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Dansylation of unactivated alcohols for improved mass spectral sensitivity and application to analysis of cytochrome P450 oxidation products in tissue extracts

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Abstract

Chemical derivatization is useful for improving the ionization characteristics of poorly or non-ionizable analytes in liquid chromatography-mass spectrometry (LC-MS). Dansyl chloride has been widely used as a derivatizing reagent for fluorescence detection and for facilitating the MS detection of phenols and amines, but not for general alcohols. A new dansylation method for improving the mass spectral sensitivity of unactivated alcohols was developed. The dansylated derivative was formed after incubation of the test compound cholesterol and excess dansyl chloride in CH_2Cl_2 in the presence of 4-(dimethylamino)-pyridine (DMAP) plus *N,N*-diisopropylethylamine at 65 °C for 1 h, with an overall yield of 96%. The versatility of dansylation was investigated by utilizing representative lipid compounds (containing different numbers of hydroxy groups) for dansylation. All dansylated derivatives of the selected compounds were detected by LC-MS/MS in the electrospray ionization (ESI) positive ion mode. Validation of the method was established in terms of the sensitivity, stability, and repeatability of dansylation. The method was then applied to characterizing the P450 7A1 oxidation product (dansylated 7 α -hydroxycholesterol) in human liver extracts using an LC-MS metabolomics/isotopic labeling approach (Tang, Z.; Guengerich, F. P. *Anal. Chem.* 2009, 81, 3071–3078). The dansylated derivative of the product was identified, with the signal increased by 10³-fold compared with a previous method (derivatization with succinic anhydride and ESI negative ion MS). Quantitation of testosterone in human liver extracts was also done as an example of the application of the dansylation method. Thus, dansylation is a potential method of modifying many alcohols for detection by fluorescence and LC-MS analysis.

Keywords

dansylation; derivatization; alcohols; mass spectral sensitivity; P450 reactions

INTRODUCTION

Liquid chromatography-mass spectrometry (LC-MS) has been demonstrated to be a powerful analytical tool for metabolomic analysis.^{1–3} However, the need still exists for

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text (Table S-1 and Figure S-1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

more sensitivity of MS detection, especially when compounds of interest show poor ionization efficiency or only trace levels exist in biological samples (e.g., urine, plasma, tissue extracts, etc), the signals of which may be affected by components with strong signal intensities.^{4, 5}

In order to enhance the detection sensitivity and thus to expand the applicability of LC-MS metabolomics, chemical derivatization has been extensively used by converting poorly or non-ionizable analytes into easily detectable derivatives, with the introduction of either ionic or readily ionized moieties.^{6–9} This process is generally indispensable for gas chromatography (GC)-MS. For unactivated alcohols (often relevant substrates or metabolites of cytochrome P450 (P450) enzymes), various derivatization methods have been developed and applied for improving the sensitivity of MS detection.^{6–9} *S*-Pentafluorophenyl tris(2,4,6-trimethoxyphenyl)phosphonium acetate bromide was synthesized and used for derivatizing alcohols, with enhanced response in electrospray ionization (ESI)-MS.¹⁰ Derivatization of neutral sterols into picolinyl esters was done for analysis of sterol profiles in human serum by LC-ESI-MS/MS.¹¹ Chemical derivatization with 3-nitrophthalic anhydride was used for quantification of endogenous farnesol and geranylgeraniol in rat liver and testis.¹² In our previous work, succinic anhydride was utilized to derivatize 7 α -hydroxy (OH) cholesterol for substrate identification of P450 7A1 in human liver extracts.¹³ However, drawbacks of the above derivatizations (such as non-commercially available derivatization reagents, time-consuming incubation steps, harsh reaction conditions, etc.) encourage the development of novel chemical derivatization methods that are typically fast, simple, robust, and significantly improving the mass spectral sensitivity.

Dansyl chloride is a fluorescent derivatization reagent that has been widely used for fluorescence detection and for facilitating the ionization characteristics of phenols^{14–19} and amines,^{20–22} due to the introduction of a dimethylamino moiety in the structure. In recent years, dansylation has proven to be a useful tool in metabolite profiling. The application of dansyl chloride in identifying polar urinary metabolites has been reported, resulting in the formation of more hydrophobic, readily ionizable derivatives that are more sensitive for LC-MS analysis.²³ A quantitative metabolome profiling technique was established for the analysis of the metabolites containing amine or phenol group(s), based on differential ¹²C/¹³C-isotope dansylation labeling.²⁴ However, dansylation of unactivated alcohols has not been reported, which would be useful in identifying new substrates and products for elucidation of functions of novel P450 enzymes.⁴

The aim of the present work was to develop a modified dansylation method for derivatizing unactivated alcohols for improved mass spectral sensitivity and to apply it to identifying new P450 oxidation products in tissue extracts (Figure 1). Several representative lipid compounds were selected for method development and validation by investigating the sensitivity, stability, and repeatability of dansylation. The dansylation method was then applied to characterizing the P450 7A1 oxidation product (7 α -OH cholesterol) in human liver extracts using an LC-MS metabolomics/isotopic labeling approach.^{13,25} Quantitation of individual molecules in human liver extract was also done, utilizing testosterone as a test compound.

EXPERIMENTAL SECTION

Materials and Reagents

Testosterone, cholesterol, vitamin A (retinol), vitamin D₃ (cholecalciferol), 12-OH dodecanoic acid, 3-OH palmitic acid, 6 β -OH testosterone, deoxycholic acid, hydrocortisone, estrone, 7 α -OH cholesterol, 4-(dimethylamino)-pyridine (DMAP), *N,N*-

diisopropylethylamine, *tert*-butyl methyl ether, and L- α -1,2-dilauroyl-*sn*-glycero-3-phosphocholine were purchased from Sigma-Aldrich (St. Louis, MO). 5-Dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) was purchased from Invitrogen (Eugene, OR). [1,2- $^3\text{H}(\text{N})$]-Cholesterol was purchased from PerkinElmer (Boston, MA). [1 α ,2 α - ^3H]-Testosterone was purchased from American Radiolabeled Chemicals (St. Louis, MO). Human liver samples (from organ donors) were obtained from Tennessee Donor Services and stored at $-70\text{ }^{\circ}\text{C}$. Human P450 7A1²⁶ and rat NADPH-P450 reductase²⁷ were expressed in *Escherichia coli* and purified as previously reported. All other reagents and solvents were obtained from general commercial suppliers.

Dansylation Derivatization

All organic solvents used for dansylation were dried over molecular sieves (3Å) prior to use. Each dansylation incubation was carried out in an amber tube vial (Fisher Scientific, Pittsburgh, PA). Aliquots of the samples (in $\text{C}_2\text{H}_5\text{OH}$) were evaporated to dryness under a stream of N_2 , to which 100 μL of 2% (v/v) *N,N*-diisopropylethylamine in CH_2Cl_2 was added, followed by mixing with a vortex device for 30 s. To this mixture was added 100 μL of a stock solution containing dansyl chloride (10 mg/mL) and DMAP in CH_2Cl_2 (10 mg/mL). The resulting mixture was mixed with a vortex device and kept at $65\text{ }^{\circ}\text{C}$ for 1 h, with shaking at 150 rpm in an Innova 42 incubator shaker (Edison, NJ). Samples were then mixed with a vortex device, centrifuged, and dried under a N_2 stream. The residues were dissolved in 1 mL of CH_3CN , and 10 μL of the resulting solution was injected for LC-MS/MS analysis.

Yield of Dansylation Reaction

Testosterone and cholesterol (^3H -radiolabeled) were selected as model compounds for evaluation of yields of dansylation reactions, due to the lack of commercially available dansylated compounds. Dansylation incubations were done using 5 nmol of model compound (containing 0.20 μCi for testosterone or 0.27 μCi for cholesterol) under the same conditions as described above. The reaction mixture was dried under N_2 stream and the sample was dissolved in CH_3CN for radioactivity measurement by HPLC coupled to a β -RAM flow counter (IN/US Systems, Tampa, FL), with the scintillation cocktail pumped at a rate of 3.0 mL min^{-1} . HPLC separation was done using a $4.6\text{ mm} \times 75\text{ mm}$ Zorbax SB-CN column (3.5 μm ; MAC-MOD Analytical, Chadds Ford, PA). A linear gradient began with 50% (v/v) mobile phase A, 5:95 (v/v) $\text{CH}_3\text{CN}/\text{H}_2\text{O}$, increasing to 100% B, 90:10 (v/v) $\text{CH}_3\text{CN}/(\text{CH}_3)_3\text{COCH}_3$ over 5 min at a flow rate of 1.0 mL min^{-1} and was held at 100% B for 8 min.

Sensitivity of Dansylation

To evaluate sensitivity improvement for MS detection of unactivated alcohols after dansylation, lower limit of detections (LLOD) of the lipid compounds (Figure 2) with and without dansylation were determined by LC-MS/MS. The LLOD was defined as a signal-to-noise ratio (S/N) of 3.

For the samples with dansylation, the LLOD of each dansylated derivative was estimated simultaneously by directly using the mixture of the ethanolic stock solutions of the selected compounds (lipid mixture), with 0.1 nmol of estrone (internal standard), for dansylation incubations. This lipid mixture (containing the internal standard) was also used for the further studies on stability and repeatability of dansylation.

For the samples without dansylation, the LLOD of each compound was measured individually. Separate stock solutions of the lipid compounds (Figure 2) were prepared in $\text{C}_2\text{H}_5\text{OH}$ at concentrations of 1.0 mM. Aliquots of each compound (in $\text{C}_2\text{H}_5\text{OH}$) were dried

under a stream of N₂ and re-dissolved in a 95:5 (v/v) CH₃CN/H₂O mixture containing 10 mM NH₄CH₃CO₂ for LC-MS/MS analysis.

Stability of the Dansylated Derivatives

The stability of the dansylated lipid mixture (5 nmol of each compound and 0.1 nmol of the internal standard for dansylation incubations) was evaluated by analyzing the sample (stored at 4 °C) for 3 weeks. The dansylated sample was stored at -20 °C prior to and after LC-MS runs.

Repeatability of Dansylation Reaction

The intra-vial repeatability of dansylation was determined by repeated analysis (n = 6) of the same sample of the dansylated lipid mixture (5 nmol of each lipid compound and 0.1 nmol of the internal standard for dansylation incubations). The repeatability of dansylation for inter-vial comparisons was determined by measuring the samples of dansylation incubated in different vials under same conditions (n = 6), described above for dansylation derivatization.

Identifying P450 7A1 Reaction Products in Human Liver Extracts

A CHCl₃/CH₃OH (2:1, v/v) extract of human liver homogenate was prepared using the protocol described in previous work.^{13, 28} The dry liver extract was then re-dissolved in 2 mL of ethanol as a stock solution for further studies. *In vitro* P450 reaction mixtures included 1.0 mL of 100 mM phosphate buffer (pH 7.4) containing purified recombinant human P450 7A1 (1 μM), NADPH-P450 reductase (2 μM), L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine (75 μM), and 10 μL of the ethanolic stock solution of the human liver extract (1%, v/v). For isotopic labeling experiments, incubations were done using the method described previously.^{13, 25} The enzyme reaction was initiated by the addition of an NADPH-generating system including 100 μL of 100 mM glucose 6-phosphate, 50 μL of 10 mM NADP⁺, and 2 μL of a 1 mg mL⁻¹ solution of yeast glucose 6-phosphate dehydrogenase. All incubations were done at 37 °C for 20 min and quenched by adding 2 mL of CH₂Cl₂. After centrifugation at 2 × 10³ g for 40 min, the (lower) organic phase was carefully collected and dried under an N₂ stream for dansylation. The resulting mixtures of dansylated derivatives were also analyzed by LC-MS/MS.

Quantitation of Testosterone in Human Liver Extracts

Quantitation of testosterone in a complex matrix was investigated by comparing the standard curves of dansylated testosterone prepared with and without human liver extract. A series of working stock solutions of testosterone was prepared in C₂H₅OH at concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 mM. Samples containing 10 μL of different concentrations of testosterone with same amount of the internal standard (0.1 nmol estrone) were either dried directly for dansylation or spiked into 10 μL of the stock solution of human liver extract prior to dansylation. The resulting mixtures of the dansylated derivatives were submitted to LC-MS/MS assay.

LC-MS/MS Conditions and Data analysis

LC separations were performed on a Waters Acquity UPLC system equipped with an autosampler (Waters, Milford, MA) using a Phenomenex Luna C8 column (3 μm, 2.0 mm × 100 mm) at 40 °C. The injection volume was 10 μL using a partial loop with needle overfill mode. MS detections were done using a ThermoFinnigan TSQ tandem mass spectrometer (ThermoFisher, Waltham, MA), and data were acquired using a Finnigan Xcalibur software package.

Analyses of dansylated samples were carried out with a linear gradient increasing from 50% (v/v) mobile phase A (0.1% HCO₂H in a 5:95 (v/v) CH₃CN/H₂O mixture) to 100% mobile phase B (0.1% HCO₂H in a 90:10 (v/v) CH₃CN/(CH₃)₃COCH₃ mixture) over 8 min and held at 100% mobile phase B for 4 min, at a flow rate of 0.3 mL min⁻¹. MS/MS analysis of dansylated derivatives was performed using ESI source in the positive ion mode. The source spray voltage was set at 3.3 kV with the transfer capillary temperature at 270 °C. The sheath gas pressure was 33 psi and the auxiliary gas pressure was 25 psi. The multiple reaction monitoring (MRM) operating parameters are summarized in Table 1. For the dansylated sample of incubation of P450 7A1 with human liver extract, an automated search for the ¹⁸O₂/¹⁶O₂ oxidation products appearing as M/M+2 doublets was done using the MatLab-based computer program DoGEX as previously described, with an isotopic ratio range from 0.95 to 1.9 via spectral filtering.^{13, 25}

For the detection of the lipid compounds without dansylation, 10 mM NH₄CH₃CO₂ in a 5:95 (v/v) CH₃CN/H₂O mixture (A) and 10 mM NH₄CH₃CO₂ in a 95:5 (v/v) CH₃CN/H₂O mixture (B) were used as mobile phases. The LC separation was performed with an isocratic gradient of 5%A/95%B (v/v) at a flow rate of 0.3 mL min⁻¹. Selected reaction monitoring (SRM) (Supporting Information Table S-1) was used for LLOD determination for each lipid compound (Figure 2).

RESULTS AND DISCUSSION

Method Development and Optimization

A modified dansylation method for improving the sensitivity of MS detection of general alcohols was developed (Figure 1). Cholesterol was selected as an initial model to test the feasibility of dansylation. A screen of different incubation solvents (C₅H₅N, HCON(CH₃)₂, C₄H₈O, CH₃CN, and CH₂Cl₂), bases (K₂CO₃, DMAP, DMAP plus triethylamine, and DMAP plus *N,N*-diisopropylethylamine), incubation temperatures (65 °C and room temperature), and incubation times (0, 0.25, 0.5, 1, 2, 4, and 8 h) was done to achieve the best conditions. Cholesterol (5 nmol) incubated with dansyl chloride (5 mg/mL) in CH₂Cl₂ in the presence of DMAP (5 mg/mL) plus *N,N*-diisopropylethylamine (1%, v/v) at 65 °C for 1 h yielded the highest signal level of dansylated cholesterol for MS detection by direct infusion (data not shown). The mechanism of this reaction is based on the principle that DMAP is a good leaving group from the *N*-acylpyridinium intermediate (produced from DMAP with dansyl chloride), which can facilitate the subsequent nucleophilic attack of alcohol to form the dansylated derivative. To evaluate the yield of the dansylation reaction, incubations for cholesterol and testosterone were done under optimized conditions as described above. Overall yields of 96% for cholesterol and 89% for testosterone were obtained and were considered adequate for further experiments.

In order to verify the versatility of the dansylation method, representative lipid compounds (Figure 2) were selected, based on the principle of different classes of lipids containing different numbers of hydroxy groups. Estrone, a phenolic compound (easily derivatized by dansyl chloride),¹⁵ was selected as an internal standard for LC-MS analysis. In order to develop sample conditions closer to actual biological samples, a lipid mixture (Figure 2) was used for dansylation. Based on the MS fragmentation analysis, the characteristic ion fragments for dansylated alcohols and the internal standard are *m/z* 252 and 117, respectively (Supporting Information Figure S-1), which were then selected as the product ions for further LC-MS/MS analysis of the dansylated derivatives. All dansylated derivatives of the selected compounds were detected and the total ion chromatograms are shown (Figure 3), using different MRM transitions in the ESI positive mode (Table 1). The identities of all detected peaks were verified as the respective dansylated derivatives based on MS fragmentation analysis (data not shown), implying that the dansylation method is

applicable to many alcohols in addition to cholesterol and testosterone. For the compounds containing more than one hydroxyl group, the di- (for 6 β -OH testosterone and deoxycholic acid) and tri-dansylated derivatives (for hydrocortisone) were also formed, with the signal level being lower (for 6 β -OH testosterone and deoxycholic acid) or comparable (for hydrocortisone) with the mono-dansylated derivatives.

Method validation

Sensitivity, stability, and repeatability of dansylation were investigated for method validation, using the dansylated lipid mixture (Figure 2) as a whole sample for LC-MS/MS analysis.

Sensitivity

The LLOD values of the samples with and without dansylation of the selected compounds for judging the sensitivity improvement are summarized (Table 2). The signal intensity of each dansylated derivative was increased, as judged by comparison with that of each compound without dansylation—although the latter were detected separately under their own optimized conditions (Supporting Information Table S-1). For the dansylated steroids (i.e., testosterone, cholesterol, vitamin A, vitamin D₃, and 6 β -OH testosterone), the sensitivity of MS detection was dramatically improved, varying from 17- to 1000-fold, with as high as a 5,000-fold increase for 6 β -OH testosterone. With Vitamin A, the compound was not detectable without dansylation but readily analyzed after derivatization. Even for the compounds with carboxyl group in the structure (that can be easily detected in the ESI negative mode) (i.e., 12-OH dodecanoic acid, 3-OH palmitic acid, and deoxycholic acid), the MS detection sensitivity of the dansylated derivatives was also increased, although only 2- to 25-fold. These results indicate that the dansylation method can be used for improving the sensitivity of MS detection of many alcohols.

Stability

The stability of each dansylated derivative (at 4 °C) was investigated by measuring the peak area ratio of each dansylated compound to the internal standard by LC-MS/MS. All of the dansylated derivatives of the compounds in lipid mixture were stable, with no significant variation after 3-week storage (Figure 4). In order to minimum solvent evaporation, the stock solutions of dansylated samples (in CH₃CN) were subsequently kept at -20 °C for long term storage.

Repeatability

The repeatability of dansylation (in terms of intra-vial and inter-vial) was investigated for evaluation of precision of our method. The relative standard deviation (RSD) for the peak area ratio of each dansylated compound to the internal standard ranged from 1.7 to 16% for intra-vial RSD and from 2.9 to 17% for inter-vial RSD (Table 3), indicating an acceptable precision of the dansylation method.

Method Applications

A typical application of dansylation in this laboratory and others is to identify new P450 oxidation products in tissue extracts, with improved mass spectra of alcohols (*vide infra*). Further, the dansylation method can also be applied to quantitation of individual molecules in biological samples and measuring P450 enzyme activity (data not shown). Not only LC-MS but also LC-fluorescence assays (data not shown) can be employed in the analysis of the dansylated derivatives, due to the fluorescent nature of the dansyl moiety.

Identifying P450 7A1 Reaction Products in Human Liver Extracts

When LC-MS is used as the platform for analysis of biological samples, compounds with strong signal intensities may affect the detection of other trace but interesting compounds. This phenomenon may also occur in the study of identifying P450 oxidation products in tissue extracts. In principle, dansylation (employed after P450 reactions) can improve the MS detection sensitivity of some potential hydroxylated products of P450 reactions. To test this hypothesis, recombinant human P450 7A1 was selected as an example for identifying dansylated 7 α -OH cholesterol (the product of cholesterol formed by P450 7A1) using an LC-MS metabolomics/isotopic labeling approach, in which $^{18}\text{O}/^{16}\text{O}$ doublets are used as signatures for P450 products (with incorporation of oxygen from $^{18}\text{O}_2/^{16}\text{O}_2$ mixtures).^{13, 25} A doublet of m/z 636 and 638 at t_R 9.3 min was identified and judged to be dansylated 7 α -OH cholesterol by comparison (t_R , MS spectrum) with an authentic sample of dansylated 7 α -OH cholesterol (Figure 5). The signal of the dansylated 7 α -OH cholesterol (1 h incubation) in human liver extract was present at a level three orders of magnitude higher compared with a previous method (using succinic anhydride with 6 h incubation for derivatization and negative ion MS).¹³ This result demonstrates that our dansylation method is rapid and sensitive for the analysis of P450 products in tissue extracts.

Quantitation of Testosterone in Human Liver Extract

Testosterone was selected as a test compound for quantitation of dansylated derivatives of unactivated alcohols in biological samples (i.e., tissue extracts). Human liver extract was selected as the complex matrix because it contains very little of the model compound, testosterone, facilitating analysis of the dansylation system. Standard curves of the peak area ratios of dansylated testosterone to the internal standard versus different amounts of testosterone are shown for the samples of dansylated testosterone with and without human liver extract, respectively (Figure 6). Both of the standard curves show good linearity, with the slope of the curve of the sample with human liver extract being four times lower than that of the sample without human liver extract, indicating that the dansylation method is reliable to quantify representative alcohols in complex biological mixtures.

We attribute the attenuated signal intensity to signal suppression, as opposed to interference with the dansylation reaction. The presence of a biological matrix may raise the limits of detection (Table 2) but the enhanced sensitivity relative to the underivatized material is still considerable and standard curves can be generated for individual compounds, even with some suppression.

CONCLUSIONS

In summary, we developed a modified dansylation method for derivatizing unactivated alcohols. The method has been shown to be fast, simple, and robust. The dansylated derivatives can be easily formed and detected by either LC-MS or LC-fluorescence assays. The method was successfully validated by LC-MS/MS analysis of the dansylated derivatives in the ESI positive mode and showed good sensitivity, stability, and repeatability for many alcohols. For method application, characterizing the P450 7A1 oxidation product (dansylated 7 α -OH cholesterol) in human liver extracts was done, with the signal increased by 10^3 -fold compared with a previous method.¹³ Moreover, the quantitation of testosterone in human liver extracts was also done, with a good linearity of the standard curves. Therefore, dansylation is proven to be a potential method for enhancing the detection sensitivity of LC-MS analysis of unactivated alcohols. The method is also helpful for identifying new P450 reaction products in tissue extracts for elucidation of enzyme substrates and for quantifying target molecules in complex biological mixtures as well.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Reaction scheme for dansylation of unactivated alcohols.

Figure 2.
Structures of the lipid compounds and internal standard.

Figure 3.

LC-MS analysis of dansylated alcohols (5 μ M each lipid compound and 0.1 μ M internal standard, 10- μ L injection) using MRM in the ESI positive mode. The t_R values (circled with broken ovals) indicate the dansylated derivatives of the selected compounds: 6 β -OH testosterone (–2), deoxycholic acid (–2), and hydrocortisone (–3) indicate that more than one hydroxy group was dansylated, i.e. the di-, di-, and tri-dansylated derivatives, respectively.

Figure 4.

Stability of the dansylated derivatives. Dansylation incubations were carried out by adding 5 nmol of each lipid compound and 0.1 nmol of internal standard. The resulting dansylation mixture (10 μ L) was injected and analyzed by LC-MS/MS in the ESI positive mode. For the compounds containing more than one hydroxy group, the stability of the mono-dansylated derivative was measured.

Figure 5.

DoGEX results of the reaction mixture of P450 7A1 and human liver extract after dansylation. An isotopic ratio range of 0.95-1.9 was selected in the automated search for the m/z 636 and 638 doublet at t_R 9.3 min, corresponding to the mono-dansylated 7α -OH cholesterol, with the green band corresponding to a doublet with this t_R .

Figure 6. Standard curves for LC-MS analysis of dansylated testosterone with (■) and without (●) human liver extract using MRM in the ESI positive mode. Different amounts of testosterone (ranging from 0.1 nmol to 100 nmol, with 0.1 nmol of internal standard) were added for the dansylation incubations, respectively, and 10 μ L of each resulting dansylation mixture was injected.

Table 1

MRM Transitions of Dansylated Derivatives

dansylated compound	precursor ion (<i>m/z</i>)	product ion (<i>m/z</i>)	collision energy (eV)
estrone (internal standard)	504	171	40
testosterone	522	252	25
cholesterol	620	252	22
vitamin A	520	252	35
vitamin D ₃	618	252	15
12-OH dodecanoic acid	450	252	25
3-OH palmitic acid	506	252	25
6β-OH testosterone	538 (1-OH) ^a	252	25
	386 (2-OH) ^b	252	15
deoxycholic acid	626 (1-OH)	252	15
	430 (2-OH)	252	15
	596 (1-OH)	252	25
hydrocortisone	415 (2-OH)	252	45
	355 (3-OH) ^c	252	15

^a One hydroxy group is dansylated and the *m/z* of precursor ion is that of the singly-charged mono-dansylated compound.

^b Two hydroxy groups are dansylated and the *m/z* of precursor ion was deduced from that of the doubly-charged di-dansylated compound, divided by 2.

^c Three hydroxy groups are dansylated and the *m/z* of precursor ion was deduced from that of the triply-charged tri-dansylated compound, divided by 3.

Table 2Comparison of Sensitivities of LC-MS Detection of Each Lipid Compound with and without Dansylation^a

compound	with dansylation	without dansylation
	LLOD (pg)	LLOD (pg)
testosterone	2.9	72
cholesterol	3.9	3860
vitamin A	8.6	- ^b
Vitamin D ₃	12	3840
12-OH dodecanoic acid	22	540
3-OH palmitic acid	27	136
6 β -OH testosterone	3.0	15200
deoxycholic acid	20	39
hydrocortisone	109	1810

^aFor the compounds containing more than one hydroxy group, mono-dansylated derivatives were selected for measurement.^b-, not detected.

Table 3

Repeatability of Dansylation Reaction

compound	precursor ion of dansylated derivatives (<i>m/z</i>)	intra-vial RSD (%) (n = 6)	inter-vial RSD (%) (n = 6)
testosterone	522	1.8	3.9
cholesterol	620	7.3	4.2
vitamin A	520	4.3	7.0
vitamin D ₃	618	4.2	12
12-OH dodecanoic acid	450	4.6	6.1
3-OH palmitic acid	506	4.0	5.1
6 β -OH testosterone	538 (1-OH)	16	3.6
	386 (2-OH)	1.7	2.9
deoxycholic acid	626 (1-OH)	3.3	8.1
	430 (2-OH)	8.0	8.2
	596 (1-OH)	4.1	17
hydrocortisone	415 (2-OH)	- ^a	-
	355 (3-OH)	5.3	12

^a -, not detected.