of 10 μL was injected into the detecting system. Chromatography was performed at room temperature with a flow rate of 0.2 mL·min^{-1}. The gradient was designed as following: 50% B (methanol containing 0.1% formic acid) for 0.3 min, then a linear gradient to 100% B in 2.2 min and held for 3.0 min, finally dropped to 50% within 0.1 min followed by equilibration for 1.5 min. Chromatograph conditions employed when confirmation using Q-TRAP was the same as the above.

To eliminate interference from in-source fragmentation, strict criteria were used. Firstly, the signal/noise ratio of peak heights of proposed metabolites was above 5 and the retention times were identical with authentic compounds by LC-MS/MS. Secondly, their fragmentation profiles detected by API 4000 Q-TRAP system were consistent with those of authentic compounds. Lacking authentic compounds, FAEG and RFAEG in urine were validated by their typical fragments, apparently attributed to their substrates FA and RFA, and their common ligand glucuronic acid.

4.4. Metabolism relationship in dog and rat hepatocytes

To better understand fenofibrate metabolism in dogs as well as its potential species differences, hepatocytes were used to determine its metabolism roadmap. Dog and rat hepatocytes were primarily isolated by a typical two-step *in-situ* perfusion protocol as previously reported with minor modifications (Seglen 1976). After determination using trypan blue dye exclusion, hepatocytes with a viability above 85% were used. A total of 2×10^6 viable hepatocytes were seeded in 24-well plates pre-coated with collagen in DMEM and then incubated at 37 °C in a humidified incubator. Medium replacement was performed 24 h after seeding when hepatocytes adhered well and metabolism experiment was performed 48 h after seeding when cell viability recovered.

Synthesized FA, A, AR, B, X, RFA, FAT, RFAT and fenofibrate were dissolved in DMSO and added to dog hepatocytes with final concentrations of 10 μM (0.1% DMSO). After metabolism for 24 h, the medium was collected and mixed with 2 volumes of iced acetonitrile with IS. Wells without hepatocytes were treated in the same way and used as control. After sonic disruption for 10 min, all the samples were centrifuged at 16, 000 g 4 °C for 30 min to remove particulates and proteins. A total of 10 μL of the supernatant was then injected into LC-MS/MS for the detection of all these compounds with the same method as metabolite identification. Bezafibrate can be well detected in both negative mode (*m/z* 360.1/274.4) and positive mode (*m/z* 362.1/138.9). So it was used as internal standard (IS) to calculate relative abundance of metabolites and then decide their production. Data from hepatocytes of three dogs (two male and one female) were combined for metabolism pathway analysis. Metabolites were considered to be produced in hepatocytes if the peak area ratios of metabolite/IS were 2 times higher than those in control.

4.5. Comparison of fenofibrate metabolism in dogs and rats

Plasma samples of rats collected in a previous study and those of dogs collected in this study following fenofibrate administration were subject to LC-MS/MS determination. The relative abundance of secondary metabolites in dogs was calculated as signal responses of metabolites *versus* that of FA, which was then normalized to their abundance in rats. As an example to explore biotransformation from FA to RFA in two species, four concentrations of FA (final concentrations 0.4, 2 and 10 μM) were incubated in dog and rat hepatocytes. Reactions were terminated at predefined time intervals (0–70 min) by adding 2 volumes of iced acetonitrile using bezafibrate as IS. After pre-treatment similar with the above, 10 μL of the supernatant was injected into LC-MS/MS for relative quantification of RFA, by normalization to abundance of FA.

Acknowledgements: This work was supported by Ningbo Natural Science Foundation [Grant 2011A610059], Zhejiang Provincial Natural Science Foundation of China [Grant Y2110016] and National Natural Science Foundation of China [Grant 81273582].

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