Derivatization-based High-throughput Bioanalysis by LC-MS

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Liquid chromatography coupled with mass spectrometry (LC-MS) is one of the most prominent analytical techniques due to its inherent selectivity and sensitivity. LC-MS is currently the first choice for high-throughput bioanalysis due to the advancements in MS instruments and the analytical software. Based on this situation, we are developing various types of derivatization reagents, including chiral reagents for MS and/or MS/MS detection. These developed reagents are adopted for the detection of biomarker candidates related to diseases. The biomarker candidates include not only achiral molecules, but also chiral ones. Although determining the already-identified chiral molecules is relative easy, it is very difficult to identify and/or determine unknown enantiomer(s) in real samples. To solve this difficulty, we proposed a new strategy to identify unknown enantiomeric biomarkers related to diseases. This review paper deals with the development of derivatization reagents for amines and carboxylic acids in LC-MS analysis and their application to bioanalysis.

Keywords Derivatization reagents, bioanalysis, chiral metabolomics, biomarker related to disease, liquid chromatography, mass spectrometry

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1 Introduction

The determination of biologically-active molecules is very important in order to understand their function in biological systems. The types and concentrations of molecules are of great variety. Not only various achiral molecules, but also chiral metabolites are contained in the specimens. Furthermore, many molecules are hydrophilic and/or have a low mass. Therefore, the high-throughput determination of each molecule in a complex matrix is essentially difficult by reversed-phase liquid chromatography (LC). In such a case, the derivatization technique is adopted, because the hydrophilicity and water solubility of the original molecules are improved with the derivatization using a suitable reagent. The detection sensitivity also increases with the derivatization by providing a sensitive moiety, such as fluorophore. Based on this background, we synthesized various types of fluorogenic and fluorescent derivatization reagents for the labeling of the target compounds.¹⁻⁶

The fluorescence analysis is specific and sensitive for the target molecules, and thus generally provides good expected results in a real sample analysis. Fluorometry has been accepted as a major technique for specific and sensitive analyses for over

a half century. However, the trace analysis of bioactive molecules in complex matrices, such as plasma and urine, is not always satisfactory by fluorometric analysis.

Mass spectrometry (MS) is an alternative technique for the selective and sensitive determination of biological components,⁷⁻¹³ the same as fluorescence analysis. The cost of

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Table 1 Derivatization reagents for amines and carboxylic acids analysis

Reagent	Reaction condition	LC separation		MS detection	Analyte	Sample	LOD	Ref.		
		Column	Mobile-phase							
DBD-F	0.1 M Borax (pH 9.3), 60°C & 30 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	ESI ⁺ , [M+H] ⁺	TOF-MS	ORN, DAP, PUT, CAD, SPD, SPM	Human hair of healthy persons	2 – 5 fmol	18
	0.1 M Borax (pH 9.3), 60°C & 30 min	Nano-flow chip LC	Zorbax 300SB-C18 (150 mm × 75 μm, i.d., 5 μm)	0.1% FA in H ₂ O-CH ₃ CN-MeOH	ESI ⁺ , SRM	Q-TOF-MS	ORN, DAP, PUT, CAD, SPD, SPM, N-AcPUT, N8-AcSPD, N1-AcSPM	Fingernail of lung cancer patients		22
	0.1 M Borax (pH 9.3), 60°C & 30 min	UPLC	ACQUITY BEH C18 (50 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	ESI ⁺ , SRM	Tandem-MS/MS	Di-AcSPM, Di-AcSPD	Fingernail of lung cancer patients		23
	0.1 M Borax (pH 9.3), 60°C & 30 min	Column-switching HPLC	Mightsil RP-18GP (100 × 2 mm, i.d., 3 μm) & Wakopak Navi C30-5 (150 × 2.1 mm, i.d., 5 μm)	0.1% TFA in H ₂ O-CH ₃ CN & 20 mM AcONH ₄ -CH ₃ CN	ESI ⁺ , SRM	Iontrap MS	Histamine	Human hair (root & shaft) & plasma		21
	0.1 M Borax (pH 9.3), 60°C & 45 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	20 mM FANH ₄ in H ₂ O-CH ₃ CN	ESI ⁺ , [M+H] ⁺	TOF-MS	Histamine and the metabolites	Mice hair		19
	0.1 M Borax (pH 9.3), 60°C & 45 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	20 mM FANH ₄ in H ₂ O-CH ₃ CN	ESI ⁺ , [M+H] ⁺	TOF-MS	Histamine and the metabolites	C3H/HeNCrj mice hair		20
	0.1 M Borax (pH 9.3), 60°C & 30 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	ESI ⁺ , SRM	Tandem-MS/MS	Polyamine & NAc-polyamines	Saliva of breast cancer patients	9 – 43 amol	25, 26
	0.1 M Borax (pH 9.3), 60°C & 30 min	HPLC	Capcel Pack C18 MGIII (100 × 2.0, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN-MeOH	ESI ⁺ , SRM	Tandem-MS/MS	Polyamines, NAc-polyamines	Fingernail of lung cancer patients	5 – 100 fmol	24
SPTPP	0.1 M Borax (pH 9.5), 40°C & 10 min	HPLC	Mightsil RP-18GP (100 × 2 mm, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN	ESI ⁺ , [M+H] ⁺	Tandem-MS/MS	Amino acids, 3-chloro-L-Tyr, 3-nitro-L-Tyr, GABA, Aliphatic amines,	Rat plasma	0.02 – 0.43 fmol	32, 33
HP	DPDS & TPP, 60°C & 10 min	HPLC	J'sphare ODS-H 80 (150 × 2 mm, i.d., 4 μm)	10 mM FANH ₄ in H ₂ O-MeOH	ESI ⁺ , SRM	Tandem-MS/MS	PGE2, γ-CEHC, HVA, 5-HIAA	Saliva	2.9 – 11 fmol	55
HP	DPDS & TPP, 60°C & 10 min	HPLC	J'sphare ODS-H 80 (150 × 2 mm, i.d., 4 μm)	0.1% FA in H ₂ O-MeOH	ESI ⁺ , SRM	Tandem-MS/MS	CDCA, GCDCA	Saliva	5.1 – 6.7 fmol	55
HP	DPDS & TPP, 60°C & 10 min	HPLC	Cadenza CD-C18 (75 × 2 mm, i.d., 3 μm)	10 mM FANH ₄ in H ₂ O-MeOH	ESI ⁺ , SRM	Tandem-MS/MS	CDCA, GCDCA	Saliva	25 – 50 pg/mL	56
PA	DPDS & TPP, 60°C & 10 min	HPLC	J'sphare ODS-H 80 (150 × 2 mm, i.d., 4 μm)	10 mM FANH ₄ in H ₂ O-MeOH	ESI ⁺ , SRM	Tandem-MS/MS	PGE2, γ-CEHC, HVA, 5-HIAA	Saliva	1.5 – 2.8 fmol	55
PA	DPDS & TPP, 60°C & 10 min	HPLC	J'sphare ODS-H 80 (150 × 2 mm, i.d., 4 μm)	0.1% FA in H ₂ O-MeOH	ESI ⁺ , SRM	Tandem-MS/MS	CDCA, GCDCA	Saliva	2.5 – 5.6 fmol	55
APBQ	DCC & pyridine, 60°C & 10 min	HPLC	Xbridge C18 (150 × 2.1, i.d., 3.5 μm)	0.1% FA in H ₂ O-CH ₃ CN	ESI ⁺ , [M+H] ⁺	Tandem-MS/MS	Fatty acids, bile acids	Human plasma & saliva	0.2 – 0.5 fmol	57

MS instrumentation has gradually decreased and the analytical software is also good enough for efficient determinations. Thus, the method using MS is currently the first choice for bioanalysis. Although highly sensitive detection is generally carried out by the method using MS, the detection sensitivity is dependent on the target molecule, and high sensitivity is not always guaranteed. To increase the sensitivity and specificity, we synthesized several derivatization reagents for MS detection and used them for the determination of amines and carboxylic acids. Furthermore, the derivatization reagents for the separation and detection of the chiral molecules by MS/MS were also synthesized and adopted for the determination of biological specimens. These reagents were successfully applied to the trace analysis of bioactive molecules in real samples.

This review paper summarizes the properties of the synthesized derivatization reagents including chiral molecules for LC-MS/MS analysis and their application to biological samples. The derivatization reagents described in this report have been developed by our groups.

2 Reagents for Amines and Carboxylic Acids

In 1989, 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), possessing a benzofurazan structure, was developed by our group as a thiol labeling reagent.^{14,15} The reagent has no fluorescence itself but fluoresces after labeling with thiol compounds. The resulting derivatives emit a strong fluorescence in the long-wavelength region (ex. *ca.* 390 nm, em. *ca.* 520 nm). This reagent was successfully used for the sensitive determination of thiol compounds. However, it is reactive not only for thiols, but also for amines due to its strong electrophilicity. Thus, DBD-F is used worldwide for the derivatization of primary and secondary amines in various specimens.^{16,17} Although the DBD-amine derivatives are highly fluorescent, they are also applicable for determination by LC-MS (Table 1). For instance, histamine and its metabolites in hair were easily labeled with DBD-F and determined by LC separation and time-of-flight (TOF)-MS detection.^{18–23} The polyamines in the fingernail of lung-cancer patients were also

Fig. 1 Derivatization reagents for achiral molecules by LC-MS/MS.

determined after labeling with DBD-F and detected by LC-TOF-MS.²⁴ DBD-F was also applied to the determination of polyamines, including its *N*-acetylated form in the saliva of breast-cancer patients by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).²⁵ The increase and decrease of specific polyamines were identified in breast-cancer patients. The diagnosis of breast cancer using saliva is now possible by this method.²⁶

Although efficient determinations of amines, such as histamine and polyamine, were performed by the labeling with DBD-F, further sensitive and specific determinations may be required for very low-concentrations of molecules. Based on this consideration, reagents for MS analysis were planned and synthesized (Fig. 1 and Table 1). In general, positively charged reagents are predominant for increasing the detection sensitivity.²⁷⁻²⁹ The quaternary amine structure is typical for positively charged reagents, such as *p*-*N,N,N*-trimethylammonioanilyl-*N'*-hydroxysuccinimidyl carbamate iodide (THAS).^{30,31} However, we synthesized a new type of positively charged reagent, (succinimidoxymethyl-5-oxopentyl)triphenylphosphonium bromide (SPTPP), possessing a quaternary phosphonium ion structure.^{32,33} This reagent reacts with amines to produce positively charged derivatives under mild conditions. As expected, the detection sensitivity increased. For instance, γ -aminobutyric acid (GABA) (a neurotransmitter) and *p*-nitrotyrosine (an oxidation stress marker) in rat plasma were successfully determined at a trace level.³² The sensitivity was increased by one-to-two orders of magnitude as compared to the non-labeled native molecules.

MS possesses high performance for the selective and sensitive detection of various compounds.³⁴⁻⁵¹ However, the sensitive determination of negatively charged molecules, such as carboxylic acids, is not always promising in spite of the highly sensitive MS detection.^{52,53} In such a case, the derivatization technique, which generally uses a basic reagent possessing amine(s), is effective for increasing the detection sensitivity and separation efficiency of reversed-phase chromatography,⁵⁴ because the resulting derivatives are easily charged as a cation by acidic mobile-phase containing formic and acetic acids. Although the use of positively charged reagents is furthermore effective for sensitive detection, the peak-to-peak separation of the derivatives is not always easy because of their high hydrophilicities.

The detection response of carboxylic acids by LC-ESI-MS/MS can be increased by derivatization using 2-hydrazinopyridine (HP) and 2-picolylamine (PA). HP and PA rapidly reacted with

biologically and/or clinically important carboxylic acids, *e.g.*, chenodeoxycholic acid (CDCA), glycochenodeoxycholic acid (GCDCA), prostaglandin E2 (PGE2), 2-(β -carboxyethyl)-6-hydroxy-2,7,8-trimethylchroman (γ -CEHC), α -lipoic acid, homovanillic acid (HVA) and 5-hydroxyindole-3-acetic acid (HIAA), in the presence of 2,2'-dipyridyl disulfide (DPDS) and triphenylphosphine (TPP).^{55,56} The resulting HP- and PA-derivatives were highly responsive to ESI-MS operating in the positive-ion mode, and gave characteristic product ions during MS/MS, which enabled sensitive detection using selected reaction monitoring (SRM). Among the two reagents, PA has been more suitable for practical use⁵⁵ because of the higher sensitivity. The detection responses of the PA-derivatives were increased one-to-two orders of magnitude (9–158-fold) over the intact carboxylic acids and the limits of detection (LOD) were in the low femtomole range (1.5–5.6 fmol) on the column. The PA derivatization method was successfully applied to a biological sample analysis. The derivatization followed by LC-ESI-MS/MS enabled the detection of trace amounts of bile acids, γ -CEHC and HVA in human saliva with a simple pretreatment.

The use of MS is excellent for qualification analysis due to appearance of the *m/z* peak corresponding to the protonated molecule $[M+H]^+$. To easily identify the analyte by the *m/z*, a reagent possessing a bromine atom (Br) in the structure was newly developed. 1-(3-Aminopropyl)-3-bromoquinolinium bromide (APBQ) is a derivatization reagent having a bromoquinolinium moiety for the detection and identification of carboxylic acids.⁵⁷ The carboxylic acids were labeled with APBQ in the presence of activation reagents, *i.e.*, dicyclohexyl carbodiimide (DCC) and pyridine. The resulting derivatives gave the characteristic twin *m/z* peaks on the mass spectra, which are based on isotopes (79 and 81) of Br. Therefore, free fatty acids and cholic acids in plasma were easily identified by their mass spectra.⁵⁷ Of course, the derivatives were quantitatively determined by extracted ion chromatography (EIC) based on $[M+H]^+$.

3 Reagents for Chiral Amines and Carboxylic Acids

The reagents described in the previous sections are useful for the sensitive and specific analysis of amines and carboxylic acids by fluorometry⁵⁸⁻⁶³ and MS.⁷⁻¹³ However, the determination of each enantiomer of chiral molecules is essentially impossible because there is no chiral recognition moiety in the reagent structures. Therefore, chiral-derivatization reagents for the separation and detection of a pair of enantiomers were also developed in our laboratories (Fig. 2).⁶⁴⁻⁷⁰

The enantiomers of 4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N,N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole ((*R*)-DBD-PyNCS), which are chiral fluorescent labeling reagents for amines, were applied to the enantiomeric determination of amino acids (AAs) in various samples. Several D-AAs were detected in foodstuffs (*e.g.*, yogurt and other fermented foods) and/or biological samples (*e.g.*, plasma).⁷¹ The use of DBD-PyNCS enantiomers (*R* and *S* forms) seems to provide significantly advanced analyses in the field of D-AAs.

For the peptide analysis in a complex matrix, such as tissue, however, no successful results could be obtained from the fluorometric analysis using (*R*)-DBD-PyNCS, because the determination in LC is disturbed by coeluted intrinsic components, which are also labeled with (*R*)-DBD-PyNCS. Therefore, the determination using LC-MS/MS is recommended

Fig. 2 Derivatization reagents for chiral molecules by LC-MS/MS.

in such cases, although the derivatives are a fluorescent.⁷²⁻⁷⁶ As an example, the analysis of the racemization and isomerization of *N*-terminal Asp in amyloid- β ($A\beta$) peptides in the brain homogenates of Alzheimer's disease (AD) patients were accomplished by (*R*)-DBD-PyNCS labeling and LC-MS/MS analysis (Table 2).⁷⁷ The post-translational racemization and isomerization might induce the density and localization of plaque desposition in the brain tissue, because AD is related to the formation and aggregation of $A\beta$ peptide plaques in the human brain. The isomerization of Asp in the tryptic peptides of α,β -chrySTALLIN was also possible to be detected by labeling with (*R*)-DBD-PyNCS.⁷⁸

As shown in peptide analysis using (*R*)-DBD-PyNCS, a chiral reagent suitable for MS is required for the sensitive and specific determination of a pair of enantiomers. However, the number of chiral reagents for the LC-MS/MS analysis is very limited. Based on these observations, we tried to develop chiral derivatization reagents for the enantiomeric determination of

chiral amines and carboxylic acids. We first searched the reagents for chiral carboxylic acids. The applicability of commercially-available chiral amines as derivatization reagents, *e.g.*, (*R*)-(+)-4-(3-aminopyrrolidin-1-yl)-7-(*N,N*-dimethylamino-sulfonyl)-2,1,3-benzoxadiazole ((*S*)-DBD-PyNCS), (*S*)-(+)-1-(2-pyrrolidinylmethyl)-pyrrolidine ((*S*)-PMP), (*3R*)-(+)-3-(trifluoroacetamido)-pyrrolidine ((*3R*)-TFAP), and (*R*)-(-)-1-aminoindan ((*R*)-AI), was tested for the enantiomeric separation of chiral carboxylic acids using non-steroidal anti-inflammatory drugs (NSAIDs) as representative carboxylic acids.⁷⁹ The chiral reagents were evaluated in terms of tagging easiness, separation efficiency by reversed-phase chromatography, and detection sensitivity by the SRM. Among them, (*S*)-DBD-PyNCS, (*S*)-PMP, (*R*)-AI, and (*3R*)-TFAP were applicable as labeling reagents. The resolution (*R*_s) values and the LOD of the derivatives were in the range of 1.29 – 3.85 ((*S*)-DBD-APy, 2.12 – 3.85; (*S*)-PMP, 1.35 – 1.76; (*R*)-AI, 1.54 – 2.85; (*3R*)-TFAP, 1.29 – 2.36) and 0.57 – 0.96 fmol ((*S*)-DBD-APy, 0.57 –

Table 2 Derivatization reagents for chiral amines and carboxylic acids analysis

Reagent	Reaction condition	LC separation			MS detection		Analyte	Sample	LOD	Ref.	
		Column	Mobile-phase	Rs	Tandem MS/MS						
(R)-DBD-PyNCS	2% TEA, 70°C & 4 h	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	50 mM FANH ₄ in H ₂ O-0.01% FA in CH ₃ CN	RT: L-Asp 28.5 min, D-Asp 24.3 min, L-isoAsp 25.0 min, D-isoAsp 21.3 min	ESI ⁺ , [M+H] ⁺	Asp isomers in Aβ peptide	AD brain homogenate		77	
(R)-DBD-PyNCS	2% TEA, 70°C & 4 h	HPLC	Capcell Pak ADME (100 × 2.1 mm, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.53 – 4.19	ESI ⁺ , SRM	Asp isomers in tryptic peptide	Crystallin		78	
(S)-DBD-Apy	DPDS & TPP, RT & 90 min	HPLC	Mightsil C18 (100 × 2.0 mm, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN	2.12 – 3.85	ESI ⁺ , SRM	NSAIDs	Rat plasma spiked	0.57 – 0.75 fmol	79	
(S)-PMP	DPDS & TPP, RT & 90 min	HPLC	Mightsil C18 (100 × 2.0 mm, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.35 – 1.76	ESI ⁺ , SRM	NSAIDs	Rat plasma spiked	0.62 – 0.76 fmol	79	
(S)-PMP	DPDS & TPP, RT & 90 min	HPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	4.91 – 9.37	ESI ⁺ , SRM	LA, HA	Saliva of DM	5 – 12 fg	80	
(R)-AI	DPDS & TPP, RT & 90 min	HPLC	Mightsil C18 (100 × 2.0 mm, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.54 – 2.85	ESI ⁺ , SRM	NSAIDs	Rat plasma spiked	0.65 – 0.96 fmol	79	
(R)-AI	DPDS & TPP, RT & 90 min	HPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.12 – 2.64	ESI ⁺ , SRM	LA, HA	Saliva of DM	33 fg	80	
(3R)-TFAP	DPDS & TPP, RT & 90 min	HPLC	Mightsil C18 (100 × 2.0 mm, i.d., 3 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.29 – 2.36	ESI ⁺ , SRM	NSAIDs	Rat plasma spiked	0.76 – 0.91 fmol	79	
(S)-ANA	DMT-MM, RT & 5 min	HPLC	YMC-Pack Pro (150 × 2.0mm, i.d., 3 μm)	10 mM FANH ₄ in MeOH, 10 mM FANH ₄ in CH ₃ CN	RT: 3-OH-PA (11.4 & 12.3 min), γ-CHEC (7.4 & 8.2 min), ETO (10.4 & 11.3 min)	ESI ⁺ , SRM	3-OH-PA, γ-CHEC, ETO	3-OH-PA in DBS, γ-CHEC in saliva	1.8 – 11 fmol	81	
(R)-PyT-C	DPDS & TPP, 60°C & 60 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	3.07 – 4.75	ESI ⁺ , SRM	<i>m/z</i> 137	NEA, AA-methyl esters	Saliva spiked	0.55 – 1.89 fmol	82
(S)-PyT-N	DPDS & TPP, 60°C & 60 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	3.54 – 6.00	ESI ⁺ , SRM	<i>m/z</i> 137	IBP, NAc-AAAs	Saliva spiked	0.72 – 1.46 fmol	82
(S)-PCP2 (3 and 4)	DPDS & TPP, RT & 60 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN (NSAIDs), 20 mM FANH ₄ in CH ₃ CN (biological carboxylic acids)	2.52 – 6.07	ESI ⁺ , SRM	<i>m/z</i> 70	NSAIDs, biological carboxylic acids	Saliva	49 – 260 amol (NSAIDs), 32 – 520 amol (biological carboxylic acids)	84
(S)-PCP2-Me	DPDS & TPP, 60°C & 60 min	UPLC	ACQUITY HSS T3 (100 × 2.1 mm, i.d., 1.8 μm)	20 mM FANH ₄ in CH ₃ CN	1.67 – 2.95	ESI ⁺ , SRM	<i>m/z</i> 70	NSAIDs	Authentic	15 – 72 amol	83
DMT-(S)-Pro-OSu	100 mM TEA, RT & 40 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm), Capcell Core ADME (100 × 2.1 mm, i.d., 2.7 μm)	0.1% FA in H ₂ O-CH ₃ CN, 0.1% FA in H ₂ O-MeOH, <i>etc.</i>	1.2 – 9.0 (AA), 1.3 – 2.6 (amines)	ESI ⁺ , SRM	<i>m/z</i> 209	AA, amines, Ad, Nad	Saliva	0.044 – 1.2 fmol (AA), 0.02 – 2.9 fmol (amines)	86
DMT-3(S)-Apy	DPDS & TPP, 60°C & 90 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	2.45 – 5.14 (NSAIDs), 1.65 – 1.98 (biological carboxylic acids)	ESI ⁺ , SRM	<i>m/z</i> 226	NSAIDs, biological carboxylic acids	Saliva of DM	3.2 – 5.9 amol (NSAIDs), 5.8 – 658 amol (biological carboxylic acids)	85
DMT-3(S)-Apy	DPDS & TPP, 60°C & 90 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.82	ESI ⁺ , SRM	<i>m/z</i> 209 & 226	LA	DSS of healthy & DM	30 amol	36
L-PGA-OSu	10 mM TEA, 60°C & 10 min	UPLC	ACQUITY BEH C18 (100 × 2.1 mm, i.d., 1.7 μm)	0.1% FA in H ₂ O-CH ₃ CN	1.95 – 8.05	ESI ⁺ , SRM	<i>m/z</i> 84	AA	Human serum & yogurt	0.5 – 3.2 fmol	89, 90

0.75 fmol; (*S*)-PMP, 0.62 – 0.76 fmol; (*R*)-AI, 0.65 – 0.96 fmol; (3*R*)-TFAP, 0.76 – 0.91 fmol), respectively. The (*S*)-PMP was also used as the derivatization reagent for high-throughput determinations of biological chiral carboxylic acids, *i.e.*, lactic acid (LA) and 3-hydroxybutyric acid (HA). The (*S*)-PMP efficiently reacted with the carboxylic acids under mild conditions, and the resulting PMP derivatives were highly responsive to MS operating in the positive-ion mode, and gave characteristic product ions during MS/MS. The *R_s* values of

DL-LA and DL-HA were 4.91 and 9.37, respectively. The LODs on an ODS column were in the low femtogram range (5 – 12 fg). The proposed procedure was successfully used for the determination of the D- and L-isomers of LA and HA in the saliva of diabetes mellitus (DM) patients and healthy volunteers.⁸⁰

(*S*)-Anabasine ((*S*)-ANA), which is a pyridine and piperidine alkaloid found in the Tree Tobacco (*Nicotiana glauca*) plant, was next tested for applicability as a derivatization reagent for

carboxylic acids, *i.e.*, 3-hydroxypalmitic acid (3-OH-PA), γ -CEHC and etodolac (ETO), in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM).⁸¹ Although various reagents, such as DCC and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), are possible to use for the condensation reaction of amine and carboxylic acid, care should be taken for possible epimerization during the reaction. Possible epimerization was negligible in this reaction using (S)-ANA, and these carboxylic acids in the presence of DMT-MM. The resulting ANA-derivatives were highly responsive during ESI-MS in the positive-ion mode and gave characteristic product ions during the MS/MS. The detection responses of the ANA-derivatives increased by 20 – 160 fold over those of the intact carboxylic acids; the LOD was in the low femtomole range (1.8 – 11 fmol on the column). ANA derivatization was effective for the enantiomeric separation of chiral carboxylic acids. The *R_s* values were 1.92, 1.75, and 2.03 for 3-OH-PA, γ -CEHC, and ETO, respectively. The derivatization procedure was successfully used for the trace determination of 3-OH-PA in a neonatal dried blood spot (DBS) and γ -CEHC in human saliva.

To obtain further high sensitivity and separation efficiency, chiral derivatization reagents for amines and carboxylic acids by LC-MS/MS analysis were designed and synthesized (Table 2). The ideal reagent has to possess (1) a chiral recognition moiety, (2) a highly sensitive detection moiety (*i.e.*, positively-charged moiety or the structure of easy protonation), (3) a moderate molecular mass suitable for tandem quadrupole MS/MS analysis, (4) a high reactivity to the target compound, and (5) the same cleavage position in the derivatives suitable for SRM. As the derivatization reagents possessing these properties, several chiral compounds were synthesized and used for the enantiometric determination of chiral amines⁸² and carboxylic acids.⁸³

As the ideal labeling reagents for the enantioseparation, chiral compounds possessing a pyridylthiourea structure were developed.⁸² (*R*)-*N*-(3-Pyridylthiocarbamoyl)pyrrolidine-2-carboxylic acid ((*R*)-PyT-C) and (*S*)-3-amino-1-(3-pyridylthiocarbamoyl)pyrrolidine ((*S*)-PyT-N) are chiral reagents for enantiomeric determination of chiral amines and carboxylic acids in high-throughput LC-ESI-MS/MS, respectively. The chiral amines and carboxylic acids were respectively labeled with (*R*)-PyT-C and (*S*)-PyT-N at 60°C for 60 min in the presence of DPDS and TPP as activation reagents. The resulting diastereomers were completely separated by reversed-phase chromatography using an ODS column (*R_s* = 3.54 – 6.00 for carboxylic acids and *R_s* = 3.07 – 4.75 for amines). Highly-sensitive detection at the sub-fmol level was also obtained from SRM chromatograms used for monitoring a single ion *m/z* 137.0 (0.72 – 1.46 fmol for carboxylic acids and 0.55 – 1.89 fmol for amines). The proposed procedure using (*R*)-PyT-C and (*S*)-PyT-N was used for the determination of chiral amines and carboxylic acids spiked in human saliva. Because the same product ion at *m/z* 137.0 was obtained from the collision-induced dissociation (CID) of the protonated molecule of all the derivatives, the proposed procedure using both reagents (*i.e.*, (*R*)-PyT-C and (*S*)-PyT-N) seems to be useful for chiral the identification of metabolites having selected functional groups (*i.e.*, amines and carboxylic acids).

A series of prolylamidepyridins, *i.e.*, (*S*)-*N*-pyrrolidine-2-carboxylic acid *N*-(pyridin-2-yl)amide ((*S*)-PCP2), (*S*)-*N*-pyrrolidine-2-carboxylic acid *N*-(pyridin-3-yl)amide ((*S*)-PCP3) and (*S*)-*N*-pyrrolidine-2-carboxylic acid *N*-(pyridin-4-yl)amide ((*S*)-PCP4), were synthesized for the enantioseparation of chiral carboxylic acids, and evaluated in terms of the separation

efficiency and the detection sensitivity by ultra-performance liquid chromatography (UPLC)-MS/MS.⁸⁴ The peak separation and peak shape, derived from these reagents ((*S*)-PCP2, (*S*)-PCP3 and (*S*)-PCP4), were slightly different between the derivatives obtained from NSAIDs, such as ibuprofen (IBP). The separation efficiency seems to be dependent on the three-dimensional structure of the diastereomers, judging from stable structure analysis by molecular modeling (MM2). Among the three, (*S*)-PCP2 was the most efficient chiral derivatization reagent in terms of the enantioseparation of carboxylic acids. The *R_s* values and the LODs of the derivatives of the NSAIDs were in the range of 2.52 – 6.07 and 49 – 260 amol, respectively. The sensitive detection of biological carboxylic acids (LOD, 32 – 520 amol) was carried out by the proposed method using (*S*)-PCP2 and UPLC-MS/MS. The (*S*)-PCP2 was applied to the determination of carboxylic acids in human saliva. Several biological carboxylic acids, such as LA and HA, were clearly identified in saliva.

To obtain an even higher sensitivity, derivatization reagents having a triazine structure, *e.g.*, 2,5-dioxopyrrolidin-1-yl-1-(4,6-dimethoxy-1,3,5-triazine-2-yl) pyrrolidine-2-carboxylate (DMT-(*S*)-Pro-OSu) and 1-(4,6-dimethoxy-1,3,5-triazine-2-yl)pyrrolidine-3-amine (DMT-3(*S*)-Apy), were synthesized and evaluated for chiral amines and carboxylic acids. The couples of the enantiomers of the NSAIDs, *i.e.*, IBP, Naproxen (NAP) and Loxoprofen (LOX), were completely separated (*R_s* > 2.45) and sensitively detected (LOD < 5.9 amol) after derivatization with DMT-3(*S*)-Apy.⁸⁵ The high separation efficiency and detection sensitivity are due to the 3(*S*)-aminopyrrolidine moiety in the reagent and the amide structure of the resulting derivatives, which are easily cleaved by the CID. The chiral and achiral carboxylic acids in the saliva of healthy persons and diabetic patients were labeled with DMT-3(*S*)-Apy and determined by the SRM of UPLC-MS/MS. Several chiral carboxylic acids (*e.g.*, LA and HA) and achiral carboxylic acids (*e.g.*, citric acid and succinic acid) were clearly identified in saliva. Furthermore, the D/L-ratio of LA in diabetic patients was higher than those in the healthy subjects. Based on these results, DMT-3(*S*)-Apy seemed to be a useful chiral derivatization reagent for the determination not only of chiral, but also achiral carboxylic acids in real samples.

On the contrary, the enantiomers of AAs were easily labeled with DMT-(*S*)-Pro-OSu at room temperature (RT) within 40 min in an alkaline medium containing trimethylamine (TEA).⁸⁵ The diastereomers derived from proteolytic AAs, except serine, were well separated under isocratic elution conditions by reversed-phase chromatography using an ODS column (*R_s* = 1.2 – 9.0). DL-Serine was separated by using an ADME column (SHISEIDO Co. Tokyo, Japan), which has a relatively more polar surface than the conventional ODS column. The characteristic product ions, *i.e.*, *m/z* 195.3 and 209.3, were detected from all of the diastereomers based on the CID of the protonated molecule. A highly sensitive detection at the amol – fmol level was obtained from the SRM chromatogram. The chiral amines, *e.g.*, adrenaline (Ad) and noradrenaline (Nad), labeled with DMT-(*S*)-Pro-OSu were also well separated and sensitively detected by the present procedure. The method using DMT-(*S*)-Pro-OSu was used for the determination of DL-AAs in the human saliva of healthy volunteers. Various L-AAs were identified in the saliva. Furthermore, D-alanine (D-Ala) and D-proline (D-Pro) were also detected in a relatively high content ratio (>5%). Since the difference in the D/L-ratio of Ala during one day was not very high, the effect of food and beverage intake seemed to be negligible. The DL-AA analysis was also performed using the pyroglutamic acid succinimidyl

ester (L-PGA-OSu).⁸⁶ However, total analysis of all the DL-AAs also failed using this chiral reagent. Although these reagents are suitable for individual AA analyses, the simultaneous separation of the enantiomers of all AAs is currently difficult, because the enantiomeric separations of many AAs, at least 18 kinds of proteolytic AA, have to be performed by one chiral reagent. Therefore, various types of reagents are needed for total analyses of the DL-AAs.

The DMT-(S)-Pro-OSu and DMT-3(S)-Apy seem to be useful chiral derivatization reagents for the determination of amines and carboxylic acids in biological samples. Furthermore, the present methods using DMT-(S)-Pro-OSu and DMT-3(S)-Apy may be applicable for the determination of chiral metabolomics, because the resulting derivatives produce the same product ions without relation to the compounds, and show a highly-sensitive detectability in the SRM mode.

4 Detection of Biomarker Candidates Related to Disease

The determination of biomarker molecules concerning various diseases is being actively performed based on a metabolome study using LC-MS.^{87,88} However, the detection of chiral biomarker molecules possessing an asymmetric carbon in the structure has not yet been investigated in detail because of the difficulty of enantiomeric separation using a conventional column. As described in a previous section, chiral metabolites related to diabetes mellitus (DM) were discovered based on the labeling of chiral derivatization reagents, such as (S)-PCP2⁸⁴ and DMT-3(S)-Apy.⁸⁵ However, precise and reliable results could not always be obtained from real biological specimens. This is due to the difference in the matrix of the samples between patients and healthy persons. To cancel the matrix effect and obtain more accurate results, the isotopic labeling strategy using light and heavy reagents was recommended (Fig. 3).^{89,90} This isotopic labeling method minimizes the possible errors associated with the poor run-to-run reproducibility caused by ionization suppression during the electrospray, and/or retention time differences between the runs. The proposed strategy using isotopic variants seems to be preferable for the differential analysis of different sample groups, such as a healthy person and a patient. Based on this consideration, several heavy reagents labeled with deuterium (DMT-3(S)-Apy-*d*₆ and L-PGA-OSu-*d*₅) were synthesized and evaluated using biological samples. When the light and heavy DMT-3(S)-Apy were used for the analysis of the saliva of a healthy person or patient, respectively, a clear difference in the D/L ratios of LA was observed without requiring any calibration curves. These good results are due to cancelling of the matrix effect, because both reacted samples were premixed, then determined in a single chromatographic run.

If the target molecule related to diseases has already been identified by other methods, the determination of chiral compounds is relatively easy, as previously described. When the chiral molecule(s) related to disease are unknown, the identification and determination have been very difficult, until today. To solve this difficulty, we proposed a new strategy using a pair of enantiomeric derivatization reagents. The strategy includes a novel approach for chiral metabolomics fingerprinting and chiral metabolomics extraction (Fig. 4).⁹¹ The strategy is based on the labeling of a pair of enantiomers of chiral derivatization reagents (*i.e.*, DMT-(S and R)-Pro-OSu and DMT-3(S and R)-Apy) and precursor ion scan (PIS) chromatography of the derivatives. Multivariate statistics is also required for this

Fig. 3 Differential analysis using light and heavy reagents.

strategy. The chiral metabolomics fingerprinting (Step 1) was performed as follows: (1) the amines or carboxylic acids are labeled with an enantiomer of the corresponding derivatization reagent, (2) the labeled molecules are selectively determined by the PIS using the LC-MS/MS system, and (3) the labeled molecules detected from the PIS chromatogram in different sample groups are compared by multivariate statistics, such as principal-component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The biomarker candidates, which are not only chiral molecules, but also achiral ones, are picked out by Step 1. Therefore, the extraction method of the chiral molecules in the samples is required for the chiral metabolomics. The chiral metabolomics extraction method (Step 2) is operated as follows: (i) the mixed solution of different samples is divided into two groups, (ii) one sample group is labeled with an enantiomer of the derivatization reagent, whereas the other one is also labeled with the opposite enantiomer of the derivatization reagent, (iii) the labeled molecules in the two divided samples are determined by the PIS chromatogram, then compared by multivariate statistics using the same procedures of (2) and (3) in Step 1, and (iv) the chiral molecules are only extracted as increased and decreased markers. The proposed method was verified by determining the chiral carboxylic acids in the DM patient's saliva, and also applied to the detection of amines and carboxylic acids in the AD brain homogenates.⁹¹

As already described, different identifications of the chiral biomarker candidate(s) in the two groups is possible by the proposed procedures (Steps 1 and 2). However, elucidations of the exact structure of the unknown candidates, whose authentic standards are not commercially available, is generally very difficult, because the *m/z* values by MS and MS/MS analyses are not very exact in the tandem quadrupole MS instrument used in this study. To alleviate this disadvantage, we replaced the instrument from the Tandem Quadrupole MS by the Orbitrap MS, because high-resolution accurate mass data are obtained by the Orbitrap MS. These results will be reported elsewhere in the near future.

Fig. 4 Strategy for the detection of chiral biomarker candidates.

5 Conclusion and Prospect

This review paper focuses on the development of various new derivatization reagents for amines and carboxylic acids for the LC-ESI-MS analysis and the application of these reagents to biological specimens. The use of SRM in the LC-MS/MS is steadily increasing in the fields of bioanalysis over the past decade. Although the high-throughput determination of various molecules by LC-MS is generally promising, the selectivity and sensitivity essentially depend on the target molecule, and thus satisfactory results are not always obtained. In such a situation, a derivatization technique using a suitable reagent including a chiral reagent is recommended. The derivatization also increased the hydrophobicity of the analytes, and the separation efficiency by LC is improved as a result. However, there are few reagents for MS detection. The chiral reagents for enantiomeric determination are especially rare. To identify the chiral molecules related to diseases, many chiral reagents are required because an optimized reagent, which can separate all chiral molecules, has not yet been discovered. Consequently, the development of various reagents is still needed for a chiral metabolomics study. I hope that a bioanalysis study based on derivatization will be expanded worldwide by the participation of many scientists.

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7 Conflict of Interest

The author declares no conflict of interest.

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