表1-A 本研究に用いた日本人健常ボランティアの情報と検体の種類

Groups	[JMY] Japanese Young male	[JFY] Japanese Young female	[JMO] Japanese Elderly male	[JFO] Japanese Elderly female	Statistical significance
Gender	Male	Female	Male	Female	
Number	15	15	15	15	
Median age [range]	32 [25-35]	32 [25-35]	60 [55-64]	60 [55-65]	
Median Weight (Kg) [range]	64 [53-72]	54 [44-73]	65 [53-83]	51 [43-61]	[JMY] vs. [JFY] (p=0.0054), [JMO] vs. [JFO] (p<0.0001), [JMY] vs. [JMO] (N.S), [JFY] vs. [JFO] (N.S)
Median height (cm) [range]	171 [165-180]	162 [151- 175]	167 [161-182]	156 [143- 161]	[JMY] vs. [JFY] (p=0.0001), [JMO] vs. [JFO] (p<0.0001), [JMY] vs. [JMO] (N.S), [JFY] vs. [JFO] (p=0.0039)
Median BMI [range]	22 [19-24]	20 [19- 25]	23 [20-25]	21 [18- 25]	[JMY] vs. [JFY] (N.S.), [JMO] vs. [JFO] (p=0.0265), [JMY] vs. [JMO] (p=0.0161), [JFY] vs. [JFO] (N.S.)
matrices	plasma	plasma	plasma	plasma	

N.S: not significant

表1-B 本研究に用いた若年男性ボランティア(日本人、黒人、白人)の情報と検体の種類

Groups	[JMY] Japanese Young male	[AMY] African Young male	[CMY] Caucasian Young male	Statistical significance
Gender	Male	Male	Male	
Number	15	15	15	
Median age [range]	32 [25-35]	29 [26-33]	29 [25-33]	
Median Weight (Kg) [range]	64 [53-72]	74 [54-130]	78 [52.2-113.9]	[JMY] vs. [AMY] (p=0.0037), [JMY] vs. [CMY] (p=0.0225), [AMY] vs. [CMY] (N.S.)
Median height (cm) [range]	171 [165-180]	180 [165- 196]	172.7 [154.9-185.4]	[JMY] vs. [AMY] (p=0.0037), [JMY] vs. [CMY] (p=0.0062), [AMY] vs. [CMY] (p=0.0379)
Median BMI [range]	22 [19-24]	22 [18- 34]	26.2 [18.0-36.6]	[JMY] vs. [AMY] (N.S.), [JMY] vs. [CMY] (p=0.0062), [AMY] vs. [CMY] (N.S.)
matrices	plasma	plasma	plasma	

N.S: not significant

表2 UPLC-TOFMSのネガティブイオンモードで検出された脂質代謝物のクラスと主な分子種

Lipid Class	Lipid subclass	Abbreviation	Number of detected species
Glycerophospholipids	Lysophosphatidylcholine	LPC	6
	Phosphatidylcholine	PC	35
	Ether PC	ePC*	19
	Phosphatidylethanolamine	PE	7
	Ether PE	ePE*	16
	Phosphatidylinositol	PI	9
Sphingolipids	Sphingomyelin	SM	26
	Ceramide	Cer	4
	Cerebroside	CB	6
Total count			128

*エーテルPCに関しては、アルキルエーテル型及びアルケニルエーテル型を含む

表3　グリセロリン脂質・スフィンゴ脂質のうち性差・年齢差・人種差を示した代謝物の内訳とその増減

Statistical Comparisons						
Mann whitney t -Test	Gender Comparison: 14h fast, AM collection		Age comparison: 14h fast, AM collection		Ethnic comparison: 14h fast, AM collection	
	JFY JMY	JFO JMO	JMO JMY	JFO JFY	CMY JMY	AMY JMY
Total biochemicals $p \leq 0.05$	10 (7.8%)	20 (15.6%)	40 (31.3%)	31 (24.2%)	45 (35.2%)	60 (46.9%)
Biochemicals (Increase | Decrease)	10 | 0 (7.8%) | (0%)	18 | 2 (14%) | (1.6%)	37 | 3 (29%) | (2%)	24 | 7 (19%) | (5%)	15 | 30 (12%) | (23%)	39 | 21 (30%) | (16%)
LPC (6 in total)	0 | 0	0 | 2	1 | 0	2 | 0	1 | 1	4 | 1
PC (35 in total)	3 | 0	3 | 0	16 | 0	6 | 0	4 | 6	9 | 5
ePC (19 in total)	2 | 0	0 | 0	1 | 3	1 | 6	3 | 5	10 | 2
PE (7 in total)	1 | 0	3 | 0	1 | 0	3 | 0	2 | 2	4 | 2
ePE (16 in total)	0 | 0	1 | 0	5 | 0	5 | 0	3 | 6	9 | 5
PI (9 in total)	3 | 0	0 | 0	8 | 0	1 | 0	2 | 2	2 | 1
SM (26 in total)	1 | 0	11 | 0	1 | 0	6 | 0	0 | 6	1 | 5
Cer (4 in total)	0 | 0	0 | 0	4 | 0	0 | 0	0 | 1	0 | 0
CB (6 in total)	0 | 0	0 | 0	0 | 0	0 | 1	0 | 1	0 | 0
Common Biochemicals (Increase | Decrease)	1 | 0 (0.8%) | (0%)		13 | 3 (10%) | (2%)		15 | 19 (12%) | (15%)	

表4 薬物性肝障害患者12名の内訳と臨床検査値等

臨床病型	人数	性別 (男/ 女)	年齢 中央値(範囲)	ALT (U/L) 中央値(範囲)	ALP (U/L) 中央値(範囲)	T.bil (mg/dL) 中央値(範囲)	2004DDW-Jスコア 中央値(範囲)
肝細胞障害型	9	(4, 5)	63 (33-80)	1362 (80-4093)	360 (219-1398)	1.38 (0.5-6.2)	7 (4-10)
混合型	2	(0, 2)	50 (29-70)	341 (290-391)	1225 (1057-1398)	2.57 (1.28-3.85)	6 (5-7)
不明	1	(0, 1)	77	U.N	U.N	U.N	U.N

U.N, unknown

図1-A　PLS-DA解析による日本人若年・老年男女の判別

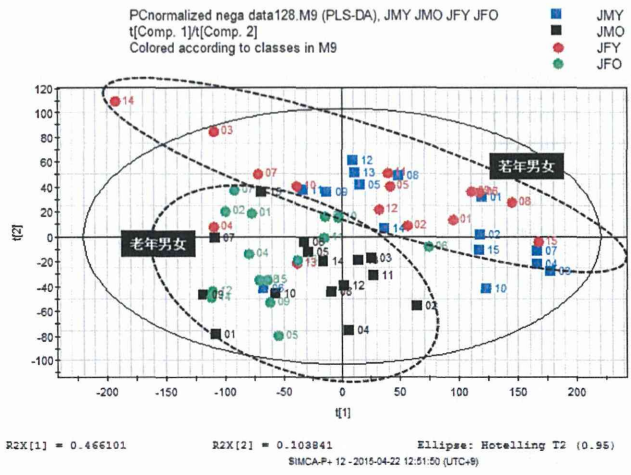


図1-B　PLS-DA解析による日本人・白人・黒人の若年男性の判別

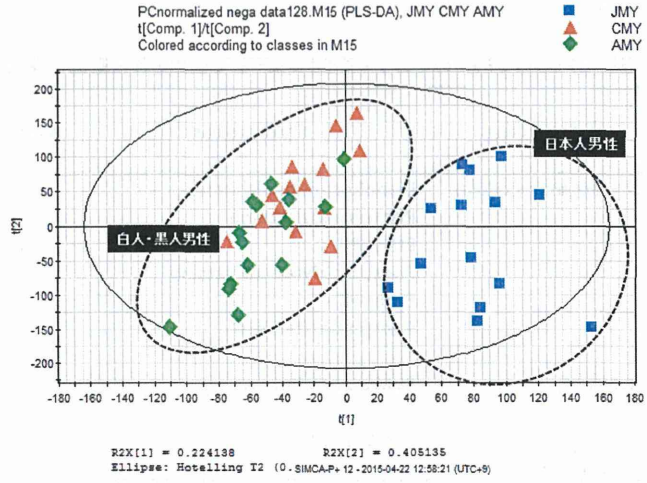
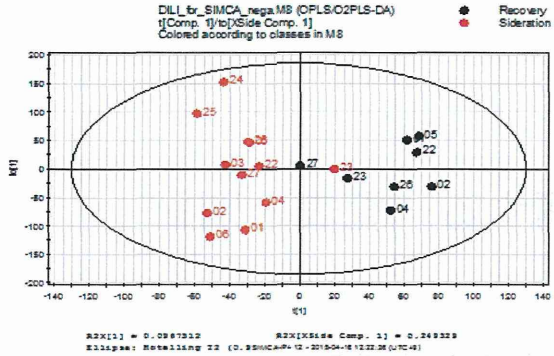
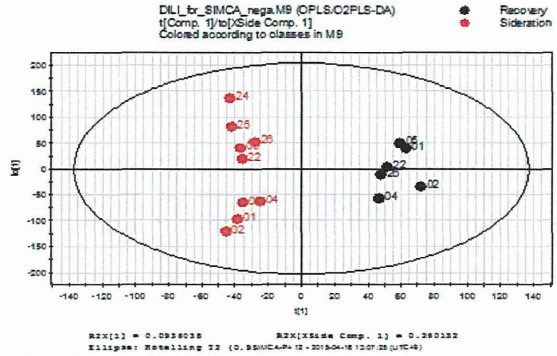


図2 薬物性肝障害患者の急性期と回復期における血漿脂質メタボロームのOPLS-DA解析による判別

(A) 12患者20サンプル
全臨床病型を含む（肝細胞障害型・混合型・不明）



(B) 9患者15サンプル
（肝細胞障害型のみ）



(C) 9患者15サンプル（肝細胞障害型のみ）によるSプロット

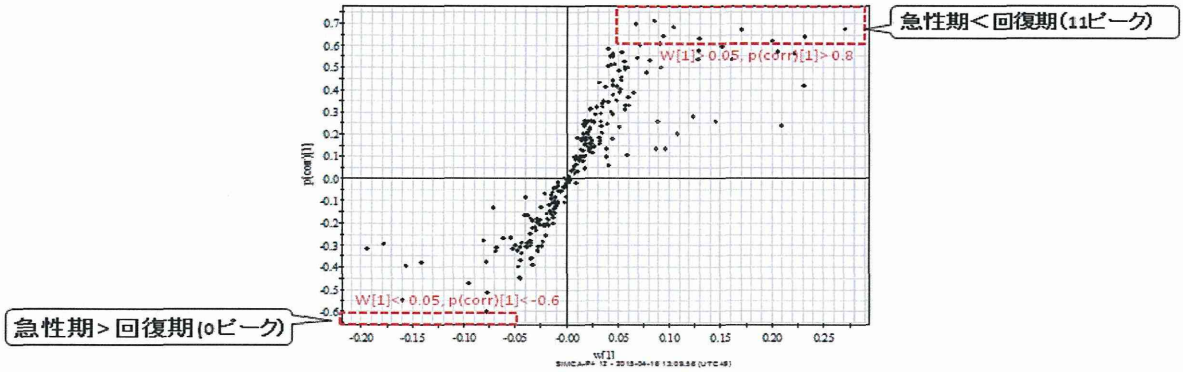
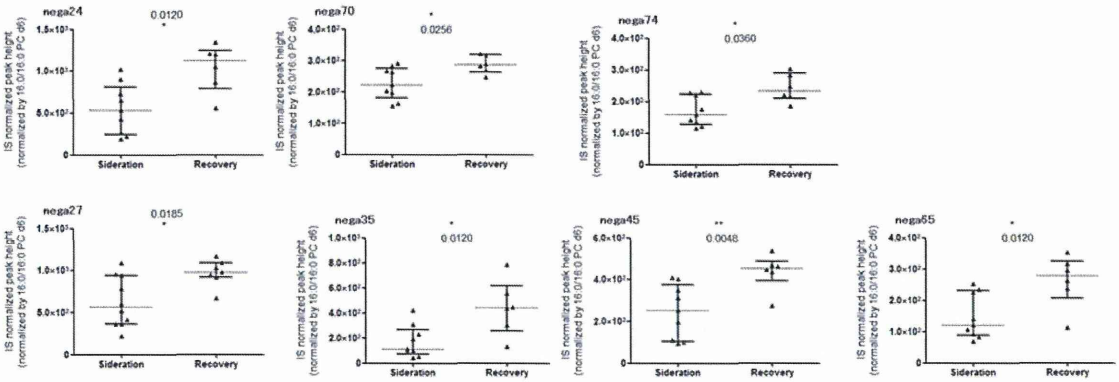


図3 OPLS-DAプロットより見出された肝細胞障害型の薬物性肝障害の急性期と回復期を判別するバイオマーカー候補（リン脂質、スフィンゴ脂質）



III. 研究成果の刊行に関する一覧表と別刷

研究成果の刊行に関する一覧表

書籍

著者氏名	論文名	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
鈴木孝昌	コンパニオン診断薬の現状と課題		最先端バイオマーカーを用いた診断薬/診断装置開発と薬事対応	技術情報協会	東京	2015	271-275

雑誌

著者氏名	論文名	雑誌名	巻	ページ	刊行年
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	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	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、斎藤嘉朗	薬物性肝障害の遺伝的素因	別冊「医学のあゆみ」内科領域の薬剤性障害　肝・肺を中心に	2014.11	11-18	2014
Nishikawa K, Iwaya K, Kinoshita M, Fujiwara Y, Akao M, Sonoda M, Thiruppathi S, Suzuki T, Hiroi S, Seki S, Sakamoto T.	Resveratrol increases CD68+ Kupffer cells co-localized with adipose differentiation- related protein (ADFP) and ameliorates high-fat-diet-induced fatty liver in mice.	Mol Nutr Food Res.	in press		

第10章　体外診断薬、コンパニオン診断／POCT／遺伝子検査の開発・評価例

第1節　コンパニオン診断薬の現状と課題

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(株)技術情報協会　発刊書籍
「最先端バイオマーカーを用いた診断薬/診断装置開発と薬事対応」(2015.1.30 発刊)
抜刷

第1節　コンパニオン診断薬の現状と課題

はじめに

個の医療の実現に向け，新規医薬品とコンパニオン診断薬が原則同時に承認申請を行うこととなり，コンパニオン診断薬の重要性は増している。従来の単なる病気の診断という役割から一歩踏み込んで，医薬品の効果副作用を予測し，その有効性を担保する役割を担う立場へと進化し，新薬開発成功の鍵を握る存在となっている。こうしたパラダイムシフトともいうべき急激な変化を遂げるコンパニオン診断薬の開発と規制に関する現状と今後の課題について，レギュラトリーサイエンスという観点から解説を加える。

1.　コンパニオン診断薬の現状

本邦のコンパニオン診断薬に関する規制，及び開発動向は，欧米に比べてやや出遅れていたが，2013年7月1日に厚生労働省医薬食品局審査管理課長通知として「コンパニオン診断薬及び関連する医薬品の承認申請に係る留意事項について」（薬食審査発0701第10号）が発出され，コンパニオン診断薬に関する基本的な考え方が示され，FDAと同様に原則コンパニオン診断薬は関連する医薬品との同時に承認申請がなされるべきであるという方向性が明確となった。また，この通知に先んじて，クリゾチニブに対するALK融合遺伝子検出キット（Vysis ALK Break Apart FISH）とモガムリズマブに対するCCR4検出キット（ポテリジオテスト）がコンパニオン診断薬としての承認を受けており，日本国内においても，コンパニオン診断薬の開発と承認がスタートラインについたと言える。さらに，がんの分子標的薬に代表されるように，治療標的自身がバイオマーカーとして診断対象となる場合や，薬物代謝酵素の多型が薬効の個体差を規定するバイオマーカーになる例など，さまざまなケースで医薬品の有効性，安全性の予測のためにいわゆるコンパニオン診断薬が使用されるケースが出てきているが，新薬と同時開発されたコンパニオン診断薬として承認を得ている例はまだ少ない。これには，従来型の診断薬とは全く違ったアプローチによる開発が必要である点，ガイドライン等の承認申請をめぐる指針がまだ整備されていなかった点も影響しているが，規制に関しては，2013年12月24日にPMDAより「コンパニオン診断薬及び関連する医薬品開発に関する技術的ガイダンス」及びそのQ&Aが発表され，今後はコンパニオン診断薬としての臨床応用へ向けた動きが加速すると予測される。こうしたコンパニオン診断薬を取り巻く国内外の状況について，開発および規制という二つの観点から解説する。

1.1　コンパニオン診断薬の開発動向

既に開発が行われ，承認を受けたコンパニオン診断薬の事例を，対象となる薬剤とともにまとめたのが表1である。広くコンパニオン診断薬を，薬の有効性安全性を予測して患者を選択するための診断薬と位置づけた場合，初期においては既存のマーカーが指標となるものや，薬が先行して後付けで診断薬が作られるケースもあったが，最近の承認例を見ると，薬剤の開発と平行してその診断薬が開発される，真の意味でのコンパニオン診断薬の事例が増えており，今後は規制の動向を受けて，新薬との同時開発が一般的となることが予想される。特に分子標的薬の領域では，治療のターゲットとなるバイオマーカーがそのまま診断指標となるケースは多く，抗体医薬品の場合には，そのまま診断薬用の抗体として応用できる可能性も考えられるため，コンパニオン診断薬の開発が容易であると考えられる。

診断技術（方法）という観点から既存のコンパニオン診断薬を分類した場合，抗体を用いて標的タンパクの発現を免疫染色等で調べる方法，遺伝子多型や変異遺伝子をRT-PCR等を使って検出する方法，また転座融合遺伝子などの染色体変化をFISH法を用いて検出する方法に大別される。また今後は，DNAマイクロアレイや，次世代シークエンス技術などを応用した手法など診断技術も多様化することが予想される。

表1　コンパニオン診断薬および関連医薬品の承認事例

承認	医薬品	適応	薬剤標的	診断標的	診断薬	手法
1998年9月（米国） 2001年5月（日本）	Herceptin （trastuzumab） 【Genentech】	HER2-positive breast cancer	HER2	HER2 over-expression	PathVysion (Abbott Molecular) 【1998年12月（米国） 2001年1月（日本）】 HercepTest (Dako) 【2010年10月（米国） 2001年5月（日本）】 他	FISH IHC
2011年8月（米国）	Zelboraf （vemurafenib） 【Genentech】	Metastatic melanoma with BRAF mutation	BRAF kinase	BRAF mutation （V600E）	Cobas 4800 BRAF V600 Mutation Test（Roche Molecular Systems） 【2011年8月（米国）】	RT-PCR
2011年8月（米国） 2012年3月（日本）	Xalkori（crizotinib） 【Pfizer】	NSCLC with ALK rearrangement	ALK kinase	EML4-ALK fusion	Vysis ALK Break Apart FISH Probe Kit（Abbott Molecular） 【2011年8月（米国） 2012年2月（日本）】	FISH
2012年3月（日本）	ポテリジオ（モガリズマブ） 【協和発酵キリン】	再発または難治性のCCR4陽性成人T細胞白血病リンパ腫（ATL）	CCR4	CCR4	ポテリジオテスト（協和メディックス） 【2012年3月（日本）】	IHC FCM
2001年5月（米国） 2001年11月（日本） 2003年7月（日本）	Gleevec（imatinib） 【Novartis】	Chronic myeogenous leukemia（CML） KIT positive GIST	Bcr-Abl kinase KIT kinase	Bcr-Abl translocation c-Kit（CD177）	c-Kit pharmDX（DAKO） 【2012年11月（米国）】	IHC
1994年1月（日本） 1998年10月（米国）	カンプト，Camptosar （irinotecan） 【ヤクルト，Pfizer】	非小細胞肺癌，小細胞肺癌，子宮頸癌，卵巣癌	topoisomerase	UGT1A1多型 （UGT1A1*28, *6）	Invador UGT1A1 Molecular Assay（Third Wave Technologies） 【2005年8月（米国）2008年6月（日本）】	Invador
2002年7月（日本） 2003年5月（米国） 2013年7月（米国）	Iressa（gefinitib） 【Astra Zeneka】 Gilotrif（afatinib） 【Boehringer Ingelheim】	NSCLC	EGFR	EGFR変異	therascreen EGRF RGQ PCR kit（Qiagen） 【2013年7月（米国）2012年1月（日本）】	RT-PCR
2004年11月（米国） 2007年10月（日本）	Tarceva（erlotinib） 【OSI Pharmaceuticals】	NSCLC	EGFR	EGFR変異	Covas EGFR Mutation Detection Test（Roche Molecular Systems） 【2013年5月（米国）】	RT-PCR
2004年2月（米国） 2008年7月（日本）	Erbitux（cetuximab） 【Imclone】	Colorectal cancer	EGFR	KRAS変異 EGFRタンパク	therascreen KRAS RGQ PCR kit（Qiagen） 【2012年7月（米国） 2011年4月（日本）】 EGRF pharmDX kit（DAKO） 【2006年9月（米国）】	RT-PCR IHC
2014年7月（日本）	アレセンサ（アレクチニブ） 【中外製薬】	NSCLC	ALK kinase	ALK融合遺伝子	ヒストファインALK iAEPキット（ニチレイバイオサイエンス） 【2014年7月（日本）】	IHC

1.2　コンパニオン診断薬に対する規制動向

米国においては保険制度の影響もあって，診断薬としてFDAの承認をとらずにCLIA認証を受けたラボにて行ういわゆるLDTがコンパニオン診断薬として使われるケースもあったが，品質，有効性に関する懸念とこれからは新規医薬品の承認のために使われるという位置づけで，FDAとしてもきちんと承認申請を経た体外診断薬として規制を行いたいという意図から，2011年7月にコンパニオン診断薬に関するガイダンス案[1]を発表し，2014年8月に正式版となったが，これにより原則的にコンパニオン診断薬とそれを利用する新薬は同時承認とするという姿勢を公表した。そして，FDAにおいても薬剤と診断薬の審査部門が相互に連携を図りながら審査に当たることが示された。今後は，コンパニオン診断薬と関連したLDTを巡る米国での規制動向が注目される。

一方で，欧州薬品庁（EMA）も同様にコンパニオン診断薬に関するガイドライン案[2,3]を2010年6月に出しているが，こちらはFDAほどには積極的な姿勢を打ち出しておらず，少し温度差がある。これには，欧州では各国の事情があって統一的な規制がしづらい点や，CEマークを取得すれば販売が可能となる点などが影響していると考えられる。開発における注意点や，医薬品開発の早期段階からの開発を開始することの重要性を指摘している点で，審査というよりも開発のためのガイドラインという色彩が強い。いずれにしても，欧米においてこのようなガイドラインが公表されたことは，個の医療の実現に向けたコンパニオン診断薬の重要性を規制当局が認識していることを反映するもので，日本においても同様のガイドラインの作成が望まれていた。

これを受けてPMDAにおいてコンパニオン診断薬プロジェクトが立ち上がり，2013年7月のコンパニオン診断薬に関する厚生労働省の課長通知[4]発出へとつながった。これはおおむねFDAのガイドラインに沿った内容となっており，コンパニオン診断薬の承認申請は，それを利用する医薬品の承認申請と原則として同時期に行われるべきとされている。そして，開発側とあわせ審査を行う側においても，医薬品と診断薬の各部門が緊密に連携をしていくことの重要さが改めて示されている。

これらガイドライン（通知）においては，開発や審査における基本的な姿勢が示されているものの，コンパニオン診断薬のとしての承認のための具体的な技術要件に関しては記載されていない。そこで2013年12月24日にPMDAより「コンパニオン診断薬及び関連する医薬品開発に関する技術的ガイダンス」及びそのQ&Aが発出された[5]。ここでは，臨床試験において，バイオマーカーにより患者を特定する際の留意点として，バイオマーカー陰性例の取り扱い，前向きな臨床試験の必要性と実施時期，コンパニオン診断薬としてのバリデーション試験の実施時期等について述べられている。また，コンパニオン診断薬の評価に関して，臨床的意義，同等性試験，及び分析法バリデーションに関する考え方が示されている。さらに最近では，コンパニオン診断薬の承認書記載に関する留意事項に関する医療機器審査管理室長通知も発出されている[6,7]。

こうしたコンパニオン診断薬としての特徴を考慮しつつ，体外診断薬自体としての評価を行う場合には，従来ある技術的要件を適応すれば評価は可能であるが，今後問題となるのは，DNAチップ等の新規診断手法を伴う次世代型の診断薬としての評価基準であり，新しいコンパニオン診断薬の評価においては，こうした新規の技術の評価が課題となることが予想される。合わせて，コンパニオン診断薬の開発においては，当該医薬品の最終的な臨床試験においてその有効性が確立されている必要があるため，承認申請用の臨床試験データが得られる前に，それまでの限られたデータから開発者自身がその評価を確定させなければならず，そのための指針となるべき開発のためのガイドラインの整備も望まれている。

2.　コンパニオン診断薬に関する課題

2.1　何が今までと違うのか

診断薬として必要とされる性能及び技術要件は従来の診断薬の場合と変わらないものの，コンパニオン診断薬の特徴としては，医薬品との同時承認が前提となる点が大きく異なる。このためには，診断薬の開発は医薬品開発のかなり早期から相互に連携をとりながら行われる必要があり，医薬品メーカーと診断薬メーカーとの関係は従来型からの大きな転換を迫られており，新しいビジネスモデルの構築が急務である。診断薬部門を自社グループ内に持つメーカーはその

点有利であるが，他社との連携が必要とされる場合が多く，利益性の低い診断薬サイドの開発中止を含めた利益保証をどのように行うかは，直面する大きな課題となっている。

2.2 規制に関する課題

コンパニオン診断薬に関する厚生労働省の課長通知が発出され，本邦においてもコンパニオン診断薬と関連する医薬品に対する審査側の姿勢が明らかとなり，技術的ガイダンス等も示されたことにより今後の方向性が明らかとなった。基本的には米国と同様に原則医薬品との同時承認が原則となるが，もともと日本においては保険償還のために承認申請が前提となっていた点でLDTとしての診断薬が普及していた米国ほどの混乱はないと考えられる。

医薬品とそのコンパニオン診断薬の臨床性能とは相互依存しており，選択した患者群での医薬品の有効性が示されれば，医薬品と同時に診断薬としての性能も担保されたことになる。このためには遅くともPhaseIII，できればPhaseII臨床試験に入る前に申請を行う製品版の体外診断薬が準備されている必要があり，これを用いて臨床試験の患者選択を行う必要がある。臨床試験の途中における開発品から製品版への変更もあり得るが，この際に異なる診断薬で取得したデータ間のブリッジングをいかに保証するか，さらにそれを審査においてどのように評価するかも，今後の重要な課題となりうる。さらに，同一のバイオマーカーを測定するキットの取り扱いや，目的とする医薬品のコンパニオン診断以外の診断への応用なども課題となることが予想される。

また，有効性のある患者群を選択して投薬を行う場合，無効であると予測された患者群（バイオマーカー陰性例）を臨床試験においてどう扱うかも新たな課題となることが予想される。無効であると予測された患者群に対して薬剤が無効であることを証明するためには，それらの患者に対しても投薬を行う必要があり，倫理的側面も含めてどの程度そのようなデータを要求するかは規制側の課題となる。無効とされた患者群に対して他の薬剤の選択肢がある場合には良いが，それがない場合に，患者が投薬を受ける権利を奪われることをどう考えるかも，今後重要な課題となることが予想される。

また，副作用が予測される患者群を選別する場合においては，そうした可能性のある患者群に投薬して副作用を確かめることは無理であるため，診断薬としての臨床性能を医薬品の治験データから得ることは難しく，後ろ向き試験や動物実験のデータから評価をせざるを得ない。この場合，臨床的有効性というよりも，対象となるバイオマーカーを正しく診断しているかどうかが評価において重要となると考えられる。

今後本邦におけるコンパニオン診断薬の開発を促進するためには，開発初期段階において診断薬としての性能に関する評価を固める必要があり，そのためのガイドとなるべき指針の整備が望まれる。前述の通知の中で，関連する医薬品の治験の届出に際しては，使用するコンパニオン診断薬に関しても記載することが求められており，この段階である程度の基準をクリアしていることを要求されると考えられるため，その際の評価基準ともなりうる。

2.3 開発に関する課題

コンパニオン診断薬にとって最も重要な課題となるのは，その開発のためのビジネス戦略であると考えられる。

第一の課題として，体外診断薬自体に対する利益が医薬品ほど望まれず，コンパニオン診断薬を独立採算とした場合にはビジネスとして成り立たない可能性が大きい。さらに，開発中の医薬品がドロップアウトした場合には，コンパニオン診断薬としても成立しないわけであり，このリスクをどう扱うのかも大きな問題である。こうしたことを考えた場合，利益とリスクを含めたコンパニオン診断薬の採算性は，医薬品側である程度補償する必要があると言える。コンパニオン診断薬としての保険点数を上げるという方向性もあるが，新規医薬品の使用に不可欠であるという観点から診断薬は医薬品の一部であると考え，医薬品の販売利益の一部を診断薬側に償還する形のビジネスモデルを構築することが望ましい方向性ではないかと予測している。

第二の課題として，治験のどの段階で最終的な製品版の診断薬を準備し，どの段階でどの程度の性能評価を行うべきかという問題が挙げられる。同時承認に向けては，なるべく早い段階で製品版の診断薬を準備しその性能を確認しておくことが望まれるが，開発初期においてはドロップアウトのリスクも考えると必ずしもそこまでは必要なく，医薬品開発の進行に沿って診断薬も開発品から最終製品へと進化させていく戦略がとられるであろう。

その他の課題としては，開発初期や治験の初期の限られた臨床データから診断薬としての性能を評価しないといけない点，そのために，後ろ向き試験や，過去の試験データ，および動物実験や in vitro の試験データをいかに活用するかという点，メカニズムに基づいて診断標的としてのバイオマーカーの有効性を示せるか，倫理的問題を含めて臨床試験におけるバイオマーカー陰性例の患者をどう扱うかなどの項目があげられ，こうした問題をうまくクリアしていくことが，コンパニオン診断薬およびそれを利用する医薬品の開発を成功に導き，個の医療の実現へと結びつけるための課題となる。

3. 個の医療の実現に向けて

これまではとかく脇役的存在であった診断薬が，コンパニオン診断薬の登場により医薬品と同等に主役的存在へとなりつつあり，個の医療の実現に向けて不可欠な役割を担っていると言える。政府の「健康・医療戦略」における最先端の技術に関わる取り組みにおいても，コンパニオン診断薬は，分子標的薬や個別化医療とともにキーワードとして取り上げられており，コンパニオン診断薬と新薬の同時審査の体制を整えるとともに，その評価指標に関する研究を推進するとされている。コンパニオン診断薬による患者の選択が適切に行われてこそ，薬剤の有効性が正しく発揮されるわけであり，新薬開発の成功の鍵を握っている。医薬品との同時承認を前提とするわけであるから，コンパニオン診断薬が承認されないと医薬品としても承認されないことになる。よって，これまで以上に体外診断薬としての性能評価を承認申請時に整備しておく必要があり，追加データを要求されれば医薬品自体の承認の遅れにもつながるため，それを防ぐための企業努力も重要となる。また，承認申請をスムーズに行うためには，開発段階での治験相談等を通じて開発側および審査側（PMDA）と連携を図ることが重要であり，この点は先の通知にも述べられている。今後日本発のコンパニオン診断薬およびそれを利用した医薬品の承認事例が増えることを期待しながら，個の医療の実現に向けたレギュラトリーサイエンスの推進に協力していきたい。

文　　献

1) Draft guidance for industry and FDA staff, In Vitro Companion Diagnostic Devices. 14 July, 2011
2) Reflection paper on co-development of pharmacogenomic biomarkers and assays in the context of drug development (draft). 24 June, 2010
3) Reflection paper on methodological issues associated with pharmacogenomic biomarkers in relation to clinical development and patient selection (draft). 9 June, 2011
4) コンパニオン診断薬等及び関連する医薬品の承認申請に係る留意事項について　薬食審査発 0701 第 10 号　平成 25 年 7 月 1 日
5) コンパニオン診断薬及び関連する医薬品に関する技術的ガイダンス等について　薬機発第 1224029 号 平成 25 年 12 月 24 日
6) コンパニオン診断薬等に該当する体外診断用医薬品の製造販売承認申請に際し留意すべき事項について　薬食機発 0219 第 4 号 平成 26 年 2 月 19 日
7) コンパニオン診断薬等体外診断用医薬品の製造販売承認申請に際し留意すべき事項についての質疑応答集　薬食機発 0328 第 7 号

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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

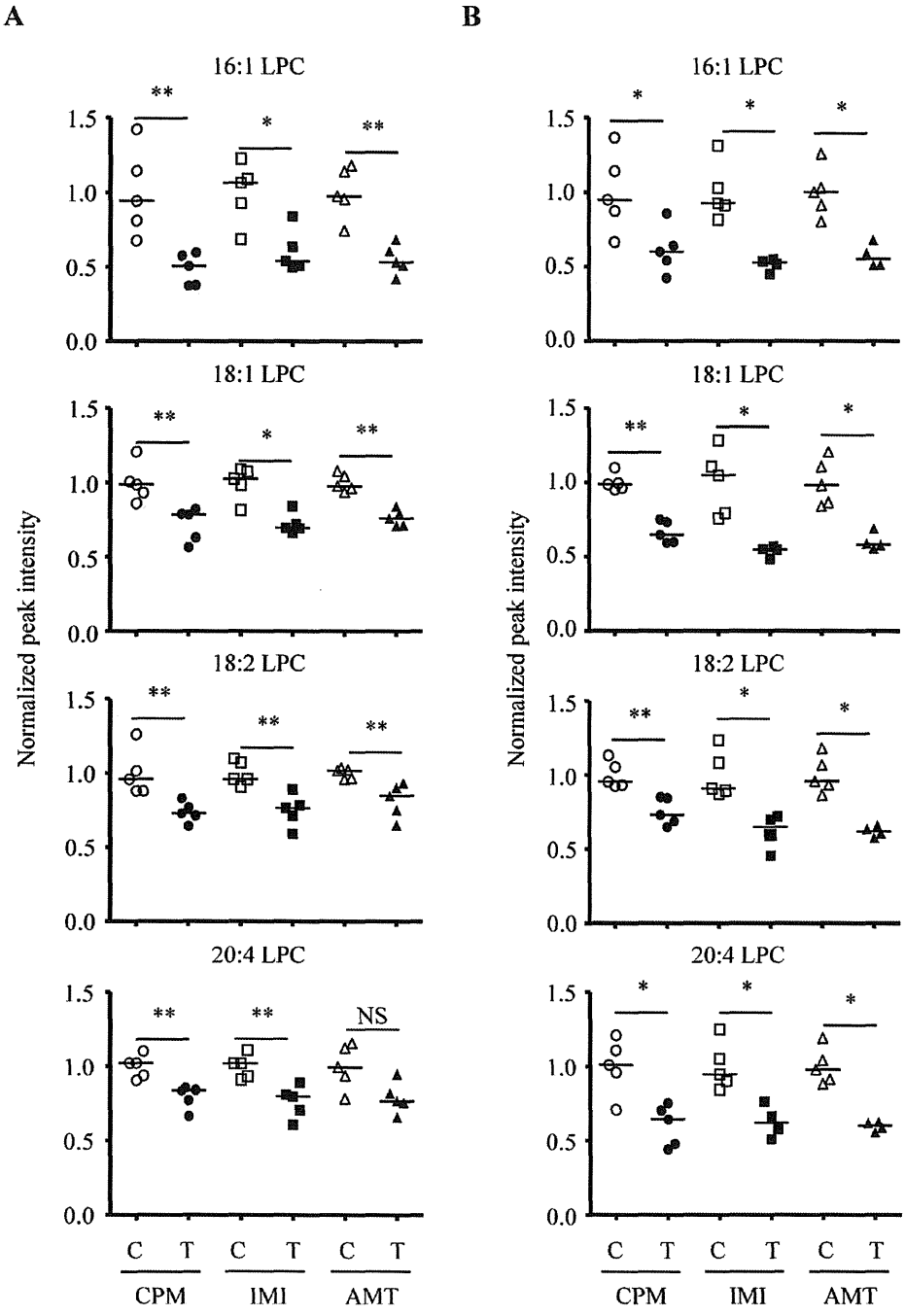

FIG. 1. Plasma levels of LPCs in rats treated with tricyclic antidepressants. Lipid extracts were prepared from the plasma of rats treated with indicated drugs (black) or control solvents (white) for 3 (A) or 28 days (B). The determined ion peak levels of the identified LPCs were normalized to that of 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

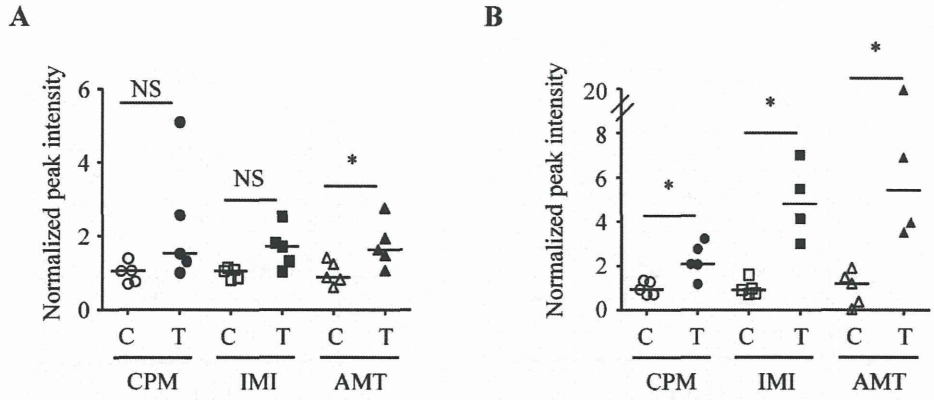

FIG. 2. Plasma levels of 42:1 HexCers in rats treated with tricyclic antidepressants. Lipid extracts were prepared from the plasma of rats treated with indicated drugs (black) or control solvents (white) for 3 (A) or 28 days (B). The determined ion peak levels of 42:1 HexCer were normalized to that of 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.

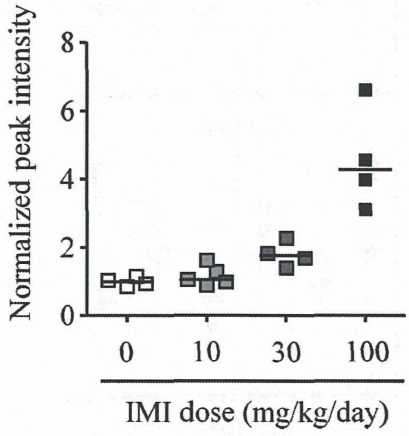

FIG. 3. Dose-dependent effect of imipramine treatment on plasma 42:1 HexCer levels. Lipid extracts were prepared from the plasma of rats treated with an indicated dose of imipramine for 28 days. The determined ion peak levels of 42:1 HexCer were normalized to that of the internal standard (12:0/12:0 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.

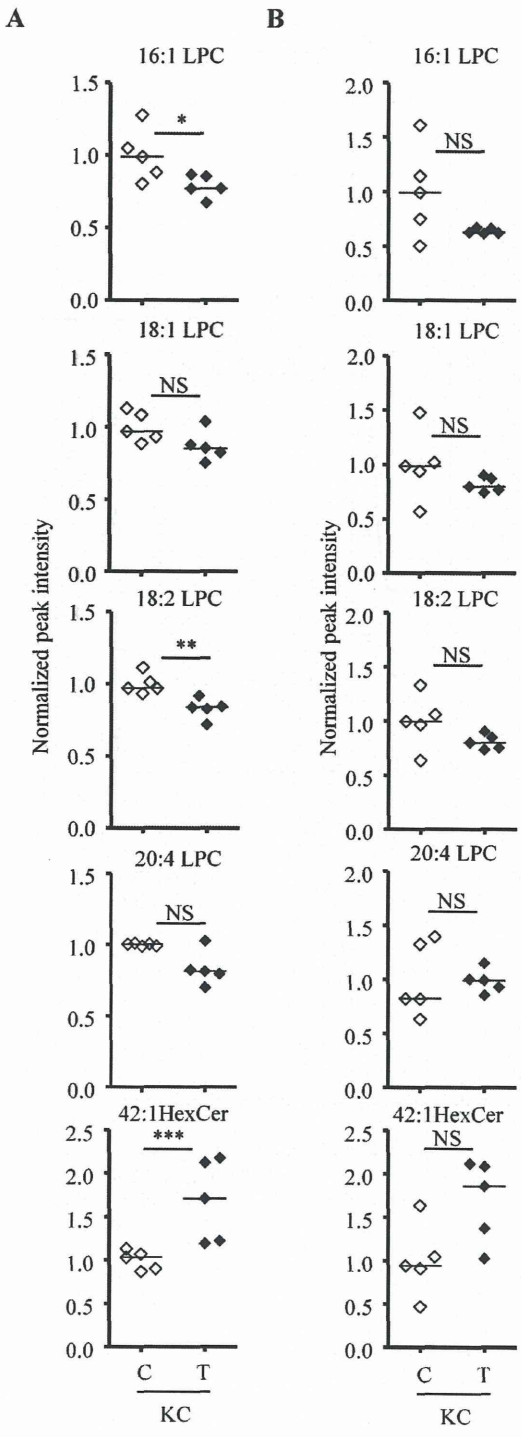

FIG. 4. Plasma levels of LPCs and 42:1 HexCers in rats treated with ketoconazole. Lipid extracts were prepared from rat plasma treated with ketoconazole (black) or control solvents (white) for 3 (A) or 28 days (B). The determined ion peak levels of indicated LPCs and 42:1 HexCer were normalized to that 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.

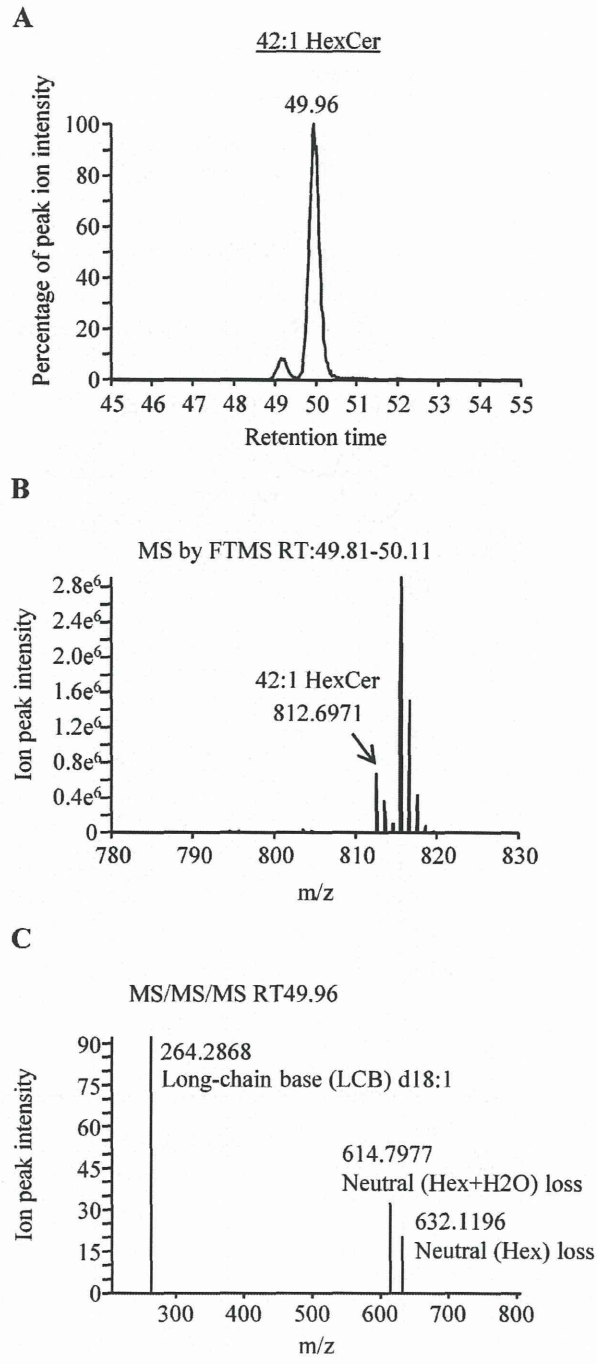

FIG. 5. Extracted ion chromatogram (A), mass spectrum (B), and mass/mass/mass spectrum (C) of the identified 42:1 HexCer. A neutral loss of 18 Da (neutral [H_2O] loss from M + H) from the precursor ion was used as a trigger to obtain the mass/mass/mass spectrum. In the mass/mass/mass spectrum, the deduced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

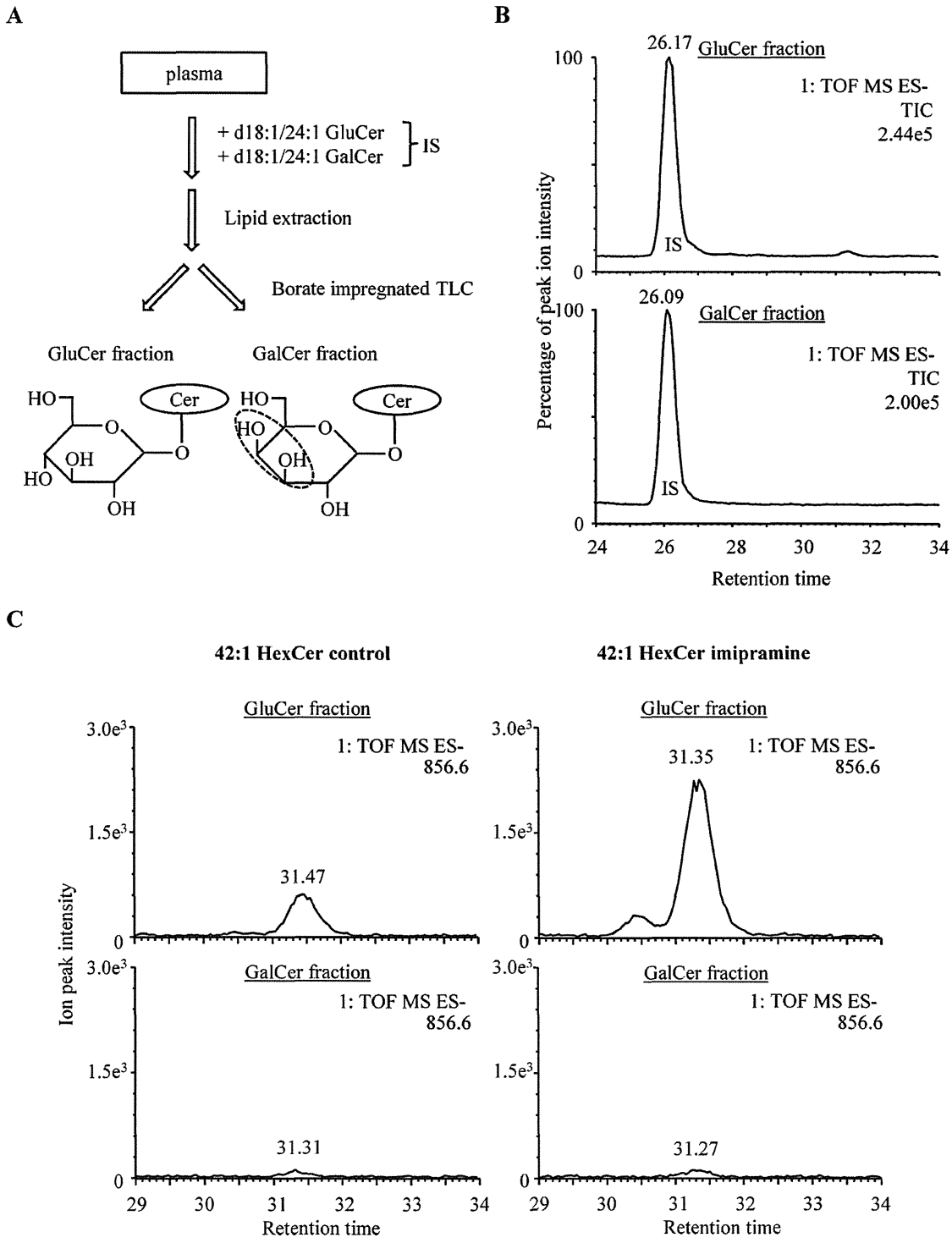

FIG. 6. Differentiation of GluCer from GalCer to the 42:1 HexCer identity. (A) Scheme for separation of GluCer and GalCer by borate-impregnated TLC. Dotted circle indicates a putative site forming the borate complex. (B) Ion chromatograms of the standards of the GluCer and GalCer fractions. (C) Ion chromatogram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

Abe, A., Hiraoka, M. and Shayman, J.A. (2007). A role for lysosomal phospholipase A2 in drug induced phospholipidosis. *Drug Metab. Lett.* **1**, 49–53.

Anderson, N. and Borlak, J. (2006). Drug-induced phospholipidosis. *FEBS Lett.* **580**, 5533–5540.

Baronas, E.T., Lee, J.W., Alden, C. and Hsieh, F.Y. (2007). Biomarkers to monitor drug-induced phospholipidosis. *Toxicol. Appl. Pharmacol.* **218**, 72–78.

Bu, Q., Huang, Y., Yan, G., Cen, X. and Zhao, Y.L. (2012). Metabolomics: A revolution for novel cancer marker identification. *Comb. Chem. High Throughput Screen.* **15**, 266–275.

Carroll, M. (1981). Genetic heterogeneity of membrane-bound beta-glucosidase in Gaucher's disease. *J. Inherit. Metab. Dis.* **4**, 11–13.

Cheng, J., Ma, X., Krausz, K.W., Idle, J.R. and Gonzales, F.J. (2009). Rifampicin-activated human pregnane X receptor and