Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry for biomedical analysis

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ABSTRACT: Liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) is one of the most prominent analytical techniques owing to its inherent selectivity and sensitivity. In LC/ESI-MS/MS, chemical derivatization is frequently used to enhance the MS/MS detectability. The derivatization improves the separation and ionization efficiency. Moreover, the generated derivatives give particular product ions by CID (collision induced dissociation), which allow for the sensitive detection. In this review, we present an overview of the derivatization reagents which have been applied to LC/ESI-MS/MS, focusing on the applications involving small molecules in biomatrices.

Key Words: LC/ESI-MS/MS (liquid chromatography/electrospray ionization tandem mass spectrometry), derivatization, reagent

Introduction

The development of sensitive and selective determination methods of trace level compounds is essential to elucidate their biological roles and functions in living systems. Recently, liquid chromatography/mass spectrometry (LC/MS) is frequently utilized for this purpose. In particular, high-performance liquid chromatography/tandem mass spectrometry (LC/MS/MS) equipped with electrospray ionization (ESI) ion source is the most prominent method, as it requires lower temperature for ionization compared with other ionization methods, and thus it can be used for thermally unstable compounds. In addition the low background noise level in MS/MS technology enables sensitive detection of the targeted compounds.

However, all the compounds can not be favorably analyzed by LC/ESI-MS/MS. For example, the ionization efficiencies are often extremely low and such compounds cannot be sensitively detected. An analyte should have the following properties to be sensitively analyzed by LC/ESI-MS/MS. Firstly, it must be in its ionic form in the solution phase or be chargeable through adduct formation in gas-phase reaction. Secondly, the analyte must have a non-polar region, since hydrophobic compounds can be well separated from salts and interfering compounds possessing suppression effects (*1*). And the non-polar ions prefer the droplet-air interface and reside at the droplet surface. Consequently these ions enter the gas phase more readily than those in the droplet interior and show a higher response (*2-5*). Thirdly, it is desirable that the target analyte fragments efficiently upon collision induced dissociation (CID) and generates an intense product ion for the sensitive MS/MS detection.

Chemical derivatization of the analyte is often used to enhance the detection sensitivity in ESI-MS. In the past decade, it was reported that the chemical derivatization of the analyte by the chargeable compounds improved ESI-MS responses of the target analytes (*6-9*). Since then, several reagents originally utilized in ultraviolet or fluorescence detection were borrowed for this purpose followed by the synthesis of others to be specifically used in LC/ESI-MS. These reagents were summarized in several review papers (*10-13*). These regents are aimed to enhance the ESI response but they are not designed to generate a particular product ion by CID. Therefore, the fragment patterns of the derivatives and the generation of a particular product ion depended on the structures of the generated derivatives, not on the reagents. On the contrary, the reagents developed specifically for LC/ESI-MS/MS were designed to carry a structure suitable for MS/MS detection. They are efficiently fragmented by CID to generate particular product ions. In this review, we present an overview of the derivatization reagents which have been applied to LC/ESI-MS/MS,

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Received October 4, 2007
Accepted October 17, 2007

Table 1. Derivatization reagents and analytes in LC/ESI-MS/MS

Reagent	Analyte	Detection limit	Ref
for ketones and aldehydes			
hydroxylamine	testosterone (human serum)	0.035 nmol/L	*14*
hydroxylamine	altrenogest (horse urine)	13 pg/mL	*15*
hydroxylamine	succinylacetone (human urine)	0.063 μM (LOQ)	*16*
DNPH	aliphatic aldehydes (environmental water)	μg/L range	*17*
cyclohexanedione	aliphatic aldehydes (plasma)	20-100 pg	*19*
5,5'-dimethyl-1,3-cyclohexanedione	aliphatic aldehydes (human brain tissue)	5 pg (LOQ)	*20*
Gir P	17-hydroxyprogesterone (dried filter paper blood)	10 ng/mL	*21*
Gir T	5-formyl-2'-deoxyuridine (Hela-S3 cell)	3-4 fmol	*22*
Dns-Hz	succinylacetone (dried blood spot, urine)	0.005 μM	*23*
HMP	testosterone, 5α-dihydrotestosterone (prostatic tissue)	1.0 ng/g tissue	*24*
HMP	testosterone, 5α-dihydrotestosterone (human prostate)	1.0 fmol for testosterone	*25*
HMP	testosterone (rat brain, serum)	0.06 ng/g tissue	*26*
HMP	dehydroepiandrosterone (human silva)	25 pg/mL	*27*
HMP	5α-reduced pregnane type neurosteroids (rat brain, serum)	0.25 ng/g tissue	*28*
HP	5 kinds of steroids such as testosterone	2 fmol	*29*
DAABD-MHz	aliphatic aldehydes	30-60 fmol	*30*
for alcohols and phenols			
Dns-Cl	17β-testosterone (mouse plasma, brain)	50 pg/mL	*31*
Dns-Cl	estrone, 17β-estradiol; estriol, 17α-ethinylestradiol (water sample)		*32*
Dns-Cl	17α-ethinylestradiol (aqueous environmental sample)	1.0 ng/mL	*33*
Dns-Cl	15 kinds of endogenous estrogen (human urine)	2 pg	*34*
Dns-Cl	ethinylestradiol (monkey plasma)	0.2 fg/mL	*35*
Dns-Cl	17β-estradiol (human serum)	0.6 pg	*36*
Dns-Cl	norethindrone, ethinylestradiol (human plasma)	2.5 pg/mL for ethinylestradiol	*37*
Dns-Cl	ethinyl estradiol (human plasma)	2.5 pg/mL	*38*
Dns-Cl	propfol (rat plasma)	20 ng/mL (analytical range)	*39*
Dns-Cl	1-hydroxypyrene (human urine)	20 pg/mL	*40*
pocolinic acid	7α-hydroxy-4-cholesten-3-one (human plasma)	100 fg	*41*
propionyl anhydride; benzoyl anhydride	ribosides, nucleotides (plant)	attomole-low femtomole range	*1*
NA	estrone, estradiol, androsterone	2 fmol	*29*
for carboxylic acids			
HCl-butanol	methylmalonic acid (human serum, plasma urine)	0.05 μmol/L	*56*
HCl-butanol	methylmalonic acid (human plasma, urine)	0.12 μmol/L (S/N = 40)	*57*
HCl-butanol	methylmalonic acid (human serum, plasma)		*58*
HCl-butanol	nitrotyrosine (rat plasma)	0.07 pmol	*59*
HCl-butanol	homocarnosine (cerebrospinal fluid)	20 nmol/L	*60*
trimethylamino-ethylalcohol (TAME)	very long chain fatty acids (human plasma)		*61*
4-dimethylamino-benzylamine	valproic acid (VA) and its metabolites (human plasma)	200 ng/mL for VA	*67*
DAABD-AE	C5-C6 di-carboxylic acids	0.025 μM for glutaric acid	*70*
DAABD-AE	pristanic, phytanic, C22:0, C24:0 and C26:0		*71*
for amines			
NBD-F	biogenic amines (tryptamine, histamine, agmatine, *N*-methylsalsolinol) (rat tissues)	0.6 ng/mL	*72*
NBD-F	*D,L*-amino acids (central nervous system)	95-150 ng/mL	*73*
acetic anhydride	shingosine-1-phosphate dihydrosingosine-1-phosphate (bovine serum, human endotherial cell)	less than 50 fmol	*74*
acetic anhydride pentafluorobenzyl cloride	aminonitropyrene diaminopyrene (rat urine, blood)	0.1 pg for diaminopyrene	*75*
NIT	18 kinds of volatile primary and secondary amines (air sample)	0.12-0.25 ng/μL	*76*
pentafluoropropionic acid anhydride	diamines (human urine, plasma)	0.2-0.3 fmol	*77*
nitrobenzyl chloroform	GABA agonist (rat plasma)	10 ng/mL	*78*
Dns-Cl	muscimol, iboteic acid (mushroom)	25 ppm	*79*
THAS	amino acids	atto-mole level	*80*
for miscellaneous compounds			
NBA	3,5-dinitrosalicylic acid anhydride (poultry muscle and liver)	0.04 mg/kg	*81*
NBA	nitrofuran metabolites (food samples)	0.11-0.21 μg/kg	*82*
ammonia	phenethyl isothiocyanate (human plasma, urine)	2 nmol/L	*83*
DBA	isocyanates (airsample)	10 atto mole	*84*
diazomethane	biphosphates (serum, urine)	pg/mL level	*85*
hexamethyleneimine	deoxyguanine adduct	low femto mole range	*86*

Abbreviations, DAABD-AE: 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole; DAABD-MHz: 4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole; DBA: di-*n*-butyl amine; DNPH: 2,4-dinitrophenyl hydrazine; Dns-Cl: dansyl chloride; Dns-Hz: Dansyl hydrazine; Gir P: 1-(carboxymethyl)pyridium chloride hydrazide; Gir T: (carboxym ethyl)trimethylammonium chloride hydrazide; HMP: 2-hydrazino-1-methyl-pyridine; HP: 2-hydrazinopyridine; NBD-F: 4-fluoro-7-nitro-2,1,3-benzoxadiazole; NA: isonicotinyl azide; NBA: 2-nitrobenzaldehyde; NIT: naphtyl isothiocyanate; LOQ: limit of quantification; THAS: 4-(trimethylammonium)anilyl-*N*-hydroxysuccidimidyl carbamate iodide.

focusing on the applications to low molecular weight compounds (Table 1).

Derivatization of analytes

Ketones and aldehydes (carbonyl compounds)

Ketones and aldehydes are neutral functional groups. The ionization efficiencies in ESI of these compounds are usually low. To overcome this, a chargeable moiety was introduced to these compounds to enhance the ionization efficiency. Hydroxylamine reacts with ketones to form the corresponding oximes. The derivative contains nitrogen atom and is expected to improve the ionization efficiencies of the analytes. Hydroxylamine was applied to LC/ESI-MS/MS analysis of ketones such as testosterone (*14*), altrenogest (a steroid used for the control of estrus in horse) (*15*), and succinylacetone (the hallmark of hepatorenal tyrosinemia) (*16*). A significant increase of detection sensitivity for altrenogest was observed (*15*). The product ions obtained by CID were dependant on the analyte structures.

DNPH (2,4-dinitrophenylhydrazine) was used for the determination of aldehydes and ketones in disinfected water (*17*). The derivatives were detected in the negative-ion mode. Several common product ions such as *m/z* 163, *m/z* 152, *m/z* 122, derived from the reagent skeleton, were observed. The product ion spectra were complicated by the several fragment ions generated by CID. The transitions of the $[M-H]^-$ ions to these product ions were used for MRM (multiple reaction monitoring). DNPH derivatives were also analyzed by LC/APCI (atmospheric pressure chemical ionization)-MS/MS (*18*). Cyclohexanedione was used for the determination of aliphatic aldehydes (C3-C10) including 4-hydroxynonenal and nonanal, the peroxidation products of fatty acids. Aldehydes were condensed with two molecules of cyclohexanedione in the presence of ammonia to form the tri-cyclic compounds (Hantzsch reaction). These compounds gave the common product ion at *m/z* 216 by CID, derived from the tri-cyclic structure. The transitions of $[M+H]^+$ ions of the derivatives to *m/z* 216 were used for MRM (*19*). Similarly, 5,5'-dimethyl-1,3-cyclohexanedione was used for the derivatization of biogenic aldehydes. The derivatives gave the common product ions at *m/z* 273 or 274 by CID (*20*). The transitions of $[M+H]^+$ ions of the derivatives to *m/z* 273 or 274 were used for MRM.

Girard's reagent P (1-(carboxymethyl)pyridium chloride hydrazide; Gir P) and Girard's reagent T ((carboxymethyl)trimethylammonium chloride hydrazide; Gir T) are reagents that possess a permanent cationic charge. Gir P was used for the determination of 17-hydroxyprogesterone (17-OHP), the marker for congenital adrenal hyperplasia. Several fragment ions (*m/z* 80, 93, 121) were generated by CID. The transition of *m/z* 299 ($[M]^{2+}$) to *m/z* 80 was used for MRM (*21*). Gir T was used for the determination of 5-formyl-2'-deoxyuridine, a major thymidine lesion generated by reactive oxygen species. The generated derivative gave the product ion at *m/z* 195, by the loss of a trimethylamino moiety (59 Da). The detection limit (3-9 fmol) was about 20 fold better than that for the direct analysis of the underivatized compound (*22*). Dansyl hydrazine (5-dimethylaminonaphthalene-1-sulfonyl hydrazine; Dns-Hz) was used for the analysis of succinylacetone in dried blood spot specimens. The generated dansyl hydrazone selectively gave the product ion at *m/z* 170 by CID, assigned to the cleavage of dimethyaminonaphtyl moiety originated from the reagent (Figure 1a). The product ion spectra of the derivatives were rather simple and clear. The transition of *m/z* 462 ($[M+H]^+$) to *m/z* 170 was used for MRM (*23*).

One of the most promising reagents for aldehydes and ketones is HMP (2-hydrazino-1-methyl-pyridine) (*24*). HMP has a 1-methylpyrizino group as a permanently charged moiety and a hydrazine group as a reactive site. HMP reacted with carbonyl compounds at 60ºC within 1 h. The ionization efficiencies of HMP derivatives were rather high and the generated derivatives gave a product ion at *m/z* 108 by CID, derived from 1-methylpyrizinoamino moiety (Figure 1b). HMP was applied to the LC/ESI-MS/MS analysis of keto-steroids such as testosterone, 5α-dihydrotestosterone in prostate and prostatic tissue (*24,25*), testosterone in rat serum and brain (*26*),

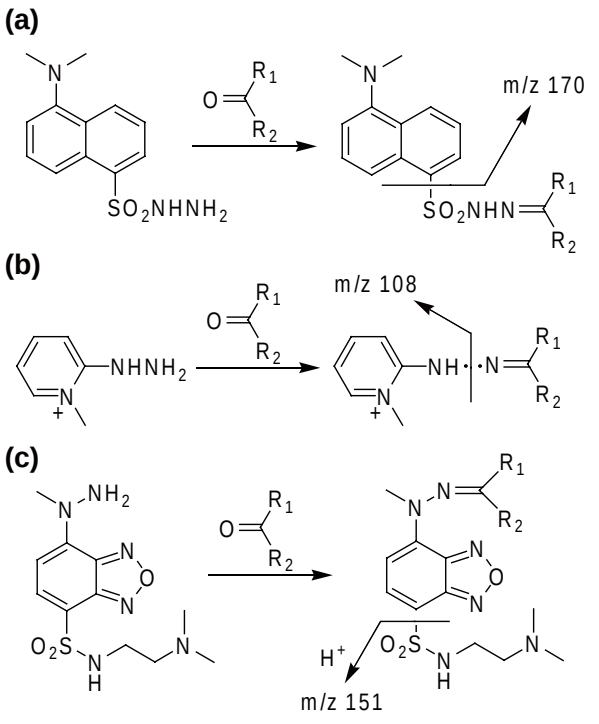

Figure 1. Derivatization reaction for ketones and aldehydes, and the product ion of the derivative obtained by CID, (a) Dns-Hz; (b) HMP; (c) DAABD-MHz.

dehydroepiandrosterone in saliva (*27*), 5α-reduced pregnane-type neurosteroids in rat brain and serum (*28*). HMP is not effective for increasing the detection responses of di-oxo-steroids. This phenomenon was due to the fact that small molecules with a multi-charge are unstable in the gas phase and provided multiple ions. To overcome these problems, HP (2-hydrazinopyridine) was used for di-oxo-steroids such as androsterone and progesterone. The generated derivatives gave the intense product ions at *m/z* 322 and 348 for the derivatives of androsterone and progesterone, respectively (*29*). Recently DAABD-MHz (4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-*N*-methylhydrazino-2,1,3-benzoxadiazole) for aldehydes was developed. It has a dimethylamino group as a chargeable moiety and a hydrazino group as a reaction site. The generated derivative showed a predominant product ion at *m/z* 151 by CID, derived from the protonated (*N*,*N*-dimethylamino)ethylaminosulfonyl moiety of the reagent (Figure 1c). The transition of $[M+H]^+$ ions to *m/z* 151 was used for MRM (*30*).

Alcohols and phenols

Alcohols and phenols are neutral compounds. Therefore, derivatization is required to enhance the ionization efficiency. One of the most often used reagents is dansyl chloride (5-Dimethylamino-1-naphthalenesulfonyl chloride; Dns-Cl). Dns-Cl has a dimethylamino group as an ionization moiety and a reactive sulfonylchloride group. It reacts with phenols at 60ºC within several minutes. The generated derivatives provided an almost single product ion at *m/z* 171 by CID, which was assigned to protonated dimethylaminonaphtyl moiety (Figure 2a). SRM (selected reaction monitoring) using the transition of quasi-molecular ion of the derivative to *m/z* 171 or 170 resulted in a sensitive detection of the derivative. So far, it was used for determination of steroids having hydroxyl group such as 17β-estradiol, estrone, 17α-ethinyl estradiol (*31-38*), propofol (veterinary medicine) (*39*), and 1-hydroxypyrene (biomarker to monitor the exposure to polycyclic aromatic hydrocarbons) (*40*).

The derivatization to picolynoyl ester was used for 7α-hydroxy-4-cholestene-3-one, a biomarker for bile acid biosynthesis. The derivative gave the product ion at *m/z* 383 by CID, due to the loss of picolinic acid moiety. The transition of *m/z* 506 ($[M+H]^+$) to *m/z* 383 was used for SRM (*41*) (Figure 2b). Propionyl and benzoyl anhydride were used for bases, ribosides, and intact nucleotides such as AMP, ADP and AMP (*1*). The ESI response was enhanced by the formation of hydrophobic derivatives. In addition, the retention on a reversed-phase column was greatly increased, and the derivatives were separated without the need for an ion paring reagent, known for its unwanted suppression effects on ionization. Propionated cytokinins were analyzed by

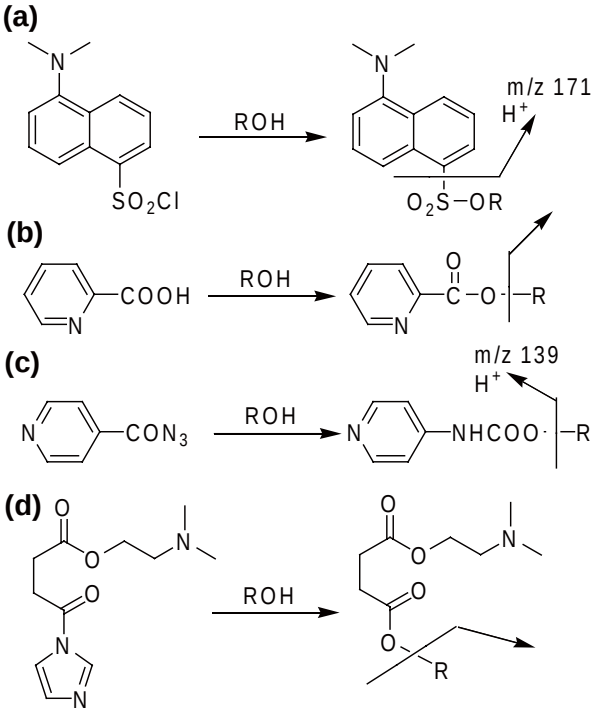

Figure 2. Derivatization reaction for alcohols, and the product ion of the derivative obtained by CID, (a) Dns-Cl; (b) picolynic acid; (c) NA; (d) MDMAES imidazole.

LC/ESI-MS/MS with detection limits in the sub femto-mole range. NA (isonicotinoyl azide) was used for the derivatization of di-hydroxysteroids such as estradiol and 5α-androstane-3α,17β-diol. NA reacted with two hydroxyl groups of estradiol at 80ºC for 30 min and the generated derivative gave the product ion at *m/z* 139 by CID, which was assigned to the protonated pyridyl carbamic acid (Figure 2c). The transition of $[M+H]^+$ ion to *m/z* 139 was usable for SRM analysis (*29*). MDMAES (mono-(dimethylaminoethyl) succinyl) imidazole was used for cholesterol and dehydrocholesterol. MDMAES imidazole reacted with hydroxyl group at 70ºC for 10 min and generated MDMAES ester. The ester gave the product ion at *m/z* 369 by CID, due to the neutral loss of MDMAES moiety (189 Da) (Figure 2d). Cholesterol and dehydrocholesterol in dried spot of plasma were analyzed by ESI-MS/MS without chromatographic separation (*42*). This reagent is usable for the derivatization of alcohol and SRM analysis.

LC/APCI-MS/MS is suitable for neutral derivatives. Acetic anhydride was used for budesonide in human plasma (*43*), and benzoyl chloride for propylene glycol in rat plasma and lung tissue (*44*). Estrone and related compounds were derivatized with pentafluorobenzyl bromide (*45*).

Carboxylic acids (including amino acids)

Carboxylic acids are detectable in the negative ESI-MS. However their sensitivity is known to be poor, because of the high background noise. In addition the mobile phases for the carboxylic acids separation are

not always compatible with ESI-MS. In one approach, carboxylic acids such as fatty acids are derivatized to their esters, and analyzed by LC/APCI-MS. Fatty acid esters are neutral or rather hydrophobic having an atom with proton affinity such as oxygen and are therefore suitable for APCI-MS detection.

One of the successful examples for ESI-MS/MS detection of carboxylic acids with derivatization is the simultaneous analysis of amino acids and acylcarnitines in dried blood spots for the diagnosis of organic acidemias and amino acidpathies developed by Rashed and coworkers, which paved the way for the reliable automated newborn screening (*46-48*). Amino acids were derivatized with butanolic HCl and the generated butyl esters were introduced to ESI-MS/MS without chromatographic separation. Most of α-amino acids butyl esters gave the intense product ions correspond to the loss of $HCOOC_4H_8$ (102 Da) by CID. Therefore, amino acids profile in biological samples can be obtained by the neutral loss scan of 102 Da. Acylcarnitines serve as marker metabolites for inherited disorders related to organic acid and fatty acid metabolism (*49*). Acylcarnitines have a quaternary ammonium group and a carboxylic group in their structure. Their butyl esters were introduced to ESI-MS/MS and gave the common product ion at *m/z* 85 by CID (*46*). Therefore, acylcarnitine profile can be obtained by precursor ion scan of *m/z* 85. These methods are currently widely used for the analysis of amino acids and acylcarnitines in urine, plasma, serum, or blood (dried blood spot). This topic was the subject of several excellent papers (*50-55*).

Butanolic HCl derivatization was also used for LC/ESI-MS/MS of carboxylic acids. Methylmalonic acid is the marker for a group of metabolic disorders caused by deficiency in methylmalonyl-CoA mutase or a defect in vitamin B_{12} metabolism. The di-butyl ester gave the product ion at *m/z* 119, due to the loss of 2 C_4H_8. The transition of *m/z* 231 ($[M+H]^+$) to *m/z* 119 was used for MRM (*56-58*). Nitrotyrosine is the tyrosine nitration product. Its butyl ester gave the product ion at *m/z* 181 or *m/z* 227, due to the loss of C_4H_8 (56 Da) or the loss of C_4H_8 and formic acid (102 Da) by CID. The transitions of *m/z* 283 ($[M+H]^+$) to these product ions were used for MRM detection (*59*). Homocarnosine is a brain specific di-peptide and the marker of heritable defect in GABA pathway. The transition of protonated homocarnosine butyl ester, *m/z* 297 ($[M+H]^+$) to *m/z* 212, derived from the loss of 85 Da, due to the loss of the aminobutyryl moiety, was used for SRM experiments (*60*).

Recently, several reagents having an ionization moiety, a reaction site, and a suitable structure for MS/MS detection were used for derivatization of carboxylic acids. TMAE (trimethylaminoethyl) ester derivatives were prepared for LC/ESI-MS/MS of very long chain fatty acids, the diagnostic markers for peroxisomal disorders (*61*). Fatty acids were treated with oxalyl chloride, dimethylaminoethanol, followed by the methylation with methyl iodide. These derivatives gave the product ion by the loss of 59 Da, derived from $(CH_3)_3N$ moiety of the derivatization reagent, and each fatty acid derivative was detected by MRM (Figure 3a). The generated derivatives were suitable for MS/MS detection. However, the three step derivatization reaction was tedious for routine assay. TMAE or DMAE (dimethylaminoethyl) ester derivatization of fatty acids and ESI-MS/MS analysis without chromatographic separation were also reported (*62-66*). Valproic acid and its metabolite were derivatized with 4-dimethyla minobenzylamine and analyzed by LC/ESI-MS/MS. The generated amide derivative gave the product ion at *m/z* 120 by CID, derived from dimethylamino phenyl moiety of the reagent (Figure 3b). The transition of *m/z* 277 ($[M+H]^+$) to *m/z* 120 was used for MRM for the determination of valproic acid (*67*). Recently, DAABD-AE, (4-[2-(*N*,*N*-dimethylamino)ethylaminosulfonyl]-7-(2-aminoethylamino)-2,1,3-benzoxadiazole) was developed as a derivatization reagent for carboxylic acids with excellent mass spectrometric charcteristics (*68,69*). DAABD-AE was used for the derivatization of dicarboxylic acids such as glutaric acid and 3-hydroxyglutarate, the marker metabolites for glutaric acidemia type 1 in human urine (*70*). The generated amide derivative gave the product ion at *m/z* 151 by CID, derived from the protonated (*N*,*N*-dimethylamino)ethylaminosulfonyl moiety of the reagent (Figure 3c). The transition of $[M+H]^+$ ions to *m/z* 151 was used for MRM. An attractive diagnostic method for peroxisomal disorders was also developed based on DAABD-AE derivatization and LC-MS/MS analysis. Compared to standard gas chromatography-mass spectrometric methods routinely used for this

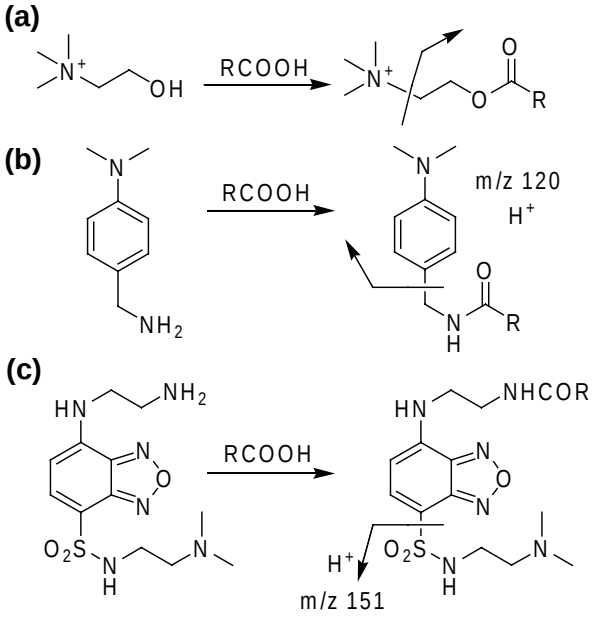

Figure 3. Derivatization reaction for carboxylic acids, and the product ion of the derivative obtained by CID, (a) trimethylaminoethyl (TMAE) alcohol; (b) 4-dimethylaminobenzylamine; (c) DAABD-AE.

purpose, this LC-MS/MS method is more simple, saves 75% of instrument time and requires one tenth of biological sample volume (*71*).

Amines

The compounds having amino group are easily protonated under acidic conditions and suitable for ESI-MS. However, the analysis of amines is often troublesome because of their high polarity, basicity, and high water solubility. Chemical derivatization makes amines more hydrophobic and the generated derivatives can be more easily separated from the interfering compounds on the reversed-phase column, and can be more sensitively detected in ESI-MS. In addition, the increase in the molecular weight decreases the background noise from the matrix, since the background is generally lower in the higher mass range.

NBD-F (4-fluoro-7-nitro-2,1,3-benzoxadiazole) was used for biogenic amines such as tryptamine, histamine, *N*-methylsalsolinol, and agmatine. The characteristic product ions were produced for each derivative by CID. In case of agmatine, the transition of *m/z* 294 ($[M+H]^+$) to *m/z* 277 was used for MRM (*72*). NBD-F was applied to the determination of *D*-amino acids. The characteristic precursor to product ion transitions, *m/z* 297 to 279 (NBD-Asp), *m/z* 269 to 223 (NBD-Ser), *m/z* 311 to 293 (NBD-Glu) were monitored for quantification (*73*). Acetyl anhydride was used for Sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate, an endogenous sphingolipid and the analog. Acetyl chloride reacted with an amino group and a hydroxyl group, and generated bi-acetylated derivatives. The transition of *m/z* 462 ($[M-H]^-$) to *m/z* 402 for S1P by CID, due to the neutral loss of acetic acid, was used for MRM (*74*). Acetyl chloride was used for polyaromatic amines such as diaminopyrenes and aminonitropyrenes, human carcinogens. The acetylated derivatives gave two sensitive MS/MS transitions, which were used for MRM, one was for quantification and the other for confirmation (*75*). Acetylation improved peak shapes and reproducibility in LC of diaminopyrene resulting in high signal to noise ratios. NIT (naphtylisothiocyanate) was used for the determination of eighteen kinds of primary and secondary amines in air samples. The derivatives of the primary amines gave the common base peak at *m/z* 144 and the fragment ion at *m/z* 127 by CID, whereas those of secondary amines gave the common base peak at *m/z* 186 and the fragment ion at *m/z* 128 by CID. This method provided the structural information of the analytes, and was suited for the analysis of complex environmental samples (*76*). Pentafluoropropionic acid anhydride was used for diamines, markers for the exposure to isocyanates. The reaction proceeded within 10 sec and the generated derivatives were analyzed by LC/ESI-MS in the negative ion mode. The $[M-H]^-$ ion was selected as precursor ions. And the product ions of $[M-H-120]^-$ or $[M-H-240]^-$, due to the neutral loss of one or two CF_3CHF_2 group, or *m/z* 119 corresponding to $CF_3CF_2^-$ ion, were monitored (*77*). Nitrobenzyl chloroformate was used for 3-amino-2(*S*)-hydroxypropylmethylphospheric acid, $GABA_B$ receptor agonist. The derivative gave the product ion at *m/z* 152, assigned to 4-nitrobenzyl alcohol anion, which was generated by the cleavage of ester bond of the reagent (Figure 4a). The transition of *m/z* 371 ($[M-H]^-$) to *m/z* 152 was used for SRM (*78*). Dansyl chloride was used for musimol and ibotenic acid, bioactive compounds in mushroom. The generated derivatives gave the product ion at *m/z* 171, derived from protonated dimethylaminonaphtyl moiety of the reagent (Figure 4b). The transitions of (M^+) ions to *m/z* 171 were used for MRM (*79*).

THAS (4-(trimethylammonium)anilyl-*N*-hydroxysuccidimidyl carbamate iodide) was the reagent designed for LC/ESI-MS/MS. It reacted with amino acids to form urea compounds. The derivatives gave the characteristic cleavage at the urea bond that is the binding position between the reagent and amino group, and produced characteristic fragment ions derived from the reagent skeleton (Figure 4c). Amino acids were analyzed with the detection limits of atto-mole level (*80*).

Amines are sometimes derivatized with reagents for ultraviolet or fluorescence detection, converted to neutral compounds, and analyzed by LC/APCI-MS. The readers should refer to the previous review (*10*).

Miscellaneous

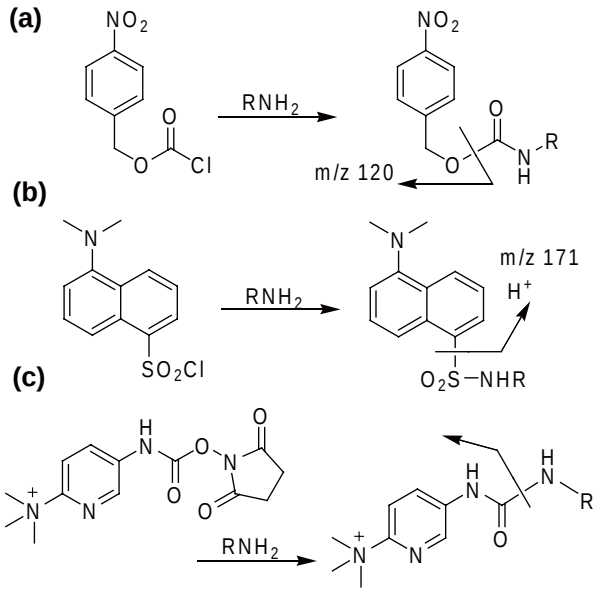

Figure 4. Derivatization reaction for amines, and the product ion of the derivative obtained by CID, (a) 4-nitrobenzyl chloroformate; (b) Dns-Cl; (c) THAS.

NBA (2-nitrobenzaldehyde) was used for the determination of 3,5-dinitrosalicylic acid hydrazide (DSH), nifursol (histomoniasis prevention compound) metabolites. DSH, generated by the acid-catalysed hydrosis of tissue-bound metabolites, was derivatized in situ with NBA and isolated by liquid-liquid extraction, and analyzed by LC/ESI-MS/MS in the negative-ion mode. The derivative gave the fragment ions at *m/z* 182, 183, and 226 by CID, derived from the analyte skeleton. The transitions of *m/z* 374 (quasi-molecular ion) to *m/z* 182 and 226 were used for MRM (*81*). NBA (2-nitrobenzaldehyde) was also used for nitrofuran metabolites, effective compounds against pathogenic bacteria. The transitions of ($[M+H]^+$) ions to the characteristic product ions were used for MRM (*82*).

Ammonia was used as a derivatization reagent for phenethyl isothiocyanate, a dietary compound present in cruciferous vegetables that has cancer-preventive properties. The derivative gave the fragment ion at *m/z* 105 due to the loss of the thiourea moiety. The transition of *m/z* 181 ($[M+H]^+$) to *m/z* 105 was used for SRM (*83*). DBA (di-*n*-butylamine) was used for the derivatization of isocyanates. The generated thiourea derivatives gave the fragment ions at *m/z* 130 and 156, assigned to $[DBA+H]^+$ and $[DBACO]^+$, respectively. The transitions of ($[M+H]^+$) ions to these fragments were used for MRM (*84*).

Diazomethane was used for the derivatization of biphosphonates such as risedronate and alendronate, the compound effective to osteoporosis and bone-related diseases. Risedronate is extremely hydrophilic and structurally similar to many endogenous phosphorylated compounds. Four hydroxyl groups of risedronate were methylated by diazomethane on silica-based anion-exchange sorbents. The derivatization made the analyte more hydrophobic, and improved chromatographic separation and ESI response. The transition of *m/z* 340 to *m/z* 214 for risedronone, and *m/z* 344 to 214 for alendronate were use for MRM (*85*). Hexamethyleneimine was used for the derivatization of deoxyguanosine monophosphate adducts, the indicator for the onset of tissue carcinogenicity. The hydrophobic derivatives showed increases in ionization efficiency and improved peak shape. The derivatization showed 3-4 fold signal enhancement compared to underivatized deoxyguanosine nucleotide (*86*).

Conclusion

The derivatization reagents applied to LC/ESI-MS/MS in biomedical analysis were reviewed. These reagents were used for the derivatization of ketones, aldehydes, alcohols, carboxylic acids, amines, and other compounds. The derivatization improved the separation efficiency, ionization efficiency and MS/MS detectability of the analytes. Some regents are aimed to enhance the ESI response but they are not designed to generate a particular product ion by CID. The fragment patterns of the derivatives and the efficiencies depended solely on the structures of the analytes. These reagents are not always useful for LC/ESI-MS/MS. In some cases, the derivatives gave particular product ions efficiently and were sensitively and selectively detectable in MRM. On the contrary, the reagents designed for LC/ESI-MS/MS have the suitable structure for MS/MS detection. They fragment easily by CID and efficiently generate particular intense product ions. Although derivatization is very useful to enhance the detectability in LC/ESI-MS/MS, it should be noticed that excess reagents can cause ionization suppression of the derivatives. Thus, separation of the derivatives from excess reagent by LC is often required. Prominent derivatization regents for LC/ESI-MS/MS are still desired in the fields of biomedical analysis.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was supported by The Uehara Memorial Foundation and by Hoansya. The authors thank Dr Chang-Kee Lim, MRC Bioanalytical Science Group for his kind suggestions and valuable discussion.

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