methods

Rapid UPLC-MS/MS method for routine analysis of plasma pristanic, phytanic, and very long chain fatty acid markers of peroxisomal disorders

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Abstract **Quantification of pristanic acid, phytanic acid, and very long chain fatty acids (i.e., hexacosanoic, tetracosanoic, and docosanoic acids) in plasma is the primary method for investigateing a multitude of peroxisomal disorders (PDs). Typically based on GC-MS, existing methods are time-consuming and laborious. In this paper, we present a rapid and specific liquid chromatography tandem mass spectrometric method based on derivatization with 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE). Derivatization was undertaken to improve the poor mass spectrometric properties of these fatty acids. Analytes in plasma (20 μl) were hydrolyzed, extracted, and derivatized with DAABD-AE in ~2 h. Derivatives were separated on a reverse-phase column and detected by positive-ion electrospray ionization tandem mass spectrometry with a 5 min injection-to-injection time. Calibration plots were linear over ranges that cover physiological and pathological concentrations. Intraday (n = 12) and interday (n = 10) variations at low and high concentrations were less than 9.2%. Reference intervals in normal plasma (n = 250) were established for each compound and were in agreement with the literature. Using specimens from patients with established diagnosis (n = 20), various PDs were reliably detected. In conclusion, this method allows for the detection of at least nine PDs in a 5 min analytical run. Furthermore, this derivatization approach is potentially applicable to other disease markers carrying the carboxylic group.**—Al-Dirbashi, O. Y., T. Santa, M. S. Rashed, Z. Al-Hassnan, N. Shimozawa, A. Chedrawi, M. Jacob, and M. Al-Mokhadab. **Rapid UPLC-MS/MS method for routine analysis of plasma pristanic, phytanic, and very long chain fatty acid markers of peroxisomal disorders. *J. Lipid Res.* 2008.** 49: **1855–1862.**

Supplementary key words peroxisomes • Zellweger syndrome • DAABD-AE • hexacosanoic acid • tetracosanoic acid • docosanoic acid • derivatization

Peroxisomal disorders (PDs) are a heterogeneous group of congenital diseases caused by defective peroxisomes. Dysfunctions of these cellular organelles disturb several metabolic pathways that mainly involve lipids and often result in a progressive multisystem disease (1–4). In the peroxisome, both anabolic and catabolic functions occur. The former include synthesis of bile acids, docosahexanoic acid, and plasmalogens. Catabolic reactions encompass α- and β-oxidation of certain fatty acids, among other reactions (1–7). PDs are categorized into two main groups: peroxisome biogenesis disorders (PBDs) and single enzyme/transporter deficiencies (PEDs). Zellweger syndrome (ZS), neonatal adrenoleukodystrophy, and infantile Refsum disease (RD), which belong to PBD group, are caused by different mutations in the same genes (7–10). The PED list was recently shortened to ten disorders after it was proven that mevalonate kinase is a cytosolic enzyme (9, 11). The wide spectrum of clinical heterogeneity of PBDs is caused by losing the diverse functions of peroxisomes. ZS, characterized by craniofacial dysmorphia, severe neurological and hepatic ab-

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Abbreviations: DAABD-AE, 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; ESI-MS/MS, electrospray ionization tandem mass spectrometry; IS, internal standard; LC-MS/MS, liquid chromatography tandem mass spectrometry; PBD, peroxisome biogenesis disorder; PD, peroxisomal disorder; PED, single enzyme/transporter deficiency; Phy, phytanic acid; Pri, pristanic acid; QC, quality control; RD, Refsum disease; SRM, selected-reaction monitoring; UPLC, ultra performance liquid chromatography; VLCFA, very long chain fatty acid; X-ALD, X-linked adrenoleukodystrophy; ZS, Zellweger syndrome.

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normalities, and early death, is the most severe. Other PDs share some of these symptoms, with widely varying severity, organ involvement, and survival (12).

Together with clinical presentation and imaging studies, biochemical assays are essential for PD diagnosis and subclassification. However, no single assay is capable of detecting all PDs. Quantification of plasma hexacosanoic ($C_{26:0}$), tetracosanoic ($C_{24:0}$), and docosanoic ($C_{22:0}$) acids, collectively known as very long chain fatty acids (VLCFAs), is the primary screening test for PBDs and a multitude of PEDs, including X-linked adrenoleukodystrophy (X-ALD), the most prevalent PD (1, 2, 13). Although additional biochemical assays in patients with normal levels of VLCFAs are seldom warranted (5), analysis of phytanic acid (Phy, $C_{20:0}$ branched) and plasmalogens is valuable when RD and all types of rhizomelic chondrodysplasia punctata are clinically suspected.

Determination of VLCFAs, with or without Phy and pristanic acid (Pri, $C_{19:0}$ branched), is traditionally carried out by GC-MS (14–16). These well-documented and highly specific assays suffer the eternal GC draw back of tedious sample preparation and lengthy chromatographic times. Methods based on positive-ion electrospray ionization tandem mass spectrometry (ESI-MS/MS) (17) and negative-ion ESI-MS (18) are limited to VLCFAs and are incapable of differentiating Phy and Pri from their straight-chain isomers. Recently, Johnson (17) improved his original method by including a liquid chromatography step and a second derivatization to form the trimethylaminoethyl ester derivative. Although this made possible the simultaneous determination of Pri, Phy, and VLCFAs, the method was hampered by the multi-step derivatization and the long chromatographic time of ~30 min (19).

Recently, we described the synthesis of 4-[2-(*N,N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole (DAABD-AE) and demonstrated its potential as a derivatization reagent for carboxylic acids (20–22). Designed specifically for ESI-MS/MS, DAABD-AE contains an ionizable moiety with high proton affinity to improve the ionization process and a hydrophobic benzofurazan structure. In this work, we describe a novel approach for the simultaneous analysis of VLCFAs, Phy, and Pri that involves derivatization with DAABD-AE and stable isotope-labeled internal standards (ISs). The method provides essential information about peroxisomal functions so that it permits identifying a multitude of PBDs and PEDs.

MATERIALS AND METHODS

Chemicals and solutions

HPLC-grade acetonitrile was purchased from Fisher Scientific (Fairlawn, NJ,). Pentadecafluorooctanoic acid was from Fluka (Buchs, Switzerland). DAABD-AE was synthesized according to the published procedure (20). The following chemicals were purchased from Sigma-Aldrich: $C_{22:0}$, $C_{24:0}$, $C_{26:0}$, Phy, arachidic acid ($C_{20:0}$ linear), nonadecanoic acid ($C_{19:0}$ linear), 4-dimethylaminopyridine, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. Pri, $^2H_3$-Phy, and $^2H_3$-Pri were from Dr. H. J. ten Brink (Vrije Universiteit Medical Center, Amsterdam, The Netherlands). $^2H_3$-$C_{22:0}$, $^2H_4$-$C_{24:0}$, and $^2H_4$-$C_{26:0}$ were from C/D/N Isotopes Inc. (Quebec, Canada). All other reagents were of analytical grade or better.

Solutions of individual fatty acids and labeled compounds at a concentration of 1 mg/ml were prepared in a mixture of chloroform-methanol (2:1; v/v). These were stored at 4°C and were stable for a minimum of 3 months. Working solutions of the desired mixtures were prepared by dilution in the same solvent. A working IS mixture containing $^2H_3$-Phy and $^2H_3$-Pri (2 μg/ml), $^2H_3$-$C_{22:0}$ and $^2H_4$-$C_{24:0}$ (0.5 μg/ml), and $^2H_4$-$C_{26:0}$ (0.1 μg/ml) was prepared in acetonitrile.

Calibrators and quality control samples

Linearity was established by analyzing plasma calibrators enriched with target analytes. Excess solvent was evaporated under N_2 at room temperature before adding the plasma. Calibrators were enriched as follows: 1, 2, 4, 10, 20, 40, and 60 μM for Pri; 0.96, 1.92, 3.84, 9.6, 19.2, 38.4, and 57.6 μM for Phy; 4.4, 8.8, 17.6, 44, 88, 176, and 265 μM for $C_{22:0}$; 4.1, 8.2, 16.4, 41, 82, 164, and 245 μM for $C_{24:0}$; and 0.25, 0.5, 1, 2.5, 5, 10, and 15 μM for $C_{26:0}$. Nonenriched plasma was included with each set of calibrators to correct for endogenous levels of fatty acids. Quality control (QC) samples were set at 2 and 20 μM for Pri, 1.92 and 19.2 μM for Phy, 8.8 and 88 μM for $C_{22:0}$, 8.2 and 82 μM for $C_{24:0}$, and 0.5 and 5 μM for $C_{26:0}$ to represent low and high concentrations. Calibrators and QCs were divided into small portions and kept at −20°C. On the analysis day, one set of calibrators and QCs was allowed to thaw at room temperature just prior to use.

Samples and sample preparation

Our institution's Internal Review Board (King Faisl Specialist Hospital and Research Centre, Riyadh, Saudi Arabia) approved this study, and all samples used were provided to the analysts after removal of all personal identification information. Reference intervals were determined by analyzing plasma specimens collected in EDTA tubes (n = 250) that were initially submitted to our lab for VLCFA analysis by GC-MS and reported as "unremarkable." Archived plasma specimens from patients with established PDs were also analyzed (n = 20). Control and patient samples were stored at −20°C for up to 1 year and 10 years, respectively, and were allowed to thaw naturally at room temperature before use.

Twenty microliters of patients plasma, QCs, or calibrators were transferred into disposable screw-capped borosilicate tubes (13 × 100 mm; Fisher Scientific) and mixed with 50 μl of 5 M HCl and 0.4 ml of the working IS. The sealed tubes were then heated at 100°C for 1 h. After cooling to room temperature, 1 ml of *n*-hexane was added to the tubes, and they were vortex-mixed (3 min) and centrifuged (3,800 rpm, 5 min). After collecting and evaporating the upper layer under N_2, the following were added successively to the residue: 25 μl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (25 mM in water), 25 μl of 4-dimethylaminopyridine (25 mM in acetonitrile), and 50 μl of DAABD-AE (2 mM in acetonitrile). Tubes were then tightly sealed and incubated at 60°C. After 45 min, the reaction was stopped by adding 0.1 ml of mobile phase A, and 5 μl was injected onto the liquid chromatography tandem mass spectrometry (LC-MS/MS) system. Stability of DAABD-AE derivatives was assessed by analyzing samples kept at 4°C in the autosampler tray at 0, 1, 2, 4, 8, 12, and 24 h post reaction.

LC-MS/MS system and operating conditions

A Waters ACQUITY Ultra Performance liquid chromatograph (Waters; Milford, MA) composed of a binary pump, an auto-

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sampler, and a thermostatted column compartment maintained at 40°C was used. All column effluent was directed into a Quattro micro atmospheric pressure ionization bench-top triple quadrupole mass spectrometer (Micromass; Manchester, UK), equipped with a Z-spray ESI source and a switching valve. MassLynx software (v 4.0; Micromass) running under Microsoft Windows XP professional environment was used to control the instruments and for data acquisition.

Separation was performed on a 2.1 × 50 mm column packed with 1.7 μm particles (ACQUITY UPLC BEH C_{18} column; Waters). Mobile phase A was 80% acetonitrile, mobile phase B was 100% acetonitrile, and both contained 0.5 g/l pentadecafluorooctanoic acid. The gradient program was as follows: 0–1 min 100% of A, 1–2 min from 100% to 50% of A, and 2–3.5 min 50% A at a flow rate of 0.4 ml/min. The column was reequilibrated with mobile phase A for an additional 1.5 min at a flow rate of 1 ml/min. The injection-to-injection time was 5 min.

The ESI source was operated in the positive-ion mode at a capillary and cone voltage of 4.0 kV and 45 V, respectively with collision energy of 30 eV. Argon was used as collision gas, and nitrogen was used as the nebulizing and desolvation gas. Ion source and desolvation temperatures were maintained at 125°C and 350°C, respectively. Scanning was in the selected-reaction monitoring (SRM), where the transitions to the common fragment at *m/z* 151 generated from the following protonated parent ions were recorded: 609 (Pri), 612 ($^2H_3$-Pri), 623 (Phy), 626 ($^2H_3$-Phy), 651 ($C_{22:0}$), 654 ($^2H_3$-$C_{22:0}$), 679 ($C_{24:0}$), 683 ($^2H_4$-$C_{24:0}$), 707 ($C_{26:0}$), and 711 ($^2H_4$-$C_{26:0}$).

Method validation

Intraday (n = 12) and interday (n = 10) imprecision was assessed by repeatedly analyzing high and low QC samples every working day over a period of 2 weeks. The reproducibility was evaluated by analyzing one normal and one abnormal plasma specimen repeatedly to generate intraday (n = 10) and interday variations (n = 5). Analytical recovery was calculated from the QC sample results according to the following equation: [Recovery (%) = 100 × (concentration measured−concentration in nonenriched plasma)/concentration added]. Potential interfer-

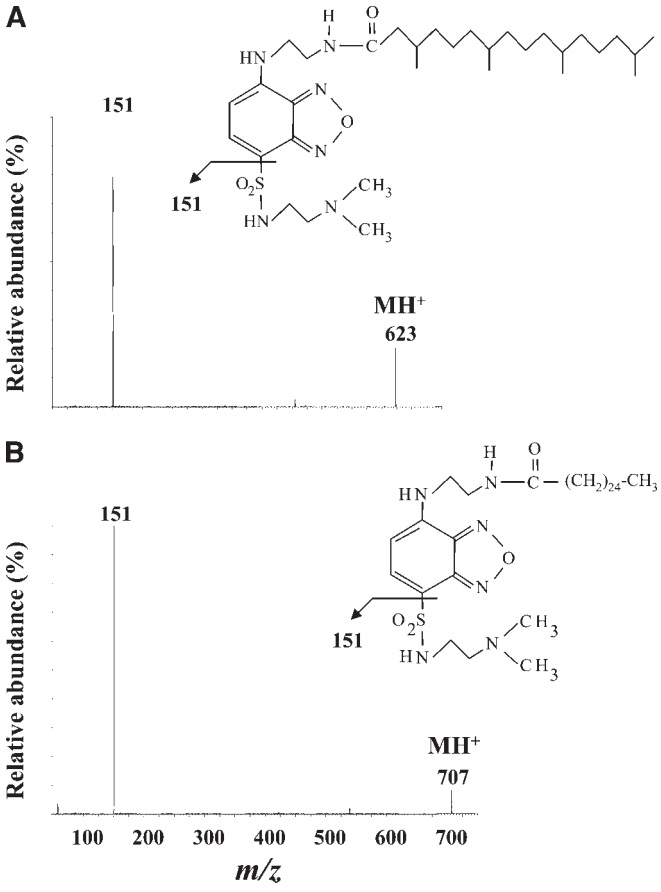

Fig. 1. The product ion spectra obtained by electrospray ionization tandem mass spectrometry analysis for the $[MH]^+$ of 4-[2-(*N*, *N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole derivatives of phytanic acid at *m/z* 623 (A) and $C_{26:0}$ at *m/z* 707 (B).

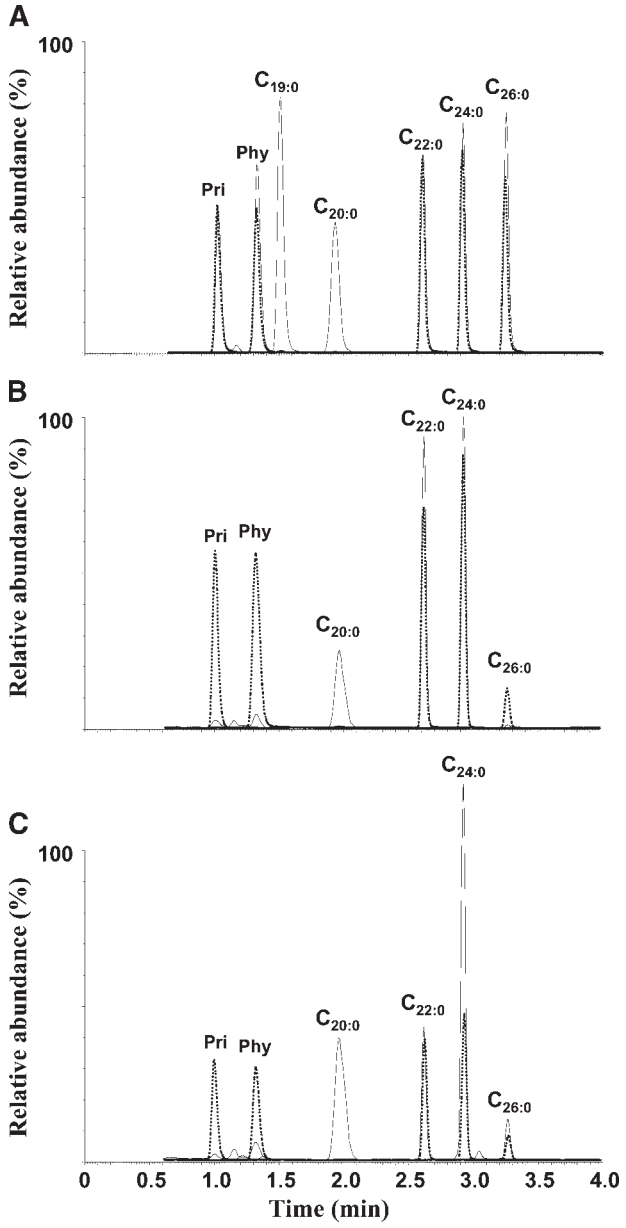

Fig. 2. Extracted mass chromatograms obtained with a standard fatty acid mixture (A), a plasma sample from a normal subject (B), and that from a patient with a peroxisome biogenesis disorder (C). Solid lines represent the analytes and dashed lines represent the deuterium-labeled internal standard. $C_{19:0}$ and $C_{20:0}$ represent nonadecanoic and arachidic acids, respectively.

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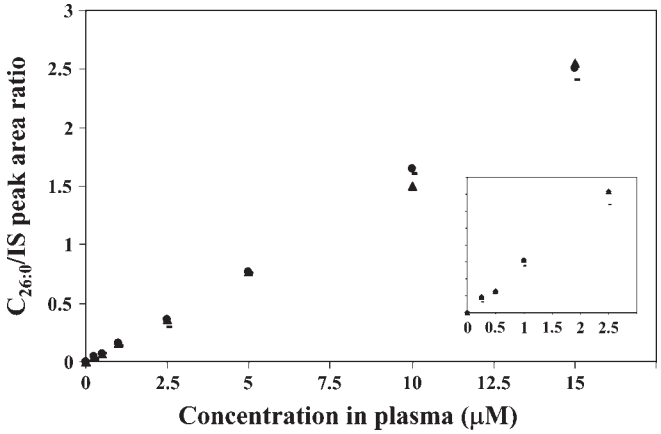

Fig. 3. Three overlaid calibration plots for $C_{26:0}$ in plasma obtained on three different days. The inset represents linearity at the lower part of the plot.

ence from isobaric compounds was evaluated by analyzing individual standard solutions. We compared the levels of VLCFAs obtained in 20 plasma samples by the current LC-MS/MS versus a routine reference method (23).

RESULTS

MS/MS and LC-MS/MS experiments

Derivatization conditions used in this work were identical to those reported earlier for dicarboxylic acid (22). Continuous infusion of reaction mixtures into the first quadrupole and the subsequent ESI-MS scanning revealed intense ions at *m/z* of 609, 623, 651, 679, and 707 corresponding to $[MH]^+$ of DAABD-AE derivatives of Pri, Phy, $C_{22:0}$, $C_{24:0}$, and $C_{26:0}$, respectively. The transmission of these ions into the collision cell and the subsequent scanning by the second resolving quadrupole for fragments revealed a simple fragmentation pattern with an intense fragment at *m/z* of 151 common to all studied analytes. **Fig. 1** shows the structures and product ion mass spectra of Phy (Fig. 1A) and $C_{26:0}$ derivatives (Fig. 1B). As anticipated, the fragmentation pattern and optimal ESI-MS/MS conditions for all analytes and their isotope-labeled ISs were identical and were used to design SRM experiments.

The chromatographic system was optimized to retain DAABD-AE derivatives on the column while allowing other substances that may have an ion-suppressing effect, including excess reagents, to elute. This was achieved by a high-organic-content mobile phase with a gradient program that increases the acetonitrile concentration from 80% to 90% (v/v) over the course of the chromatographic run. Addition of pentadecafluorooctanoic acid, a volatile additive, to the mobile phase enhanced the ESI process and improved the peak shape of Pri and Phy, which eluted early in the run. **Fig. 2** shows extracted mass chromatograms obtained with a standard fatty acid mixture (Fig. 2A), a plasma sample from a normal subject (Fig. 2B), and a plasma sample from a patient with a PBD (Fig. 2C). As shown in Fig. 2A, arachidic and nonadecanoic acids, the linear-chain $C_{20:0}$ and $C_{19:0}$, respectively, were completely resolved and did not interfere with quantification of their

TABLE 1. Precision, recovery and limits of detection of Pri, Phy and VLCFAs in plasma

		Intraday			Interday			Recovery[c]	
Compound	Concentration Added	Mean	CV	n	Mean	CV	n	Mean (CV)	LOD[d]
	μM	*μM*	*%*		*μM*	*%*		*%*	*fmol/5μl*
Pristanic	2	2.9	5.5	12	2.9	9.2	10	98.9 (6.5)	12
	20	19.3	5.3	12	19.7	4.4	10	95.1 (5.1)	
	Normal[a]	0.99	3.1	10	1.1	6.4	5		
	Abnormal[b]	1.03	4.1	10	1.2	12.4	5		
Phytanic	1.9	5.6	5.4	12	5.1	6.9	10	96.8 (9.2)	6.4
	19	20.9	1.2	12	20.5	6.0	10	95.7 (4.8)	
	Normal	5.8	4.2	10	5.9	6.8	5		
	Abnormal	2.5	7.8	10	2.2	5.8	5		
C22:0	8.8	53.4	1.7	12	54.3	6.3	10	109.1 (10.2)	0.59
	88	148.6	4.8	12	139.6	6.4	10	102.3 (5.6)	
	Normal	66.3	3.3	10	67.5	5.2	5		
	Abnormal	34.1	1.8	10	34.5	6.4	5		
C24:0	8.2	46.7	2.4	12	44.8	4.2	10	105.1 (6.9)	0.60
	82	126.4	0.5	12	119.7	4.6	10	101.7 (1.2)	
	Normal	64.7	2.6	10	63.3	3.3	5		
	Abnormal	64.6	5.9	10	65.2	4.2	5		
C26:0	0.5	1.3	1.1	12	1.1	7.7	10	91.3 (8.7)	0.45
	5	6.3	1.2	12	6.1	4.0	10	103.9 (2.4)	
	Normal	1.0	5.2	10	1.08	8.3	5		
	Abnormal	3.3	0.7	10	3.4	5.4	5		

CV, coefficient of variation; Pri, pristanic acid; Phy, phytanic acid; LOD, limits of detection; VLCFA, very long chain fatty acid.

[a] A plasma sample from a healthy individual.

[b] A plasma sample from a patient with a peroxisome biogenesis disorder (PBD).

[c] Recovery (%) = 100 × (concentration measured−concentration in nonenriched plasma)/concentration added.

[d] Limits of detection at signal-to-noise ratio of 3 in femtomoles per 5 μl injection.

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isobaric branched antipodes. Pri, the analyte with the lowest molecular weight, eluted first at ~1 min, whereas $C_{26:0}$ appeared last at 3.25 min. After each sample, the column was reequilibrated with mobile phase A for 1.5 min; therefore, the injection-to-injection time was 5 min. To avoid overloading the ESI source with potential ion-suppressing contaminants from samples or reagents, the automatic switching valve supplied with the MS/MS detector was programmed to divert the column effluent to waste for the first 0.6 min and the last 1 min of each run.

Isotopic purity of IS materials was assessed by analyzing individual solutions using MS/MS and LC-MS/MS, because contamination with nondeuterated antipodes could have led to overestimation. No contamination was observed, and this revealed that the IS materials were isotopically pure. Not unexpectedly, each analyte coeluted with the corresponding IS as a single peak (Fig. 2). Derivatives were stable for at least 24 h when kept in the autosampler tray at 4°C.

Assay performance and validation

Regression analyses of analyte-to-IS peak area ratios versus concentration in plasma revealed linear relationships in the following ranges (μM): Pri, 1–60; Phy, 0.96–57.6; $C_{22:0}$, 4.4–265; $C_{24:0}$, 4.1–245; and $C_{26:0}$, 0.25–15. Calibration was reproducible, and as a representative example, **Fig. 3** depicts three overlaid calibration plots of $C_{26:0}$ in plasma obtained on three different days. Method performance characteristics, including intraday and interday precision of QCs and clinical samples, expressed as coefficient of variation (%), analytical recovery, and limits of detection, are summarized in **Table 1**.

Fig. 4 illustrates Bland-Altman plots that show the distribution of average concentrations of VLCFAs in 20 plasma samples measured by the current method and in parallel by our standard GC-MS method versus the difference in the paired values. Pri and Phy are not measured by our GC-MS; hence, they were not included in this comparison.

Analysis of control and patient samples

Reference intervals of absolute fatty acid concentrations and ratios ($C_{24:0}/C_{22:0}$ and $C_{26:0}/C_{22:0}$) obtained in plasma from controls (n = 250) and patients with confirmed PDs (n = 20) are summarized in **Table 2**. For comparison, shown also are reference ranges obtained by GC-MS in other populations.

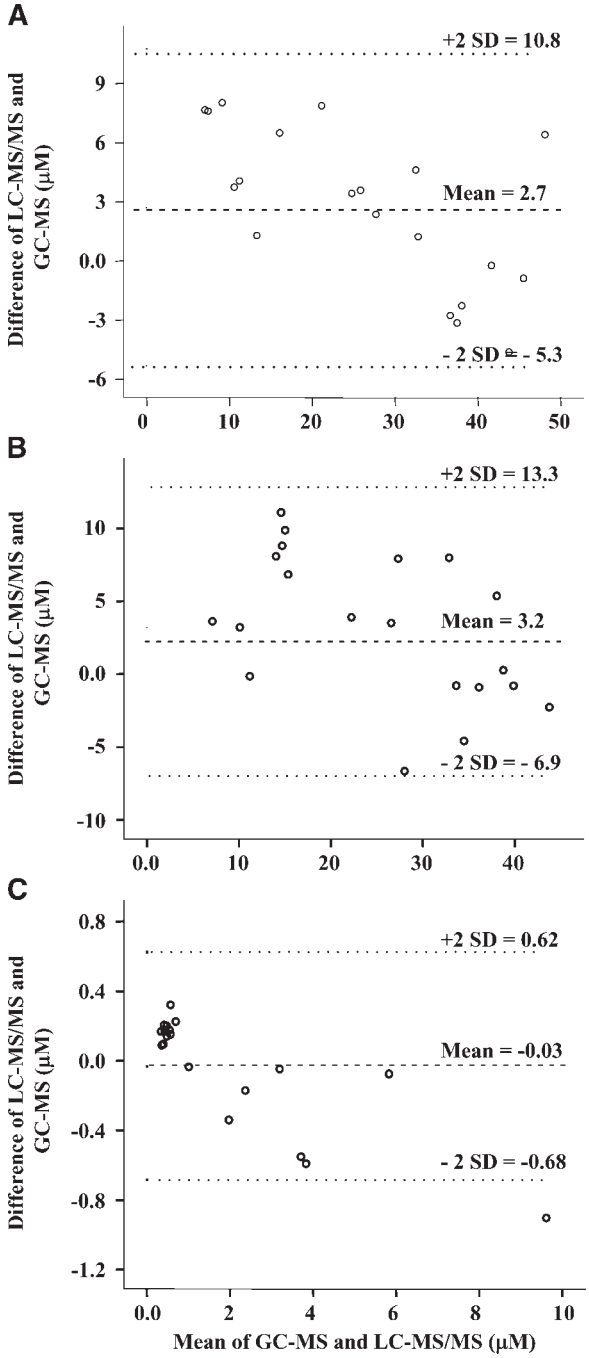

Fig. 4. Bland-Altman plotting of the mean concentrations obtained by the GC-MS and liquid chromatography tandem mass spectrometry methods versus the differences in the paired values for $C_{22:0}$ (A), $C_{24:0}$ (B), and $C_{26:0}$ (C).

DISCUSSION

The importance of measuring VLCFAs for assessing peroxisomal functions has been recognized since the 1980s (24). Quantifying these analytes in plasma is indicated when a PD is clinically evident or as part of investigating nonspecific presentations in newborns (i.e., profound hypotonia, seizures, and neuronal migration defect) or in older children (i.e., progressive neurological dysfunction and school failure). In service laboratories, methods for routine clinical analysis of Pri, Phy, $C_{22:0}$, $C_{24:0}$, and $C_{26:0}$ are often based on GC-MS (14–16, 23). These methods are well-validated and reliable, but laborious and time-consuming (18, 25). Analysis by HPLC has received limited attention, obviously due to lacking a detectable moiety like a chromophore or fluorophore (26).

LC-MS/MS is a robust technique with an increasing utilization in clinical laboratories. Nevertheless, limited attempts have been made to adopt it for Pri, Phy, and

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TABLE 2. Concentrations of Pri, Phy, and VLCFAs in plasma of controls and patients with various PDs

		Pri	Phy	C22:0	C24:0	C26:0	C24:0/C22:0	C26:0/C22:0	Remarks
		μM	μM	μM	μM	μM			
X-ALD/	Median	1.25	0.84	40.6	67.0	3.3	1.61	0.080	This study
AMN[a] (n = 4)	Range	0.34–1.79	0.32–1.77	29.7–64.3	47.6–103.4	2.0–5.6	1.57–1.75	0.068–0.089	
RD (n = 1)		0.20	269.9	51.7	38.03	0.79	0.74	0.015	This study
PBD (n = 15)	Median	1.42	1.45	26.7	38.2	8.6	1.65	0.32	This study
	Range	0.27–41.2	0.14–137.9	9.7–34.0	22.3–64.1	2.7–14.3	0.99–2.3	0.08–0.62	
Controls (n = 250)	Median	0.57	1.58	51.08	37.7	0.64	0.71	0.012	This study
	Range	0.0–3.4[b]	0.04–11.5[c]	9.6–100.5	3.4–91.7	0.04–1.46	0.15–1.15	0.001–0.028	
	95th %	1.67	6.17	86.7	66.3	1.17	0.95	0.022	
Controls	Range	0.01–2.98	0.04–9.88	17–96	N/A	0.22–1.31	N/A	0.003–0.021	Ref 28
Controls	Range[d]	0.0–1.5	0.3–11.5	41.1–90.3	37.4–74.9	0.6–1.2	0.69–1.0	0.011–0.022	Ref 15

PD, peroxisomal disorder; X-ALD, X-linked adrenoleukodystrophy; AMN, adrenomyeloneuropathy; RD, Refsum disease; N/A, not available.
[a] The adult form of X-ALD.
[b] The upper limits of Pri for age groups <1 year and 1–2 years are 1.54 and 2.0 μM, respectively.
[c] The upper limits of Phy for age groups <1 year and 1–2 years are 6.8 and 5.3 μM, respectively.
[d] 5%–95% interval.

VLCFAs analysis. This might be attributed to the fact that anions of saturated fatty acids do not fragment to a product ion with a significant intensity (25). To overcome this, Johnson, Trinh, and Oe (19) prepared a permanently charged trimethylaminoethyl ester derivative of fatty acids. Although they resolved the poor ionization, the introduction of a permanent positive charge altered the hydrophobic nature of the derivatives, complicated the chromatographic process, and necessitated a lengthy chromatographic run of 30 min, similar to those observed in GC-MS. Therefore, the high-throughput advantage often associated with LC-MS/MS was not achieved. As an alternative approach, we aimed at preparing a hydrophobic derivative with high ionization efficiency. For this purpose, we utilized our recently developed reagent, DAABD-AE, known to form stable amides upon reaction with a carboxyl group (20–22). This reagent was developed with the convenient ESI in mind, and was thus designed to contain a readily chargeable moiety and a benzofurazan skeleton. The hydrophobic nature of the latter increased the overall lipophilicity of the derivatives and hence improved the separation from early-eluting ion-suppressing compounds in the commonly used reverse-phase chromatography. Furthermore, it is known that nonpolar ions preferentially reside at the droplet-air interface and hence enter the gas phase more readily than those in the droplet interior, and this enhances the ESI process (27).

Because of structural similarities, the analytes studied produced characteristic product ion spectra with a major intense peak at *m/z* 151 that was assigned to the dimethylaminoethylaminosulfonyl fragment from DAABD-AE (Fig. 1). It is worth mentioning that the reactivity of branched-chain fatty acids was low compared with their straight-chain isomers, and this might be attributed to steric hindrance.

In LC-MS/MS, a good chromatography is critical for the overall method performance. Packed with very small particles (1.7 μm), the short ultra performance liquid chromatography (UPLC) column used in this study provided sufficient retention and allowed for a relatively high flow rate of 0.4 ml/min of a mobile phase with substantial organic content. High organic content facilitated the desolvation process in the ion source, and thus contributed to the increased detector response. Under these conditions, $C_{26:0}$, the last peak to elute, appeared at 3.25 min. The injection-to-injection time was 5 min, provided that a complete resolution of isobaric linear and branched $C_{19:0}$ and $C_{20:0}$ was achieved. Compared with published GC-MS and LC-MS/MS methods (14–16, 19, 23), our method is five to six times faster and saves at least 75% of the instrument time. During our preliminary investigations, we observed satisfactory chromatographic separation on a C8 column (150 × 2.1 mm, i.d. 5 μm; Symmetry, Waters) with a run time of ~10 min using the same mobile phase. With further optimization and validation, this should be useful to laboratories with no access to UPLC technology.

The fatty acids studied here were liberated from lipid-containing species such as triglycerides and phospholipids according to published procedures (17, 19). Extraction was achieved in a single step with *n*-hexane. Recovery was satisfactory and ranged from 91% to 109% (Table 1). Derivatization with DAABD-AE proceeded under mild conditions (60°C for 45 min), and the reaction mixture was analyzed with no further purification. As described, this method does not require special precautions such as rinsing the glassware with certain solvents (18), and analyzing a batch of 15 patients samples with the associated calibrators and QCs would require ~2 h for sample preparation and another 2 h of instrument time.

The inherent specificity of this assay, which stems from the unique design of tandem mass spectrometers, resulted in low background noise and high signal-to-noise ratio. Therefore, we were able to reliably measure fatty acids circulating at low physiological levels, such as Pri and $C_{26:0}$, using a very small volume of plasma (20 μl). This is an important consideration in pediatric testing (19) when compared with the 200 μl often required for standard GC-MS. The use of stable isotopes as IS to compensate for any deficiency during sample preparation and analysis was very effective, and the assay was robust and satisfactorily reproducible (Table 1).

Method comparison with established GC-MS demonstrated good agreement. Bland-Altman plots (Fig. 4) indi-

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cated that results of the two methods are equivalent and fall within the clinically acceptable 95% limit.

For maximum diagnostic utility, the working range should include pertinent reference intervals and pathological concentrations. $C_{26:0}$, the most decisive marker for PD diagnosis, was reliably detectable in plasma of all controls analyzed. Phy, known to be exclusively from exogenous sources, circulated at levels that varied significantly among controls (0.04–11.5 μM). This analyte was detected in all samples analyzed, whereas its α-oxidation product, Pri, was not detectable in 3% of samples. Table 2 shows the reference intervals obtained by this LC-MS/MS in our population, which are in agreement with established values obtained by GC-MS in reputable laboratories (15, 28). It is worth mentioning that plasma levels of Pri and Phy were age dependent (see Table 2 footnotes). This was similar to those observed by ten Brink and coworkers (29), who reported increased levels of these acids in older children.

The diagnostic criteria for PDs involve absolute concentrations of individual analytes and their ratios. In patients with ZS, neonatal adrenoleukodystrophy, infantile RD, and a multitude of PEDs, absolute levels of $C_{26:0}$ and $C_{26:0}/C_{22:0}$ ratios are permanently elevated, and thus are considered reliable diagnostic indices. These are preferred over unsaturated $C_{26:1}$ and $C_{26:1}/C_{22:0}$, which are generally lower, especially in X-ALD and peroxisomal acyl-CoA oxidase deficiency (28). As shown in Table 2, an isolated elevation of Phy was observed in the patient with the deficiency of phytanoyl-CoA hydroxylase that causes RD. Unfortunately; the diagnostic value of Pri could not be demonstrated here, owing to the lack of samples from patients with 2-methylacyl-CoA racemase deficiency.

During 2007, this method was applied to the prospective analysis of more than 830 clinical samples, and performance was monitored through participation in the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited Disorders of Metabolism-organized External Quality Assessment Scheme. This further established the technical feasibility and efficacy of this LC-MS/MS, which completely replaced the standard GC-MS in our lab.

In summary, we report a new approach to the simultaneous determination of Pri, Phy, and VLCFAs, the hallmarks of PDs. This LC-MS/MS allows for determination of these analytes in a small plasma volume (20 μl) within a short analytical time of 5 min. The method is convenient, with a single liquid-liquid extraction and one derivatization step. Reference intervals obtained in our population were in agreement with the literature, and the method was able to detect confirmed cases of PDs with 100% sensitivity. The current method is capable of detecting the following nine PDs: ZS, neonatal adrenoleukodystrophy, infantile RD, rhizomelic chondrodysplasia punctata type 1, X-ALD, RD, peroxisomal acyl-CoA oxidase deficiency, D-bifunctional protein deficiency, and 2-methylacyl-CoA racemase deficiency. With further validation, this simple, accurate, and high-throughput LC-MS/MS may replace the existing traditional methods, especially in busy and high-volume laboratories. Application of this derivatization approach to other disease markers carrying a carboxylic moiety is under investigation. Until it becomes commercially available, limited amounts of DAABD-AE will be made available to interested laboratories (please contact O. Y. Al-Dirbashi).

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REFERENCES

1. Gould, S. J., G. V. Raymond, and D. Valle. 2001. The peroxisome biogenesis disorders. *In* The Metabolic and Molecular Bases of Inherited Disease. C. R. Scriver, A. L. Beaudet, D. Valle, and W. S. Sly, editors. McGraw-Hill, Inc., New York. 3181–3217.
2. Poll-The, B. T., P. Aubourg, and R. J. A. Wanders. 2006. Peroxisomal disorders. *In* Inborn Metabolic Diseases Diagnosis and Treatment. J. Fernandes, J. M. Saudubray, G. van den Berghe, and J. H. Walter, editors. Springer, Berlin Heidelberg New York. 509–522.
3. Wanders, R. J. A., P. G. Barth, and B. T. Poll-The. 2006. Peroxisomal disorders. *In* Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases. N. Blau, M. Duran, M. E. Blaskovics, and K. M. Gibson, editors. Springer, Berlin Heidelberg New York. 481–508.
4. Shimozawa, N. 2007. Molecular and clinical aspects of peroxisomal diseases. *J. Inherit. Metab. Dis.* **30:** 193–197.
5. Hoffmann, G. F., W. L. Nyhan, J. Zschoche, S. G. Kahler, and E. Mayatepek. 2002. Inherited Metabolic Diseases. Lippincott Williams and Wilkins, Philadelphia. 328–333.
6. Wanders, R. J. A. 2004. Peroxisomes, lipid metabolism, and peroxisomal disorders. *Mol. Genet. Metab.* **83:** 16–27.
7. Oglesbee, D. 2005. An overview of peroxisomal biogenesis disorders. *Mol. Genet. Metab.* **84:** 299–301.
8. Wanders, R. J. A. 2004. Metabolic and molecular basis of peroxisomal disorders: a review. *Am. J. Med. Genet. A.* **126:** 355–375.
9. Wanders, R. J., and H. R. Waterham. 2006. Peroxisomal disorders: the single peroxisomal enzyme deficency. *Biochim. Biophys. Acta.* **1763:** 1707–1720.
10. Shimozawa, N., T. Tsukamoto, Y. Suzuki, T. Orii, Y. Shirayoshi, T. Mori, and Y. Fujiki. 1992. A human gene responsible for Zellweger syndrome that affects peroxisome assembly. *Science.* **255:** 1132–1134.
11. Hogenboom, S., J. J. Tuyp, M. Espeel, J. Koster, R. J. Wanders, and H. R. Waterham. 2004. Mevalonate kinase is a cytosolic enzyme in humans. *J. Cell Sci.* **117:** 631–639.
12. Baumgartner, M. R., and J. M. Saudubray. 2002. Peroxisomal disorders. *Semin. Neonatol.* **7:** 85–94.
13. Berger, J., and J. Gärtner. 2006. X-linked adrenoleukodystrophy: clinical, biochemical and pathogenic aspects. *Biochim. Biophys. Acta.* **1763:** 1721–1732.
14. Vallanve, H., and D. Applegarth. 1994. An improved method for quantification of very long chain fatty acids in plasma. *Clin. Biochem.* **27:** 183–186.
15. Vreken, P., A. E. M. van Lint, A. H. Bootsma, H. Overmars, R. J. A. Wanders, and A. H. van Gennip. 1998. Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas chromatography-electron impact mass spectrometry. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **713:** 281–287.
16. Jakobs, C., C. M. M. van den Heuvel, F. Stellaard, C. Largilliere, F. Skovby, and E. Christensen. 1993. Diagnosis of Zellweger syndrome by analysis of very long-chain fatty acids in stored blood spots collected at neonatal screening. *J. Inherit. Metab. Dis.* **16:** 63–66.
17. Johnson, D. W. 2000. A rapid screening procedure for the diagnosis of peroxisomal disorders: quantification of very long chain fatty acids as dimethylaminoethyl esters in plasma and blood spots by electrospray tandem mass spectrometry. *J. Inherit. Metab. Dis.* **23:** 475–486.
18. Valianpour, F., J. J. M. Selhorst, L. E. M. van Lint, A. H. van Gennip, R. J. A. Wanders, and S. Kemp. 2003. Analysis of very long chain

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fatty acids using electrospray ionization mass spectrometry. *Mol. Genet. Metab.* **79:** 189–196.
19. Johnson, D. W., M. U. Trinh, and T. Oe. 2003. Measurement of plasma pristanic, phytanic and very long chain fatty acids by liquid chromatography electrospray tandem mass spectrometry for the diagnosis of peroxisomal disorders. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **798:** 159–162.
20. Tsukamoto, Y., T. Santa, H. Saimaru, K. Imai, and T. Funatsu. 2005. Synthesis of benzofurazan derivatization reagents for carboxylic acids and its application to analysis of fatty acids in rat. *Biomed. Chromatogr.* **19:** 802–808.
21. Santa, T., O. Y. Al-Dirbashi, T. Ichibangase, T. Fukushima, M. S. Rashed, T. Funatsu, and K. Imai. 2007. Synthesis of benzofurazan derivatization reagents for carboxylic acids in liquid chromatography/electrospray ionization tandem mass spectrometry. *Biomed. Chromatogr.* **21:** 1207–1213.
22. Al-Dirbashi, O. Y., T. Santa, K. Al-Qahtani, M. Al-Amoudi, and M. S. Rashed. 2007. Analysis of organic acid markers relevant to inherited metabolic diseases by UPLC-MS/MS as benzofurazan derivatives. *Rapid Commun. Mass Spectrom.* **21:** 1984–1990.
23. Moser, H. W., and A. B. Moser. 1991. Measurement of saturated very long chain fatty acids in plasma. *In* Techniques in Diagnostic Human Biochemical Genetics. F. A. Hommes, editor. Wiley-Liss, New York. 177–191.
24. Moser, A. E., I. Singh, F. R. Brown, G. I. Solish, R. I. Kelly, P. J. Benke, and H. W. Moser. 1984. The cerebrohepatorenal (Zellweger) syndrome. Increased levels and impaired degradation of very-long chain fatty acids and their use in prenatal diagnosis. *N. Engl. J. Med.* **310:** 1141–1146.
25. Johnson, D. W. 2005. Contemporary clinical usage of LC/MS: analysis of biologically important carboxylic acids. *Clin. Biochem.* **38:** 351–361.
26. Hall, N. A., G. W. Lynes, and N. M. Hjelm. 1988. Ratios for very long chain fatty acids in plasma of subjects with peroxisomal disorders as determined by HPLC and validated by gas chromatography mass spectrometry. *Clin. Chem.* **34:** 1041–1045.
27. Cech, N. B., and C. G. Enke. 2000. Relating electrospray ionization response to non-polar character of small peptides. *Anal. Chem.* **73:** 2713–2723.
28. Verhoeven, N. M., W. Kulik, C. M. M. van den Heuvel, and C. Jakobs. 1995. Pre- and postnatal diagnosis of peroxisomal disorders using stable-isotope dilution gas chromatography-mass spectrometry. *J. Inherit. Metab. Dis.* **18:** 45–60.
29. ten Brink, H. J., F. Stellaard, C. M. M. van den Heuvel, R. M. Kok, D. S. M. Schor, R. J. A. Wanders, and C. Jakobs. 1992. Pristanic acid and phytanic acid in plasma from patients with peroxisomal disorders: stable isotope dilution analysis with electron capture negative ion mass fragmentometry. *J. Lipid Res.* **33:** 41–47.

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