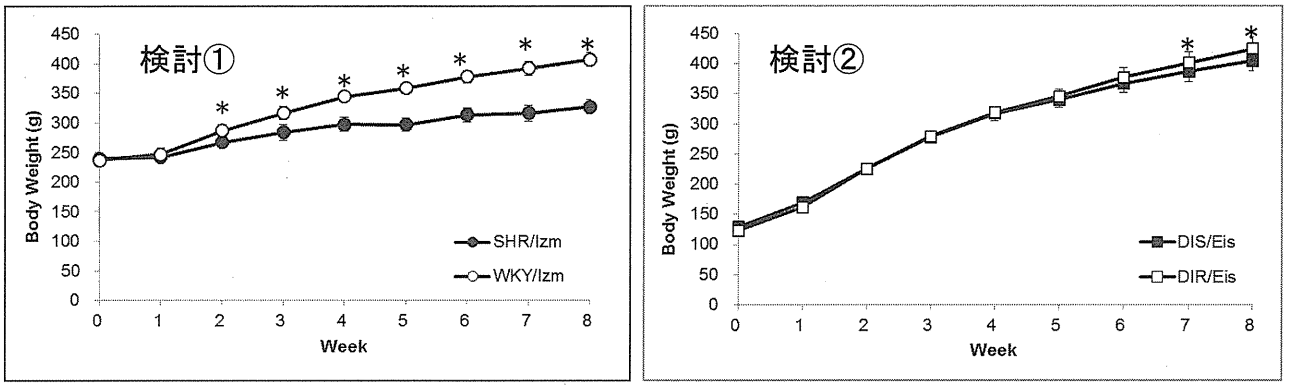


図 1 体重推移　(平均値±標準偏差, 各 n=10)　＊ p<0.05

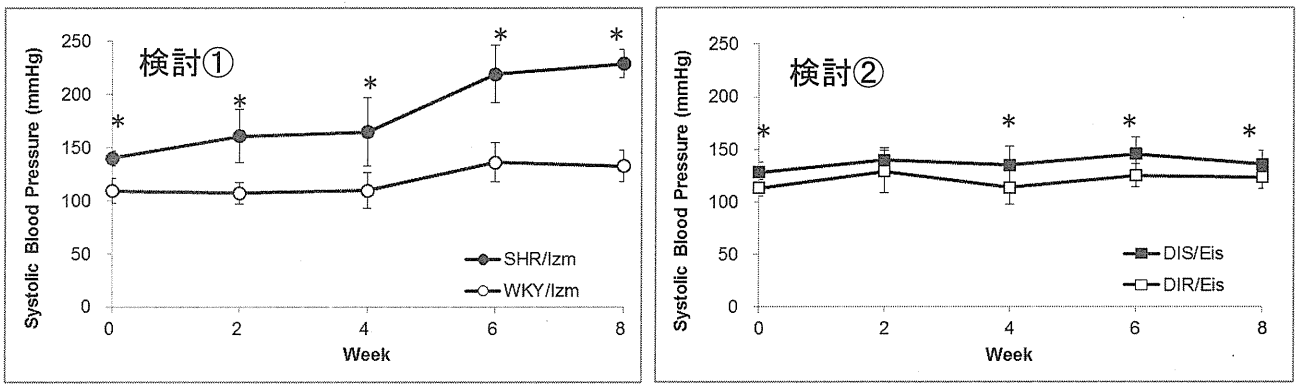


図 2 収縮期血圧　(平均値±標準偏差, 各 n=10)　＊ p<0.05

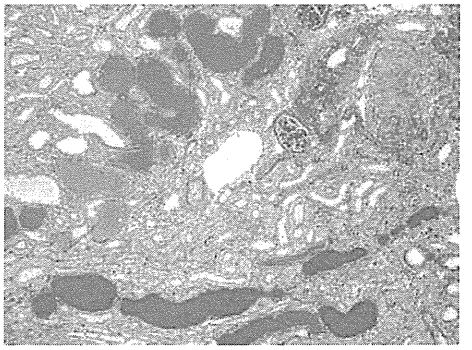


図 3 腎病理像　SHR/Izm 群(個体番号:1009)(PAS 染色 70 倍)

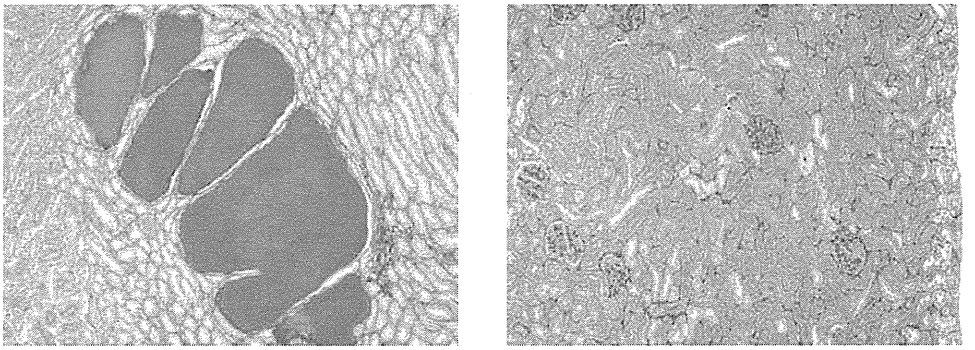
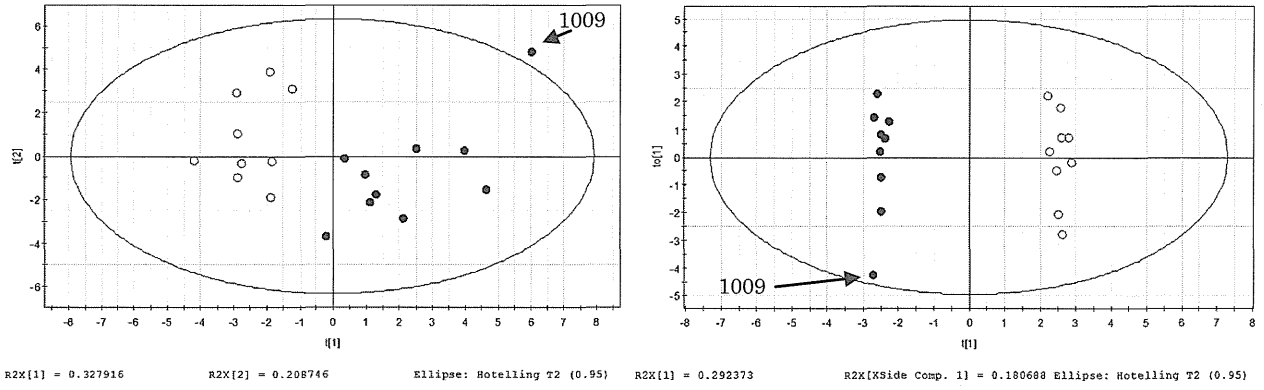


図 4 腎病理像　検討②　(左) DIS/Eis 群　(右) DIR/Eis 群　(PAS 染色 70 倍)



PCA　　O-PLS-DA

図 5 検討①　尿の ^{1}H-NMR のメタボリック・プロファイリング　(●:SHR/Izm, ○: WKY/Izm)

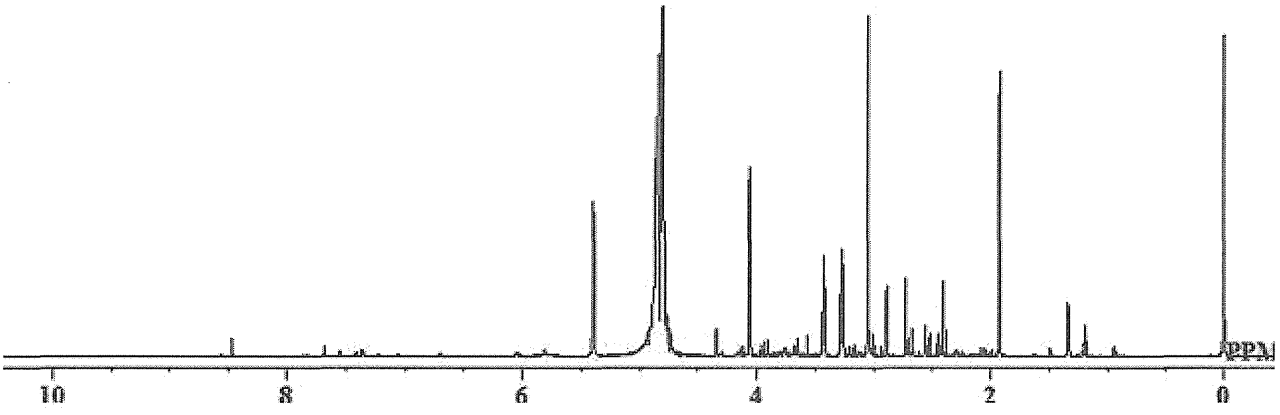
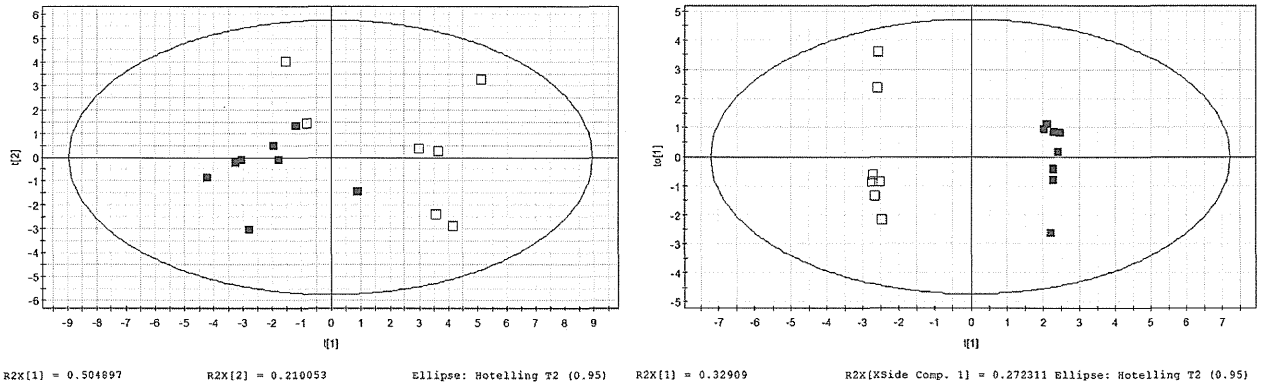


図 6 SHR/Izm (個体 No. 1009)の尿 ^{1}H-NMR スペクトル



PCA　　O-PLS-DA

図 7 検討②　尿の ^{1}H-NMR のメタボリック・プロファイリング　(■:DIS/Eis, □: DIR/Eis)

様式第１９

学　会　等　発　表　実　績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」

機関名　国立医薬品食品衛生研究所

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
Metabolomic profiles in rat blood vary between genders, ages and fasting conditions, and their qualitative comparisons with human samples. （ポスター）	Saito Y, Saito K, Ishikawa M, Urata M, Tajima Y, Inoue M, Kumagai Y, Pappan K, Maekawa K	San Diego, CA, USA (2014 AAPS Annual Meeting and Exposition)	2014. 11	国外
Metabolomic biomarker exploration highlights issues of species specificity. （口頭）	Maekawa K, Saito K, Ishikawa M, Minamino M, Kumagai Y, Saito Y.	Busan, Korea (KSCPT-JSCPT Joint symposium)	2014. 11	国外
Comparison of plasma lipidomic profile of humans with preclinical animals. （ポスター）	Saito K, Urata M, Toyoshima K, Ishikawa M, Murayama M, Tajima Y, Senoo Y, Takemoto K, Kumagai Y, Maekawa K, Saito Y.	San Francisco, CA, USA (19th North American ISSX and 29th JSSX Joint Meeting)	2014. 10	国外
Impact of gender, age, fed/fasted state of rats on their serum hydrophilic metabolites. （ポスター）	Maekawa K, Saito K, Pappan K, Ishikawa M, Urata M, Tajima Y, Murayama M, Kumagai Y, Saito Y	San Francisco, CA, USA (19th North American ISSX and 29th JSSX Joint Meeting)	2014. 10	国外
Characterization of hepatic lipid profiles of insulin-dependent NASH and following cirrhosis using	Ishikawa M, Saito K, Uebanso T, Maekawa K, Senoo Y, Murayama M,	San Francisco, CA, USA (19th North American ISSX and 29th JSSX Joint Meeting)	2014. 10	国外

mouse model. （ポスター）	Tajima Y, Nishimaki-Mogami T, Nakanishi H, Ikeda K, Arita M, Taguchi R, Fujii M, Shibazaki Y, Yoneyama H, Nammo T, Saito Y, Yasuda K.			
日本人におけるカルバマゼピン誘因性薬疹発症の危険因子 *HLA-A*31:01* のサロゲートマーカー多型を対象としたタイピング系の構築（ポスター）	前川京子，水澤精穂，北本綾，北本卓也，中村亮介，杉山永見子，上田真由美，外園千恵，池田浩子，矢上晶子， 松倉節子, 木下茂，村松正明，古谷博和，高橋幸利，松永佳世子，相原道子，関根章博，日本PGxデータサイエンスコンソーシアム，斎藤嘉朗	神戸（日本薬学会 135 回年会）	2015. 3	国内
ヒト試料を用いたバイオマーカー研究のためのレギュラトリーサイエンス（口頭）	斎藤嘉朗，齊藤公亮，児玉進，熊谷雄治、前川京子	愛媛（第 35 回日本臨床薬理学会学術総会）	2014. 12	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別
Glucosylceramide and Lysophosphatidylcholines as Potential Blood Biomarkers for Drug-Induced Hepatic Phospholipidosis	Saito K, Maekawa K, Ishikawa M, Senoo Y, Urata M, Murayama M, Nakatsu N, Yamada H, Saito	Toxicol Sci., 2014; 141: 377-386.	2014. 10	国外

	Y.			
Effects of sex, age, and fasting conditions on plasma lipidomic profiles of fasted sprague-dawley rats.	Saito K, Ishikawa M, Murayama M, Urata M, Senoo Y, Toyoshima K, Kumagai Y, Maekawa K, Saito Y	PLoS One, 2014; 9: e112266.	2014. 11	国外
Differences in metabolite profiles between blood matrices, ages, and sexes among Caucasian individuals and their inter-individual variations.	Saito K, Maekawa K, Pappan KL, Urata M, Ishikawa M, Kumagai Y, Saito Y	Metabolomics, 2014; 10: 402-413.	2014. 10	国外
薬物相互作用に影響を及ぼす遺伝子多型とその人種差	前川京子, 佐井君江	ファルマシア, 2014; 50(7) : 669-673	2014. 7	国内

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

（注２）本様式は excel 形式にて作成し、甲が求める場合は別途電子データを納入すること。

様式第１９

学　会　等　発　表　実　績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」

機関名　シミックバイオリサーチセンター

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
SD ラットのゲンタマイシン腎障害における尿中 L-FABP と他の尿中腎障害バイオマーカーの比較（ポスター発表）	鈴木 慶幸, 小松 弘幸, 門田 利人, 及川 剛, 田口 景子, 筑广 紗弥香, 菅谷 健, 齋藤 明美	神戸（日本毒性学会）	2014.7	国内
Evaluation of Urinary L-FABP As a Nephrotoxicity Biomarker in Rats (Poster presentation)	T. Kadota, Y. Suzuki, H. Komatsu, K. Taguchi, T. Sugaya	Phenix, AZ, USA（米国毒性学会）	2014.3	国外

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別

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様式第１９

学　会　等　発　表　実　績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」

機関名　東京大学大学院薬学系研究科

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
薬物トランスポーターの種差と遺伝子多型による医薬品体内動態の個人間変動（口頭）	楠原洋之	東京（第54回日本先天異常学会学術集会）	2014.7	国内
メタボロームによる薬物動態バイオマーカーの探索と薬物間相互作用の定量的解析への適用（口頭）	楠原洋之	第５回杉山特別研究室理研公開シンポジウム	2015.2	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別

（注１）発表者氏名は、連名による発表の場合には、筆頭者を先頭にして全員を記載すること。

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様式第１９

学　会　等　発　表　実　績

委託業務題目「医薬品・医療機器の実用化促進のための評価技術手法の戦略的開発」

機関名　マルハニチロ株式会社

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
LDL-コレステロールが高めの成人男性に対する魚食の脂質代謝改善効果の検証、口頭	河原﨑正貴, 鎌田彰, 矢口文, 江成宏之,根本直	日本農芸化学会 2015 年度大会	2015.3	国内

２．学会誌・雑誌等における論文掲載

掲載した論文（発表題目）	発表者氏名	発表した場所（学会誌・雑誌等名）	発表した時期	国内・外の別

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（注２）本様式は excel 形式にて作成し、甲が求める場合は別途電子データを納入すること。

別紙４

研究成果の刊行に関する一覧表レイアウト

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Saito K, Maekawa K, Ishikawa M, Senoo Y, Urata M, Murayama M, Nakatsu N, Yamada H, Saito Y.	Glucosylceramide and Lysophosphatidylcholines as Potential Blood Biomarkers for Drug-Induced Hepatic Phospholipidosis	Toxicol Sci	141 (2)	377-386	2014
Saito K, Ishikawa M, Murayama M, Urata M, Senoo Y, Toyoshima K, Kumagai Y, Maekawa K, Saito Y	Effects of sex, age, and fasting conditions on plasma lipidomic profiles of fasted sprague-dawley rats.	PLoS One	9 (11)	e112266	2014
Saito K, Maekawa K, Pappan KL, Urata M, Ishikawa M, Kumagai Y, Saito Y	Differences in metabolite profiles between blood matrices, ages, and sexes among Caucasian individuals and their inter-individual variations.	Metabolomics	10	402-413	2014
前川京子，　佐井君江	薬物相互作用に影響を及ぼす遺伝子多型とその人種差	ファルマシア	50(7)	669-673	2014

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Glucosylceramide and Lysophosphatidylcholines as Potential Blood Biomarkers for Drug-Induced Hepatic Phospholipidosis

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ABSTRACT

Drug-induced phospholipidosis is one of the major concerns in drug development and clinical treatment. The present study involved the use of a nontargeting lipidomic analysis with liquid chromatography-mass spectrometry to explore noninvasive blood biomarkers for hepatic phospholipidosis from rat plasma. We used three tricyclic antidepressants (clomipramine [CPM], imipramine [IMI], and amitriptyline [AMT]) for the model of phospholipidosis in hepatocytes and ketoconazole (KC) for the model of phospholipidosis in cholangiocytes and administered treatment for 3 and 28 days each. Total plasma lipids were extracted and measured. Lipid molecules contributing to the separation of control and drug-treated rat plasma in a multivariate orthogonal partial least squares discriminant analysis were identified. Four lysophosphatidylcholines (LPCs) (16:1, 18:1, 18:2, and 20:4) and 42:1 hexosylceramide (HexCer) were identified as molecules separating control and drug-treated rats in all models of phospholipidosis in hepatocytes. In addition, 16:1, 18:2, and 20:4 LPCs and 42:1 HexCer were identified in a model of hepatic phospholipidosis in cholangiocytes, although LPCs were identified only in the case of 3-day treatment with KC. The levels of LPCs were decreased by drug-induced phospholipidosis, whereas those of 42:1 HexCer were increased. The increase in 42:1 HexCer was much higher in the case of IMI and AMT than in the case of CPM; moreover, the increase induced by IMI was dose-dependent. Structural characterization determining long-chain base and hexose delineated that 42:1 HexCer was d18:1/24:0 glucosylceramide (GluCer). In summary, our study demonstrated that d18:1/24:0 GluCer and LPCs are potential novel biomarkers for drug-induced hepatic phospholipidosis.

Key words: phospholipidosis; biomarker; lipidomics; tricyclic antidepressant; ketoconazole

Drug-induced phospholipidosis is a lysosomal storage disorder that results in the accumulation of phospholipids in the lysosomes of the cells, organs, and tissues (Kodavanti and Mehendale,1990; Lüllmann *et al.*, 1975, 1978). Many cationic amphiphilic drugs (CADs), including clinically used antiarrhythmics (amiodarone), antibiotics (gentamycin), antidepressants (imipramine [IMI]), and antipsychotics (haloperidol), have been reported to induce phospholipidosis (Halliwell, 1997; Kodavanti and Mehendale, 1990; Reasor, 1989). Drug-induced phospholipidosis is a dose-dependent phenomenon with the accumulation of CADs along with phospholipids (Anderson and Borlak, 2006; Reasor and Kacew, 2001). Some reports have demonstrated that drug-induced phospholipidosis is associated with the inflammation and fibrosis in the liver (Lewis *et al.*, 1989; Poucell *et al.*, 1984; Rigas *et al.*, 1986), although the relationship between drug-induced phospholipidosis and the phenotypes of this adverse drug reaction remains controversial. A similar lysosomal storage disorder caused by genetic dysfunction, such as Niemann-

Pick disease type C and Gaucher disease, represents neuronal symptoms and hepatomegaly (James *et al.*, 1981; Turpin *et al.*, 1991). Therefore, drug-induced phospholipidosis is one of the concerns of risk management in clinical treatment and drug development.

The gold standard method to determine drug-induced phospholipidosis is the observation of lamellar body structures by electron microscopy (Reasor, 1989). However, the evaluation by electron microscopy requires a tissue biopsy, making this approach unsuitable for high-throughput evaluation of drug-induced phospholipidosis. Recently, a metabolomics approach, which involves the measurement of a large number of metabolites in blood plasma, serum, and urine, has emerged as a useful tool for exploring surrogate biomarkers for diseases and toxicities *in vivo* (Bu *et al.*, 2012; Gowda *et al.*, 2008; Spratlin *et al.*, 2009). Although no human study has confirmed this yet, bis(monoglycerol)phosphate (BMP) and phenylacetylglycine (PAG) have been identified as potential specific biomarkers for phospholipidosis using a rodent model of amiodarone-induced phospholipidosis (Delaney *et al.*, 2004; Mortuza *et al.*, 2003). The levels of BMP (also known as lysobisphosphatidic acid) were increased in the serum of an amiodarone-treated rat with unregulated its levels in liver, lung, and kidney. BMP levels were also upregulated in the urine of a rat treated with amiodarone or gentamycin (Baronas *et al.*, 2007; Thompson *et al.*, 2012). On the other hand, increased levels of PAG were demonstrated in the urine and plasma of an amiodarone-treated rat. However, drug-induced phospholipidosis occurs in many different organs and tissues, including the liver, kidney, lung, and lymphoid, and its occurrence varies among CADs (Halliwell, 1997; Kodavanti and Mehendale, 1990; Reasor, 1989). Thus, it remains unclear whether BMP and PAG could be useful biomarkers for all types of drug-induced phospholipidosis caused by many different CADs.

The liver plays a central role in many physiological processes such as xenobiotic metabolism and lipid and glucose homeostasis (Jungermann, 1986). The Japanese Toxicogenomics and Toxicogenomics Informatics Projects (TGP/TGP2) have created a transcriptome database of a broad range of toxic effects in the liver, including phospholipidosis, induced by about 150 drugs and biochemicals (Urushidani and Nagao, 2005). The project has used the rat liver as a model of drug-induced phospholipidosis and demonstrated that three tricyclic antidepressants (clomipramine [CPM], IMI, and amitriptyline [AMT]) induced phospholipidosis in hepatocytes and an antibiotic (ketoconazole [KC]) induced phospholipidosis in cholangiocytes (Hirode *et al.*, 2008). By profiling gene expressions in the liver of a rat treated with phospholipidosis-inducing drugs, the authors revealed that scoring of the change in 25 transcripts allows evaluation of drug-induced phospholipidosis with an accuracy of 82% (Yudate *et al.*, 2012). Although the transcriptomic approach using liver tissues can be used to assess the risk of drug-induced phospholipidosis, this approach requires liver biopsy, and thus would not be suitable for a high-throughput evaluation of phospholipidosis *in vivo*.

Because lipid molecules, such as phospholipids and sphingolipids, are not only the major components of the cellular membrane but also the signaling molecules of important biological functions of the cell, they have emerged as useful biomarkers of the biological state of the cells, organs, and tissues (Maekawa *et al.*, 2013; Tajima *et al.*, 2013). Therefore, we used a lipidomic approach, which provides a comprehensive analysis of the lipid profile, to explore common plasma biomarkers for hepatic phospholipidosis. First, we used plasma obtained from rats treated with CPM, IMI, and AMT, which induce phospholipidosis in hepatocytes. Subsequently, we analyzed plasma obtained from rats treated with KC, which induces phospholipidosis in cholangiocytes, to examine whether the potential biomarker for phospholipidosis induced in hepatocytes would also be useful in that induced in cholangiocytes. Using a nontargeting lipidomic approach with liquid chromatography (LC) and time-of-flight mass spectrometry (TOFMS), we determined the ion peak levels of lipid molecules including PC, sphingolipids, and neutral lipids. The levels of lipid molecules were subjected to multivariate statistics using an orthogonal partial least squares discriminant analysis (OPLS-DA) as models to identify potential blood biomarkers for hepatic phospholipidosis induced by each drug. We found that all three antidepressants increased the levels of hexosylceramide (HexCer) and decreased the levels of lysophosphatidylcholines (LPCs) in plasma, and those observations were consistent with KC treatment at early time point. Here, through determination of the structural features of the identified HexCer, we present experimental data to propose glucosylceramide (GluCer) and LPCs as potential low-invasive biomarkers for hepatic phospholipidosis.

MATERIALS AND METHODS

Animal specimens. The rat plasma samples used in the present study were from the TGP/TGP2 project and our previous study (Hirode *et al.*, 2008). The drug treatment and histological findings are summarized in Supplementary table 1. In brief, six-week-old male Crl:CD(SD) rats (Charles River Japan, Kanagawa, Japan) were subjected to continuous treatment with CPM, IMI, AMT, and KC for 3 or 28 days. Each drug-treatment group ($n = 5$, except for the 28-day IMI- and AMT-treatment groups [$n = 4$], see Supplementary table 1) had its own corresponding control group that was treated with vehicle ($n = 5$). For the study of the dose-dependent effects of IMI on the levels of 42:1 HexCer in plasma, the rats were divided into four groups (control and 10-, 30-, and 100-mg/kg/day IMI treatment) and treated with IMI for 28 days. The doses of the drugs that were used in the present study were shown to be the most effective doses for hepatic phospholipidosis induction in our previous study (Hirode *et al.*, 2008), except for the study examining the dose-dependent effects of IMI. The use of animal specimens was approved by the Ethics Review Committee for Animal Experimentation of the National Institute of Biomedical Innovation (Osaka, Japan) and National Institute of Health Sciences (Tokyo, Japan).

Lipid extraction. Lipid extraction was performed with the Bligh and Dyer (BD) method with minor modification. Ninety microliters of plasma was mixed with 3.8 ml of chloroform/methanol/20 mM Kpi (1:2:0.8) for 5 min and with the following internal standards: 10 nmol of 12:0/12:0 phosphatidylethanolamine (PE, Avanti Polar Lipids, Inc., Alabaster, AL) and 2 nmol of 8:0/8:0/18:2 triglyceride (TG, Larodan Fine Chemicals AB, Malmo, Sweden). After mixing, 1 ml each of chloroform and 20 mM Kpi was added, mixed for 5 min, and centrifuged at 1000 × g for 10 min. After discarding upper layers, 3 ml of chloroform/methanol/100 mM KCl (3:38:47) were added, mixed, and centrifuged at 1000 × g for 10 min to separate the lower organic layers. The lower organic layers (BD sample) were collected, dried, dissolved in chloroform/methanol (1:1), and stored at a temperature of −80°C until use.

Lipid molecule measurements and data processing. Lipid molecule measurements were performed as described previously using LC (Acquity UPLC System, Waters, Milford, MA)-TOFMS (LCT

Premier XE; Waters Micromass, Waters) (Ishikawa *et al.*, 2014). Raw data obtained by LC-TOFMS were processed using the 2DICAL software (Mitsui Knowledge Industry, Tokyo, Japan), which allows to detect and align the ion peaks of each biomolecule obtained at the specific m/z and column retention time (RT). The main parameters of 2DICAL were set as described previously with a few modifications (Ishikawa *et al.*, 2014). To extract the ion peaks of lysophospholipids (LPC), phospholipids (phosphatidylcholine [PC], PE, phosphatidylinositol [PI], phosphatidylglycerol, and BMP), and sphingolipids (sphingomyelin [SM], ceramide, and HexCer), the RT range was from 2.0 to 38.0 min in the negative-ion mode, whereas for those of neutral lipids (cholesterol ester and TG), the RT range was from 38.0 to 60.0 min in the positive-ion mode. The intensities of each extracted ion peak were normalized to those of the internal standards (12:0/12:0 PE for lysophospholipids, phospholipids and sphingolipids, 8:0/8:0/18:2 TG for neutral lipids).

OPLS-DA and lipid identification. The control and drug-treated data sets for the intensities of the extracted ion peaks from rat plasma were loaded into SIMCA-P+ 12 (Umetrics, Umea, Sweden), pareto-scaled, and analyzed using OPLS-DA to extract ion peaks that contributed to the separation of the control and drug-treated samples. To sort these ion peaks, $|w[1]| > 0.05$ and $|p(corr)| > 0.6$ in the loading s-plot of the OPLS-DA score, which represent the magnitude of contribution (weight) and reliability (correlation), respectively, were selected as cut-off values. The representative OPLS-DA score plot and loading s-plot were presented in Supplementary figure 1 (RT 2.0–38.0 and negative ion mode). Subsequently, the ion peaks identified as separating control and drug-treated rats were subjected to identification of lipid molecules as described previously (Ishikawa *et al.*, 2014). In brief, lipid molecules were identified by comparing the ion features of the experimental samples to our reference library of lipid molecule entries. Our reference library was constructed based on the RT, m/z, preferred adducts, and in-source fragments of the representative standards of each lipid class purchased from Avanti Polar Lipids, Inc. For our LC-TOFMS assay platform, we chose 10 parts per million as the acceptable range of mass accuracy in the present study.

Structural analysis. Long-chain base and fatty-acid chain of HexCer were determined by LC-Fourier transform mass spectrometry (LC-FTMS, LTQ Orbitrap XL, Thermo Fisher Scientific, Waltham, MA) as previously described with a few modifications (Ishikawa *et al.*, 2014). To identify the long-chain base of HexCer, data-dependent MS^3 analysis was performed in the positive ion mode. A specific neutral loss (H_2O; 18 Da) was obtained from HexCer with MS/MS and this was used as the trigger from MS/MS to MS/MS/MS. Because the amount of HexCers in the BD fraction used for lipid molecule measurements was not sufficient for data-dependent MS^3, a fraction containing HexCers was separated by LC, collected, and concentrated. Determination of hexosyl residue was performed by separation using borate-impregnated thin-layer chromatography (TLC) followed by extraction of the lipid fraction from the silica plate and analyzed by LC-TOFMS. Borate impregnation was performed based on the modified method by Kern (1966). Silica gel 60 TLC plates (TLC Silica gel 60, Merck, Darmstadt, Germany) were immersed in sodium tetraborate (2%) in methanol for 30 s and air-dried overnight. Plates were heated at 110°C for 30 min prior to the application of BD samples. The samples were prepared as described above using 50 μl of plasma and internal standards (2 nmol of d18:1/24:1 glucosylceramide [GluCer] and d18:1/24:1 galactosylceramides [GalCer]). The samples were concentrated to 30 μl for application and developed in chloroform/methanol/water (70:30:4). After development, the plates were sprayed with primuline (0.01%) in acetone/water (80:20) and visualized under UV light. The GluCer and GalCer spots were scraped and subjected to lipid extraction as described above. GluCer and GalCer measurements were performed by LC-TOFMS as described above.

Statistical analysis. To compare the levels of lipid molecules, the Mann–Whitney *U* test was used to assess the statistical difference in plasma lipid molecule levels between control and drug-treated rats using the R software (version 2.15.1). For the comparisons that showed statistical significance, we performed power calculations with the G*power 3 program (Faul *et al.*, 2007). The average power calculation of all of the statistically significant comparisons was 0.929, suggesting that the number of animals used in the present study was sufficient for the statistical analysis.

RESULTS

Screening for Common Biomarkers for Hepatic Phospholipidosis in Hepatocytes

To screen a broad range of lipid molecules, we used the non-targeting lipidomic approach. Phospholipids and sphingolipids were measured by LC-TOFMS with the negative-ion mode with an RT of 2.0–38.0 min, in which both classes of lipids were eluted by LC. Neutral lipids were measured by LC-TOFMS with the positive-ion mode with an RT of 38.0–60.0 min, in which neutral lipids were eluted. In total, 312 and 847 ion peaks were extracted by negative- and positive-ion modes, respectively, in plasma obtained from control or drug-treated rats.

First, we employed the data set obtained from the plasma of rats treated with tricyclic antidepressants (CPM, IMI, and AMT) to identify common biomarkers for hepatic phospholipidosis in hepatocytes. The data set of extracted ion peaks from control and each of the tricyclic antidepressants were subjected to OPLS-DA, and the score of the loading s-plot was calculated to identify ion peaks that contributed to the separation of control and drug-treated samples of plasma ($|w[1]| > 0.05$ and $|p(corr)| > 0.6$ were used as threshold values). The ion peaks were subjected to identification of lipid molecules (Supplementary table 2 for lysophospholipids, phospholipids, and sphingolipids, and Supplementary table 3 for neutral lipids). The number of identified lipid molecules is listed in Table 1. In total, 64, 56, and 42 lipid molecules (95 in total) were identified as contributing to the separation of the two groups in the plasma of rats receiving 3-day treatment with CPM, IMI, and AMT, respectively, whereas 19, 69, and 67 lipid molecules (88 in total) were identified in the plasma of rats receiving 28-day treatment with CPM, IMI, and AMT, respectively. Subsequently, we explored lipid molecules that were commonly identified in the studies of all three tricyclic antidepressants. In plasma, 22 lipid molecules were commonly identified for either 3- or 28-day treatments (Table 1). These lipid molecules include 16:1 LPC, 18:1 LPC, 18:2 LPC, 20:2 LPC, 20:4 LPC, 22:6 LPC, 32:1 PC, 32:2 PC, 38:5 PC, 38:6 PC, 40:7 PC, 40:8 PC, 34:2 PE, 36:2 PI, 34:1 SM, 42:1 HexCer, 42:2 HexCer, 54:5 TG, 56:6 TG, 56:7 TG, 56:8 TG, and 58:8 TG (Table 2). Of these lipid molecules, LPCs (16:1, 18:1, 18:2, and 20:4 LPC) and 42:1 HexCer were identified as separating the control and treated rats in both 3- and 28-day treatments. In addition, 42:1 and 42:2 HexCers showed more than a twofold increase induced by all three tricyclic antidepressants when treated for 28 days. Thus, we focused on LPCs and

TABLE 1. The Number of Identified and Qualified Lipid Molecules from OPLS-DA of Tricyclic Antidepressants

	3 days					28 days				
Drugs	LPLs	PLs	SLs	NLs	Total	LPLs	PLs	SLs	NLs	Total
CPM	7	14	6	37	64	6	6	4	3	19
IMI	5	19	4	28	56	7	22	11	29	69
AMT	5	16	6	15	42	8	21	4	34	67
Common	4	3	2	5	14	6	5	2	1	14
Total	8	32	11	44	95	8	29	12	39	88

LPL, lysophospholipid; PL, phospholipid; SL, sphingolipid; NL, neutral lipid; CPM, chromipramine; IMI, imipramine; AMT, amitriptyline.

TABLE 2. Fold Changes of Lipid Molecules Commonly Qualified OPLS-DA in All Three Tricyclic Antidepressants

				Average fold changes (/control)					
		Commonality		3 days			28 days		
Lipid classes	Lipid molecules	3 days	28 days	CPM	IMI	AMT	CPM	IMI	AMT
LPC	**16:1 LPC**	+	+	**0.49**	**0.61**	**0.55**	**0.61**	**0.51**	**0.57**
LPC	**18:1 LPC**	+	+	**0.72**	**0.72**	**0.76**	**0.67**	**0.54**	**0.60**
LPC	**18:2 LPC**	+	+	**0.74**	**0.75**	**0.82**	**0.75**	**0.62**	**0.62**
LPC	20:2 LPC	-	+	1.02	0.61	0.64	0.68	0.49	0.49
LPC	**20:4 LPC**	+	+	**0.80**	**0.76**	**0.79**	**0.60**	**0.63**	**0.60**
LPC	22:6 LPC	-	+	0.73	0.79	0.79	0.64	0.60	0.67
PC	32:1 PC	+	-	0.47	0.81	0.59	0.75	0.81	0.35
PC	32:2 PC	+	-	0.49	0.65	0.70	1.11	0.58	0.66
PC	38:5 PC	+		0.72	0.76	0.78	0.85	0.58	0.48
PC	38:6 PC	-	+	0.87	0.96	0.94	0.78	0.62	0.63
PC	40:7 PC	-	+	0.81	0.73	0.66	0.77	0.45	0.47
PC	40:8 PC	-	+	0.86	0.92	0.92	0.74	0.54	0.62
PE	34:2 PE	-	+	0.71	0.62	0.96	1.34	0.47	0.51
PI	36:2 PI	-	+	1.06	1.43	1.31	1.69	1.52	1.63
SM	34:1 SM	+	-	1.14	1.66	1.34	1.37	2.01	1.24
HexCer	**42:1 HexCer**	+	+	**2.30**	**1.69**	**1.78**	**2.27**	**4.91**	**8.19**
HexCer	42:2 HexCer	-	+	2.98	1.67	1.59	2.63	6.43	3.96
TG	54:5 TG	-	+	0.88	0.49	0.89	1.45	0.52	0.55
TG	56:6 TG	+	-	0.28	0.16	0.42	0.80	0.08	0.09
TG	56:7 TG	+	-	0.45	0.24	0.44	0.70	0.10	0.08
TG	56:7 TG	+	-	0.39	0.20	0.49	1.00	0.13	0.17
TG	56:8 TG	+	-	0.55	0.35	0.53	0.92	0.19	0.17
TG	58:8 TG	+	-	0.55	0.29	0.54	0.92	0.19	0.22

Bold letter indicates that the differences in levels of specific lipid molecule between control and drug-treatment exceeded the threshold of OPLS-DA in both 3- and 28-day drug treatment. LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyeline; HexCer, hexosylceramide; TG, triacylglyceride; CPM, chromipramine; IMI, imipramine; AMT, amitriptyline.

HexCers as potential novel plasma biomarkers for hepatic phospholipidosis and included them in the statistical analysis.

Decrease in Plasma LPCs by Drug-Induced Phospholipidosis in Hepatocytes

As shown in Figure 1A, significantly lower levels of selected LPCs (16:1, 18:1, 18:2, and 20:4), except for 20:4 LPC with AMT treatment, were observed after 3-day treatment with CPM, IMI, and AMT. In line with this finding, the levels of all four LPCs were observed to be significantly lower after 28-day treatment with CPM, IMI, and AMT when compared with controls (Fig. 1B). The extent of changes in LPCs induced by treatment was similar for all three tricyclic antidepressants and consistent between 3- and 28-day treatments. In addition, other four LPCs (16:0, 20:2, 20:5, and 22:6 LPC) tended to decrease (but not always significantly) both after 3- and 28-day treatments with all tricyclic antidepressant.

Increase in Plasma 42:1 HexCer Levels by Drug-Induced Phospholipidosis in Hepatocytes

As shown in Figure 2, significantly higher levels of 42:1 HexCer were observed for 3-day treatment only with AMT (Fig. 2A) but for 28-day treatment with all three tricyclic antidepressants (Fig. 2B). The changes in 42:1 HexCer levels were much greater with IMI and AMT than with CPM using a 28-day treatment, when phospholipidosis can be detected in a histological study. In addition, an increase in 42:1 HexCer was strengthened from 3-day to 28-day drug treatment of IMI and AMT. We also investigated a dose-dependent change of plasma 42:1 HexCer levels induced by IMI. As shown in Figure 3, 42:1 HexCer levels increased in a

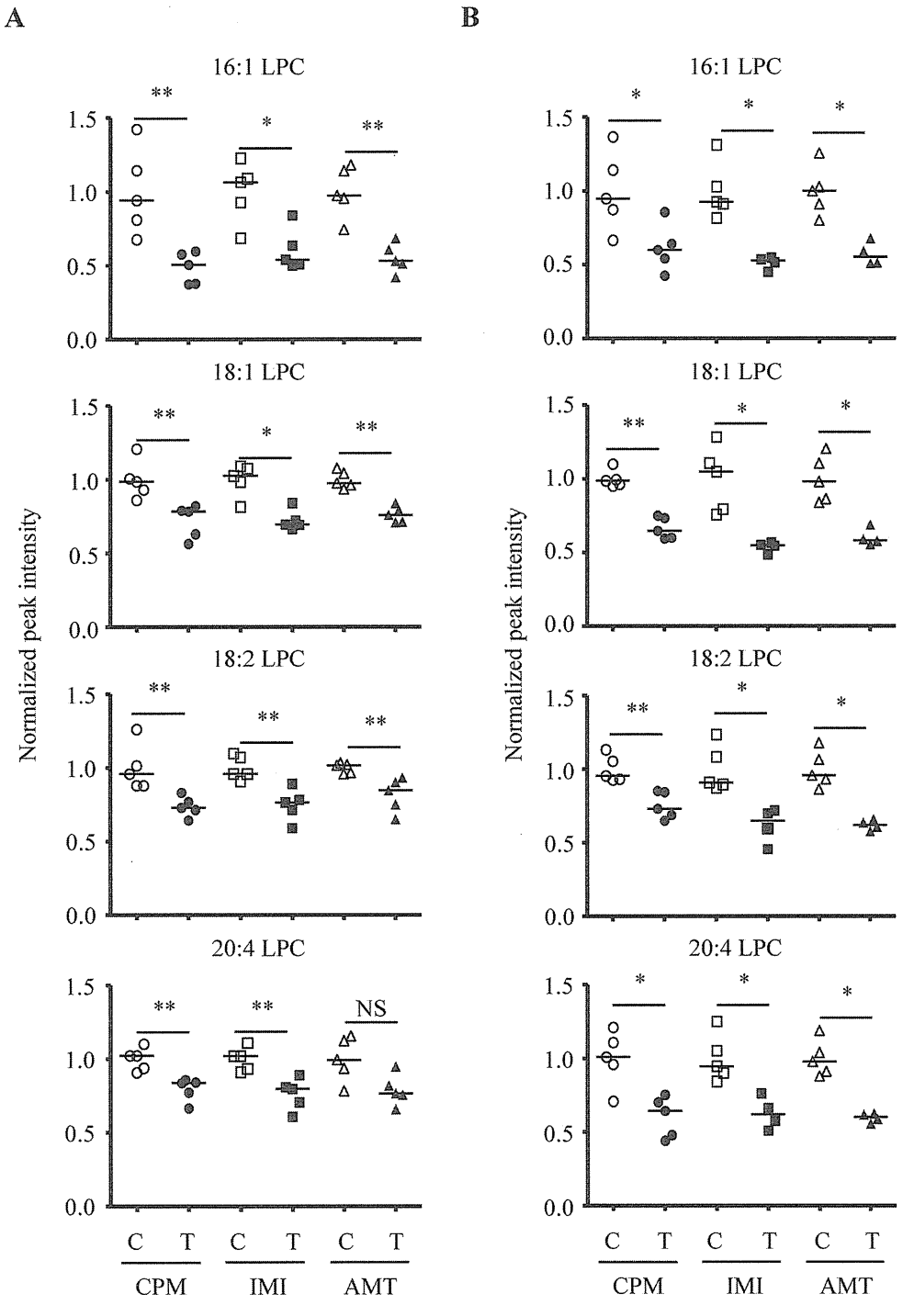

FIG. 1. Plasma levels of LPCs in rats treated with tricyclic antidepressants. Lipid extracts were prepared from the plasma of rats treated with indicated drugs (black) or control solvents (white) for 3 (A) or 28 days (B). The determined ion peak levels of the identified LPCs were normalized to that of the internal standard (12:0/12:0 PE). All data presented were normalized to the average of each control (adjusted average control value as 1). Each spot indicates the level of lipid molecules in each individual animal ($n = 5$ for the control group and $n = 4$ or 5 for the drug-treatment groups). The bar indicates the medians of each group. $^{*}p < 0.05$; $^{**}p < 0.01$ for control versus drug-treated rats. LPC, lysophosphatidylcholine; C, control; T, drug-treated; CPM, clomipramine; IMI, imipramine; AMT, amitriptyline.

dose-dependent manner. Moreover, the increasing trend of 42:1 HexCer induced by tricyclic antidepressants was also observed for other HexCers identified in our study, namely, 42:2 and 40:1 (Supplementary fig. 2).

Decreased LPC and Increased HexCer Levels in Plasma by Drug-Induced Phospholipidosis in Cholangiocytes

Because we found that decreased plasma LPC levels and increased plasma HexCer levels were common for the treatment with all three tricyclic antidepressants that induce hepatic phospholipidosis in hepatocytes, we investigated whether similar

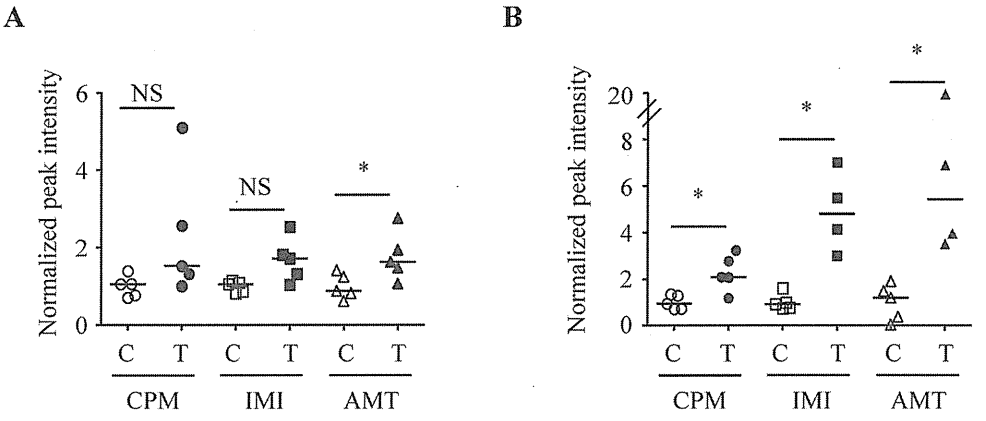

FIG. 2. Plasma levels of 42:1 HexCers in rats treated with tricyclic antidepressants. Lipid extracts were prepared from the plasma of rats treated with indicated drugs (black) or control solvents (white) for 3 (A) or 28 days (B). The determined ion peak levels of 42:1 HexCer were normalized to that of the internal standard (12:0/12:0 PE). All data presented were normalized to the average of each control (adjusted average control value as 1). Each spot indicates the level of lipid molecules in each individual animal ($n = 5$ for the control group and $n = 4$ or 5 for the drug-treatment groups). The bar indicates the medians of each group. $^*p < 0.05$ for control versus drug-treated rats. HexCer, hexosylceramide; C, control; T, drug treated; CPM, chlomipramine; IMI, imipramine; AMT, amitriptyline.

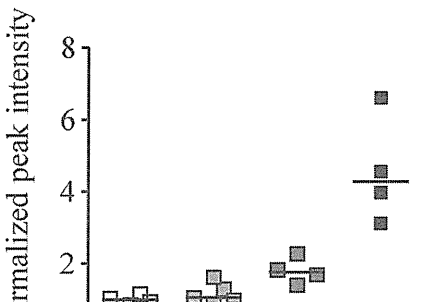

FIG. 3. Dose-dependent effect of imipramine treatment on plasma 42:1 HexCer levels. Lipid extracts were prepared from the plasma of rats treated with an indicated dose of imipramine for 28 days. The determined ion peak levels of 42:1 HexCer were normalized to that of the internal standard (12:0/12:0 PE). All data presented were normalized to the average of control (0 mg/kg/day) (adjusted average control value as 1). Each spot indicates the level of lipid molecules in each individual animal ($n = 4$ for the control group, $n = 5$ for the 10-mg/kg/day group, $n = 4$ for the 30-mg/kg/day group, and $n = 4$ for the 100-mg/kg/day group). The bar indicates the medians of each group. HexCer, hexosylceramide; IMI, imipramine.

changes could be observed for the treatment with KC, which induces hepatic phospholipidosis in cholangiocytes. Thus, we employed the data set obtained from the plasma of rats receiving 3- and 28-day treatment with KC. OPLS-DA and the same threshold values for the score of the loading s-plot led to obtain ion peaks separating control and KC-treated plasma samples. The ion peaks were subjected to the identification of lipid molecules (Supplementary table 2 for lysophospholipids, phospholipids, and sphingolipids, and Supplementary table 3 for neutral lipids). The number of identified lipid molecules is listed in Supplementary table 4. In total, 27 and 46 lipid molecules were identified in the plasma of rats receiving 3- and 28-day treatment of KC, respectively. The lipid molecules included eight LPCs (16:1, 17:0, 18:2, 20:1, 20:2, 20:4, 20:5, and 22:6 LPC) and 42:1 HexCer in the plasma of rats receiving 3-day treatment with KC, whereas only 42:1 HexCer was identified in the plasma of rats receiving 28-day treatment. A statistical analysis revealed significantly lower levels of 16:1 and 18:2 LPCs but not of 18:1 and 20:4 LPCs after 3-day treatment with KC (Fig. 4A). In addition, significantly higher levels of 42:1 HexCer were observed after 3-day treatment with KC. However, no significant changes were observed in any of the LPCs or 42:1 HexCer after 28-day treatment with KC (Fig. 4B).

Determination of Fatty Acid Side Chains of 42:1 HexCer

Because our nontargeting assay platform could only determine the total number of carbon atoms and the double-bond contents of lipid molecules, we next characterized the long-chain base and acyl-side chain of 42:1 HexCer. We determined the fatty acid side chain of sphingolipids by performing positive MS/MS/MS and characterizing the long-chain base (Ishikawa *et al.*, 2014). Because the amount of 42:1 HexCer in sample solution for lipid molecule measurements was too low to perform MS/MS/MS, we fractionized and concentrated 42:1 HexCer using LC. As shown in Figures 5A and 5B, the collection of 42:1 HexCer was verified by chromatography and mass spectrometry. The MS/MS/MS product demonstrated fragments of d18:1 long-chain base and neutral (hexose and hexose+H_2O) loss (Fig. 5C). This result indicates that the side chains of 42:1 HexCer are 18:1 long-chain base (d18:1) and 24:0 acyl-side chain.

Determination of Hexose of 42:1 HexCer

Because there are two HexCers in the body, glucosylceramide and galactosylceramide, we next tried to determine the hexose residue of the identified 42:1 HexCer. Glucosylceramide and galactosylceramide show the same molecular mass and mobility in typical reverse-phase chromatography. Thus, we employed borate-impregnated TLC (Kern, 1966), which allowed to separate glucosylceramide and galactosylceramide by preference of forming a complex with borate (Fig. 6A). Separation and collection of the GluCer and GalCer fraction were confirmed by measuring internal standards both in the GluCer and in GalCer fractions (Fig. 6B). As shown in Figure 6C, 42:1 GluCer rather than 42:1 GalCer is the major component of 42:1 HexCer in plasma. In addition, only 42:1 GluCer was increased after IMI treatment. This result indicates that 42:1 GluCer, but not GalCer, was increased by drug-induced hepatic phospholipidosis.

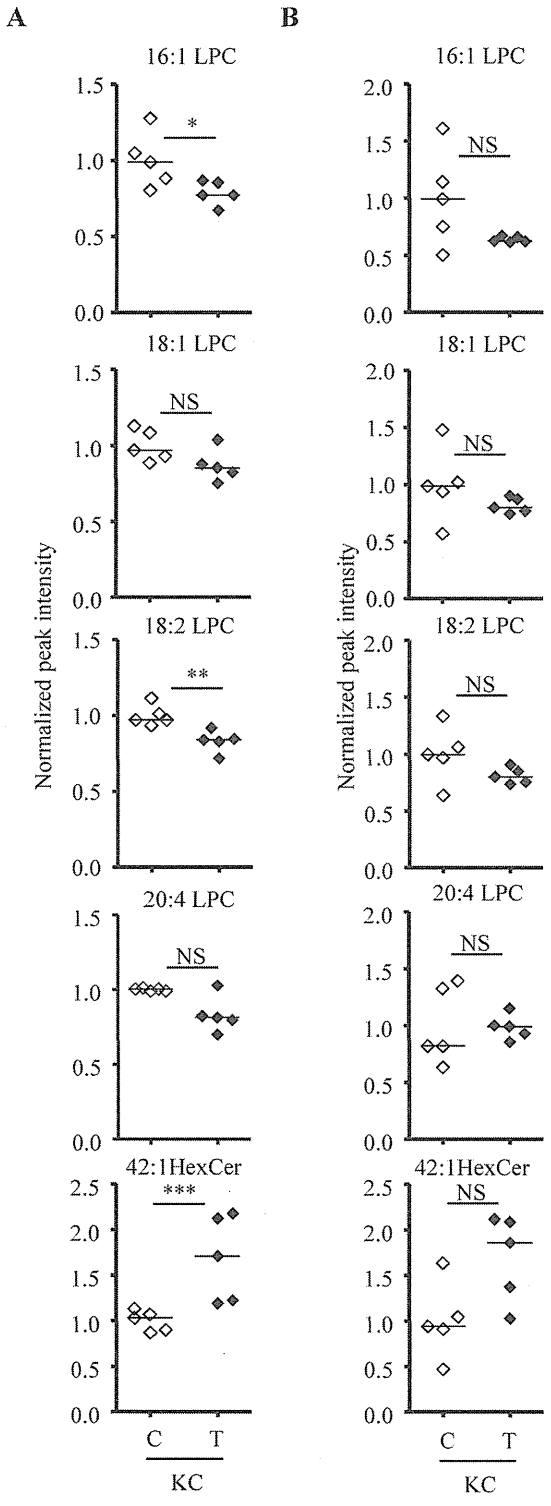

FIG. 4. Plasma levels of LPCs and 42:1 HexCers in rats treated with ketoconazole. Lipid extracts were prepared from rat plasma treated with ketoconazole (black) or control solvents (white) for 3 (A) or 28 days (B). The determined ion peak levels of indicated LPCs and 42:1 HexCer were normalized to that of the internal standard (12:0/12:0 PE). All data presented were normalized to the average of each control (adjusted average control value as 1). Each spot indicates the level of lipid molecules in each individual animal ($n = 5$ for the control group and $n = 5$ for the drug-treatment group). The bar indicates the medians of each group. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$ for control versus drug-treated rats. LPC, lysophosphatidylcholine; HexCer, hexosylceramide; C, control; T, drug treated; KC, ketoconazole.

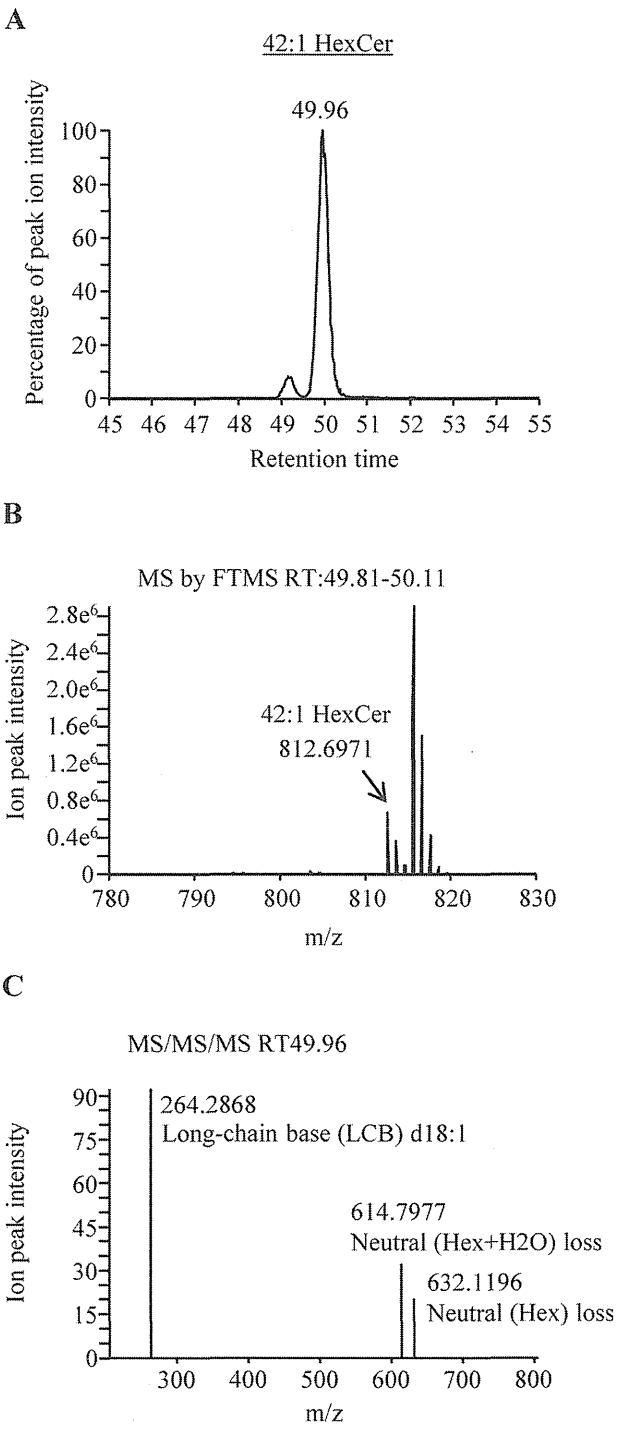

FIG. 5. Extracted ion chromatogram (A), mass spectrum (B), and mass/mass/mass spectrum (C) of the identified 42:1 HexCer. A neutral loss of 18 Da (neutral [H_2O] loss from M + H) from the precursor ion was used as a trigger to obtain the mass/mass/mass spectrum. In the mass/mass/mass spectrum, the deduced ions are described.

DISCUSSION

In the present study, we used the nontargeting lipidomic approach followed by structural characterization to identify biomarkers for drug-induced hepatic phospholipidosis. We used three tricyclic antidepressants (CPM, IMI, and AMT) as the model

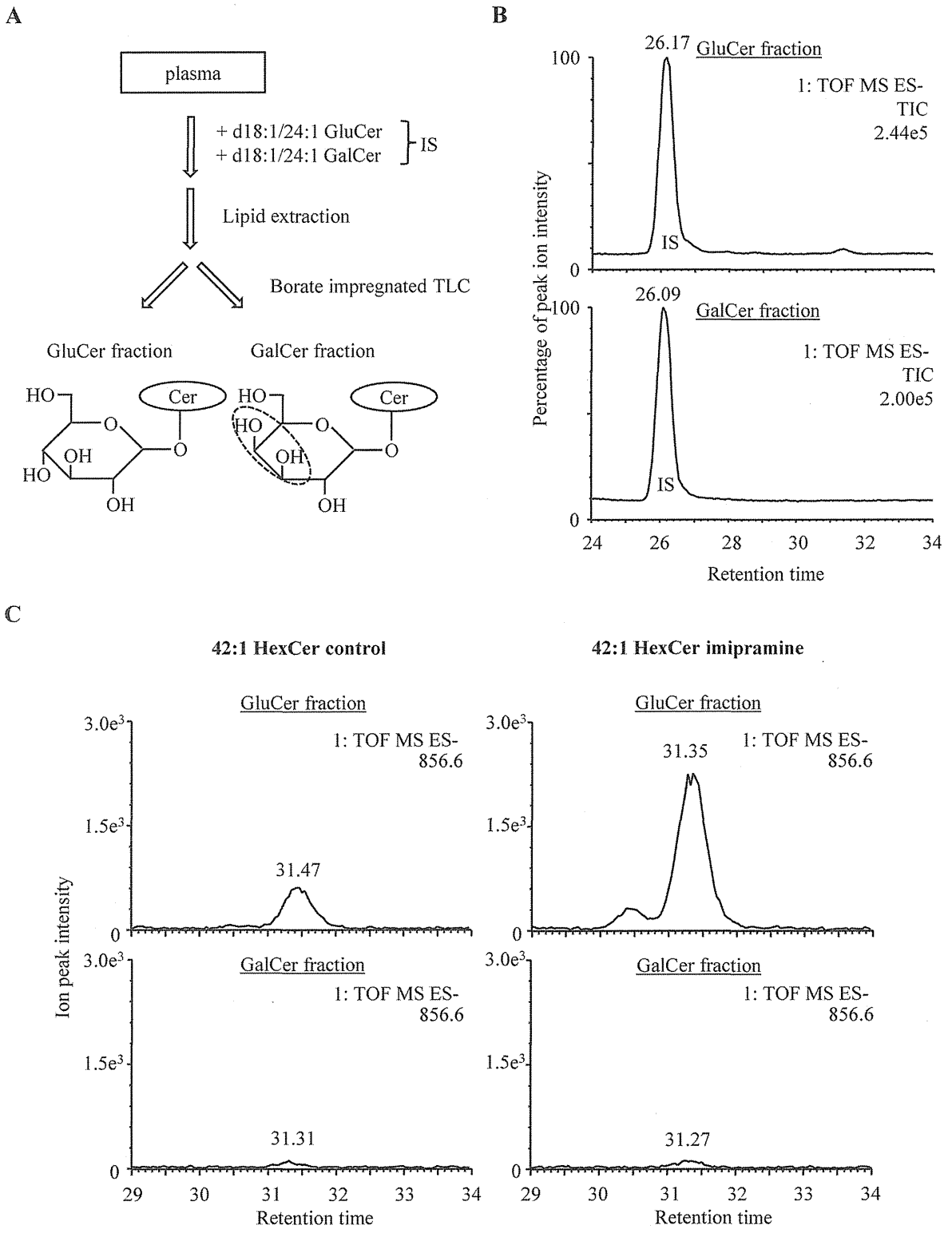

FIG. 6. Differentiation of GluCer from GalCer to the 42:1 HexCer identity. (A) Scheme for separation of GluCer and GalCer by borate-impregnated TLC. Dotted circle indicates a putative site forming the borate complex. (B) Ion chromatograms of the standards of the GluCer and GalCer fractions. (C) Ion chromatogram of 42:1 HexCer in the separated GluCer and GalCer fractions.

of hepatic phospholipidosis in hepatocytes and an antibiotic (KC) as the model of hepatic phospholipidosis in cholangiocytes. Our experiments revealed d18:1/24:0 GluCer and LPCs (especially 16:1 and 18:2 LPC) as putative blood biomarkers for drug-induced hepatic phospholipidosis. The plasma levels of d18:1/24:0 GluCer were increased by drug-induced hepatic phospholipidosis, whereas those of LPCs were decreased. These observations are a common feature of hepatic phospholipidosis among hepatocytes and cholangiocytes. However, no LPCs or d18:1/24:0 GluCer were significantly altered by 28-day treatment with KC, although d18:1/24:0 GluCer was identified to be a lipid molecule contributing to the separation of control and KC-treated rats after 28-day treatment. Thus, the application of d18:1/24:0 GluCer and LPCs as biomarkers for hepatic phospholipidosis in cholangiocytes might be limited in the early stage.

LPCs are lysophospholipids that are generally derived from PCs by the enzymatic action of phospholipase A2. Recently, LPCs have been described as blood biomarkers for several diseases and adverse drug/food effects. For example, plasma levels of 18:1 and 18:2 LPCs compared with total saturated LPC levels have been proposed as potential biomarkers for colorectal cancer (Zhao *et al.*, 2007). In addition, serum levels of 18:2 LPC have been inversely associated with the risk of diabetes (Floegel *et al.*, 2013). Moreover, acetaminophen- and D-galactosamine-induced liver injuries lead to a decrease in LPC levels, such as those of 20:4 LPC, in serum (Cheng *et al.*, 2009; Ma *et al.*, 2014). Therefore, the single use of one LPC for the diagnosis of phospholipidosis might not fully exclude other possibilities, such as colon cancer, diabetes, and liver injuries. Thus, a combinatorial application of d18:1/24:0 GluCer and 16:1 and 18:2 LPCs would be more effective for diagnosing hepatic phospholipidosis.

To date, the mechanisms underlying the decrease in LPCs in plasma remain unclear. However, it has been proposed that CADs inhibit lysosomal enzymes, including phospholipase A2, resulting in the accumulation of phospholipids in the lysosome (Abe *et al.*, 2007; Hiraoka *et al.*, 2006). Because the secretion of LPCs from the liver is quantitatively important for plasma LPC levels (Sekas *et al.*, 1985), the inhibition of hepatic phospholipase A2 might result in a decrease in the levels of LPCs in the plasma.

GluCer is a glycosphingolipid metabolized by glucocerebrosidase in lysosome (Carroll, 1981). When metabolically inert L-GluCer is injected intravenously, it is rapidly removed from the blood and most of it is taken up by the liver (Tokoro *et al.*, 1987). Subsequently, hepatic L-GluCer is excreted through the bile duct with a 3.5-day half-time. These observations suggest that blood GluCer is predominantly taken up by the liver to be metabolized by glucocerebrosidase to ceramide or be excreted from the bile duct. Thus, one possible explanation for the increased plasma d18:1/24:0 GluCer levels in drug-induced hepatic phospholipidosis is decreased GluCer clearance in the liver via the inhibition of hepatic lysosomal glucocerebrosidase in hepatocytes or biliary excretion of GluCer in cholangiocytes.

Although all tricyclic antidepressants used in the present study induced hepatic phospholipidosis, the severity of phospholipidosis varied between the drugs. All rats treated with IMI and AMT for 28 days showed hepatic phospholipidosis, with considerable severity in the case of AMT treatment, whereas only two of five rats treated with CPM for 28 days showed symptoms of hepatic phospholipidosis (Supplementary table 1). The increases in the plasma levels of d18:1/24:0 GluCer and its related HexCers (42:2 and 40:1 HexCer) were more drastic by IMI and AMT than by CPM. In addition, the dose-dependent increase in the plasma levels of d18:1/24:0 GluCer by IMI was consistent with the dose-dependent severity of the phospholipidosis that was induced by IMI treatment (Hirode *et al.*, 2008). These findings may indicate that the extent of elevation of d18:1/24:0 GluCer could reflect the severity of drug-induced hepatic phospholipidosis in hepatocytes. However, each CAD has a different preference for the formation of phospholipidosis in the tissues, and it remains unclear whether phospholipidosis progressing in the tissues other than the liver would modulate the plasma levels of d18:1/24:0 GluCer.

Unlike severity-dependent increase in d18:1/24:0 GluCer, a decrease in plasma LPC levels induced by 28-day treatment with tricyclic antidepressants was irrespective of the type of the drug. In addition, all tricyclic antidepressants resulted in a similar decrease of LPC levels irrespective of whether it was a 3- or 28-day drug treatment. Thus, the decrease in LPCs might not correlate with the severity as well as progression of drug-induced hepatic phospholipidosis in hepatocytes. The levels of LPCs (except for 20:4 LPC in AMT treatment) were significantly decreased even after 3-day treatment with all tricyclic antidepressants. Decreased LPC levels with a 3-day treatment of KC were also observed. Thus, LPCs could be useful to predict the risk of hepatic phospholipidosis.

In conclusion, d18:1/24:0 GluCer and LPCs are putative plasma biomarkers for hepatic phospholipidosis. Because a single marker, especially one LPC, is not specific for hepatic phospholipidosis, a combinatorial application of d18:1/24:0 GluCer and 16:1 and 18:2 LPCs would be more effective for diagnosing hepatic phospholipidosis. In addition, d18:1/24:0 GluCer could reflect the severity of drug-induced hepatic phospholipidosis in hepatocytes, whereas LPCs could be useful to predict the risk of both types of hepatic phospholipidosis. Future research and validation of d18:1/24:0 GluCer and LPCs as plasma biomarkers for hepatic phospholipidosis are needed to extrapolate these findings to humans.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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Effects of Sex, Age, and Fasting Conditions on Plasma Lipidomic Profiles of Fasted Sprague-Dawley Rats

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Abstract

Circulating lipid molecules reflect biological processes in the body and, thus, are useful tools for preclinical estimation of the efficacy and safety of newly developed drugs. However, background information on profiles of circulating lipid molecules in preclinical animal models is limited. Therefore, we examined the effects of multiple factors such as sex (fasted male vs. female), age (fasted 10 vs. 30 weeks old), and feeding conditions (feeding vs. fasting, 16 vs. 22 hr fasting, 10 AM vs. 4 PM blood collection), on the global profiles of lipid molecules in plasma from Sprague-Dawley rats by using a lipidomic approach. Our assay platform determined 262 lipid molecules (68 phospholipids, 20 sphingolipids, 138 neutral lipids, and 36 polyunsaturated fatty acids and their metabolites) in rat plasma. Multivariate discriminant analysis (orthogonal partial least squares discriminant analysis) and heat maps of statistically significant lipid molecules revealed that the plasma lipid profiles in rats are predominantly influenced by feeding conditions, followed by sex and age. In addition, the fasting duration (16 vs. 22 hr fasting) or the time of blood collection (10 AM vs. 4 PM blood collection) has limited or no contribution on the profiles of lipid molecules in rat plasma. Our results provide useful, fundamental information for exploring and validating biomarkers in future preclinical studies and may help to establish regulatory standards for such studies.

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Introduction

Circulating metabolites are useful tools to diagnose diseases such as lung and gastrointestinal cancer [1–3]. The advantages of diagnostic applications using circulating metabolites are non- or low-invasiveness, as well as homogeneity between humans and experimental animal models. These advantages are also shared with the application of circulating metabolites as biomarkers for estimation of the efficacy and safety of newly developed drugs. The rat is a useful and essential animal model for preclinical studies investigating drug metabolism, pharmacokinetics, and toxicological tests [4,5]. Therefore, background data of circulating metabolites in rats are useful in designing such studies.

Lipids, including fatty acids and their metabolites, phospholipids, sphingolipids, and neutral lipids, are one major class of circulating metabolites, and their hydrophobicity could allow their levels in blood to substantially reflect their levels in tissues (e.g., liver and brain tissues) [6,7]. Phospholipids and sphingolipids are major components of cell membranes, and neutral lipids serve as energy sources for the cells. The phospholipid 18:0/18:1 phosphatidylcholine (PC) acts as a circulating regulator of fatty acid uptake in muscle [8], and ceramides (Cer), a class of sphingolipids, mediate saturated fatty acid-induced insulin resistance [9]. Fatty acid metabolites such as arachidonate metabolites are signaling molecules of inflammatory response [10]. Therefore, lipids are potential biomarkers to predict the efficacy and toxicity of newly developed drugs.

Circulating lipids in human have been reported to vary with blood matrices and subjects' backgrounds, including sex and age, as well as sample collection and storage conditions. Our previous reports demonstrated that plasma and serum, two matrices obtained from blood, presented different profiles of circulating lipids [11,12]. For example, the levels of more than 100 lipid molecules showed differences between plasma and serum samples from aged women [12]. Sphingolipids are present at higher concentration in blood in female than in male individuals [12–14]. Storage temperature and freezing-thawing cycles affect the levels of many lipid species, including diacylglycerols (DGs) [12,15]. In addition, of 80 lipids circulating in the plasma, majorities were present at varying levels among different anticoagulant supplements (citrate, EDTA, and heparin) [16]. However, few comprehensive studies focusing on the factors affecting circulating lipids have been performed in preclinical animal models such as rats.

A lipidomic approach is a high-throughput measurement of a broad range of lipid molecules [17,18]. This method applies liquid chromatography for the separation of lipid molecules and mass