

Analysis of 9-Fluorenylmethoxycarbonyl Derivatives of Catecholamines by High Performance Liquid Chromatography, Liquid Chromatography/Mass Spectrometry and Tandem Mass Spectrometry

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9-Fluorenylmethoxycarbonyl (Fmoc) derivatives of catecholamines, including dopamine (DA; 3,4-dihydroxyphenethylamine), norepinephrine (NE; 2-amino-1-(3,4-dihydroxyphenyl)ethanol) and epinephrine (EPI; 1-phenyl-1-hydroxy-2-methylaminopropane) as well as 3,4-dihydroxybenzylamine (DHBA) have been analyzed using high performance liquid chromatography coupled to fluorometric detection and atmospheric pressure chemical ionization mass spectrometry at low femtomole levels. Structures of the derivatives have also been characterized by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry and electrospray/tandem mass spectrometry. Protonated molecules of Fmoc-DA and Fmoc-DHBA as well as $[M + H - H_2O]^+$ ions of Fmoc-NE and Fmoc-EPI appear in their conventional mass spectra, and abundant fragments characteristic of catecholamines dominated the spectra. Structurally diagnostic ions using several hundred femtomoles of the Fmoc derivatives of catecholamine were observed. Collision-induced dissociation (CID) of electrospray-generated protonated molecules of Fmoc-DA and Fmoc-DHBA as well as $[M + H - H_2O]^+$ ions of Fmoc-NE and Fmoc-EPI produced diagnostic product ion spectra. Liquid chromatography/atmospheric pressure chemical ionization mass spectrometric analysis of the Fmoc derivatives of catecholamines and DHBA should prove useful in the separation and characterization of these compounds from biological materials. Copyright © 1999 John Wiley & Sons, Ltd.

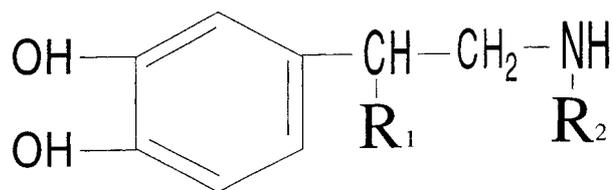
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Catecholamines (Fig. 1), including dopamine (DA; 3,4-dihydroxyphenethylamine), norepinephrine (NE; 2-amino-1-(3,4-dihydroxyphenyl)ethanol) and epinephrine (EPI; 1-phenyl-1-hydroxy-2-methylaminopropane), are a group of organic compounds with amines containing a 3,4-dihydroxyphenyl (catechol) nucleus. The catecholamines are synthesized from tyrosine in mammalian brain through the sequence:¹ tyrosine – dihydroxyphenylalanine (Dopa) – DA – NE – EPI. DA is an important neurotransmitter in the brain and its deficiency leads to the movement disorder, Parkinson's disease.^{2–4}

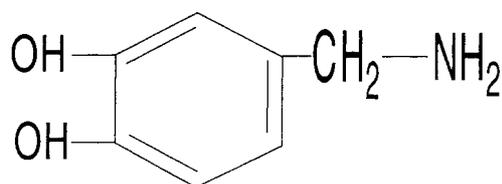
The trace analysis of catecholamines in plasma, urine and neuronal cells is highly important for the diagnosis, therapy and the prognosis of various diseases. Some methods have been published describing the assay of catecholamines, with 3,4-dihydroxybenzylamine (DHBA) as an internal standard, using high performance liquid chromatography (HPLC)

coupled to electrochemical^{5–7} or to fluorescence^{8,9} detection. Although HPLC/electrochemical methods are useful for routine analysis, overlap of catecholamines with other small molecules in complex biological samples makes these analyses difficult. The measurement of 1,2-diphenylethylenediamine derivatives of catecholamines using HPLC/fluorometric detection at low femtomole levels⁸ overlooked the structural elucidation of the derivatized catecholamines, and this method is inappropriate for the structural determination of catecholamines from new biological sources. Combined gas chromatography and mass spectrometry (GC/MS) has been used for analyzing pentafluoropropionate¹⁰ or heptafluorobutyrate¹¹ or trifluoroacetate¹² derivatives of catecholamines, but the detection limit of GC/MS is at the low picomole level. The determination of catecholamines from the immune system¹³ and oxidized products of catecholamines^{14–16} using electrospray/tandem mass spectrometry has been described. The production of DA and its metabolites including NE and EPI, if any, is expected at very low levels (femtomole level or less) in many diagnostic applications.¹⁷ A reliable approach to both qualitative and quantitative determination of catecholamines at the femtomole level has yet to be reported.

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	R ₁	R ₂
Dopamine (DA)	H	H
Norepinephrine (NE)	OH	H
Epinephrine (EPI)	OH	CH ₃



3,4-dihydroxybenzylamine (DHBA)

Figure 1. Chemical structures of dopamine (DA), norepinephrine (NE), epinephrine (EPI) and 3,4-dihydroxybenzylamine (DHBA).

In this paper we report a novel approach to the separation and structural analysis of catecholamines as their 9-fluorenylmethoxycarbonyl (Fmoc) derivatives at the femtomole level by HPLC coupled to fluorometric detection and liquid chromatography/atmospheric pressure chemical ionization mass spectrometry (LC/APCI-MS).

EXPERIMENTAL

Materials

DA, NE, EPI, DHBA and triethanolamine (TEA) were obtained from Sigma (St. Louis, MO, USA). *N*-(9-Fluorenylmethoxycarbonyl)succinimide (Fmoc-Osu) was obtained from Aldrich Chem Co. (Milwaukee, WI, USA). Acetonitrile (HPLC grade) was purchased from Burdick & Jackson, Inc. (MI, USA).

Preparation of the Fmoc derivatives

The derivatization of catecholamines and DHBA was carried out essentially as described.^{18,19} Briefly, DA, NE, EPI and DHBA (1 mg of each) in 50 μ L of water was transferred to screw-capped glass tubes (10 \times 70 mm), to which 100 μ L of acetonitrile containing TEA (pH 9) was mixed. Another 100 μ L of acetone containing Fmoc-Osu (1 μ mol/ μ L) were then added to the tubes, and the solutions were stirred at room temperature in the dark for more than 3 h. The diluted solutions (with acetonitrile) were injected into a C₁₈ HPLC column for fluorometric detection and analysis by LC/MS. The HPLC-purified derivatives (see

below) were used to produce CID spectra. Known amounts of Fmoc-DA was used for the assessment of the detection levels.

High performance liquid chromatography

The separation of the Fmoc derivatives was performed using a C₁₈ HPLC column (2 \times 150 mm, Waters Nova-Pak; Millipore, USA), with an Alliance Waters 2690 separations module and its autosampler in a Micromass Platform LC/MS system (Micromass Ltd, Manchester, UK; Waters, MA, USA). The mobile phases consisted of: solvent A: H₂O; solvent B: acetonitrile. The gradient program used was (100% A/0% B) at 0 min to 100% B at 20 min. A flow rate of 0.3 mL/min was used. The derivatives were analyzed using an HPLC/fluorescence detector (980 programmable fluorescence detector, Perkin-Elmer Applied Biosystems, CT, USA; excitation at 265 and emission at 320 nm) and positive ion APCI.

Mass spectrometry

LC/APCI-MS was performed on a Micromass Platform LC/MS system (Micromass Ltd, Manchester, UK; Waters Co., MA, USA), equipped with a APCI interface, consisting of a standard APCI source and a heated nebulizer probe, and a quadrupole mass analyzer. The probe and ion source temperature was 450 and 150 $^{\circ}$ C, respectively. LC/APCI mass spectra were acquired by scanning the mass range from 100 to 550. A C₁₈ HPLC column was used to separate the Fmoc derivatives as described above. LC/MS-SIR was performed by selected ion recording (SIR) of the fragment ions (see below) of Fmoc-EPI (m/z 166.5), Fmoc-NE (m/z 152.5), Fmoc-DA (m/z 137.5) and Fmoc-DHBA (m/z 123.5). Data acquisition and processing were controlled using MassLynx software (Micromass Ltd, Manchester, UK).

Electrospray (ES)/collision-induced dissociation (CID) experiments of the Fmoc derivatives were performed on a Q-TOF²⁰ tandem mass spectrometer (Micromass Ltd, Manchester, UK). HPLC-purified samples were diluted with acetonitrile/formic acid (100:1) and then infused into the tandem mass spectrometer at a flow rate of 4 μ L/min via a 50 μ m i.d fused silica transfer line using a Harvard Apparatus pump. For CID experiments, Q1 was set up to select precursor ions of the derivatives, pass them through a hexapole collision cell, and the TOF analyzer then scanned the product ions in the mass range from 50 to 500 DA.

RESULTS

Separation of the Fmoc derivatives by HPLC/fluorometric detection

Chromatography of the Fmoc derivatives of catecholamines and DHBA is shown in Fig. 2. This gradient program allowed the separation of Fmoc-NE (peak 1), Fmoc-DHBA (peak 2), Fmoc-DA (peak 3) and Fmoc-EPI (peak 4) from Fmoc-Osu (the most abundant peak shown in the figure, eluted from 17.8 to 18.6 min in retention time). Fmoc acid, a major by-product of the Fmoc derivatization and which eluted around 13.6 min, can overlap with Fmoc-DHBA.

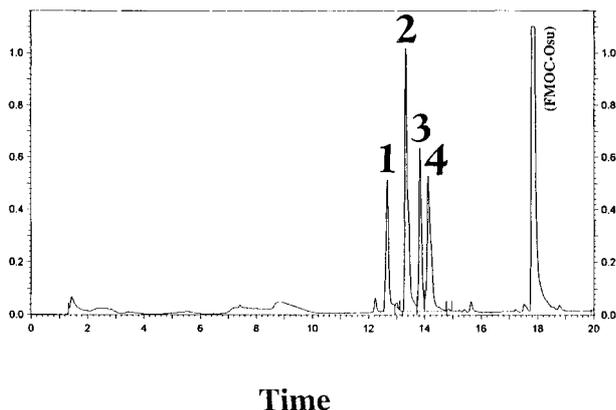


Figure 2. Elution pattern (HPLC/fluorometric detection) of FMOc-NE (peak 1), FMOc-DHBA (peak 2), FMOc-DA (peak 3) and FMOc-EPI (peak 4).

LC/APCI-MS and ES/CID of the FMOc derivatives

FMOc-NE. Figure 3 shows the mass spectrum of FMOc-NE from LC/APCI-MS (peak 1, Fig. 2). The protonated molecule of FMOc-NE is absent. An ion at m/z 374 ($[M + H - H_2O]^+$) can be observed in the spectrum and is presumably formed by the loss of H_2O from its protonated molecule, giving a stable ion which is likely quinone FMOc-DA. The trinitrobenzyl (TNB) derivative of NE was also prepared^{21,22} at pH 9 and detected by negative ion ES-MS. The presence of the deprotonated molecule ($[M - H]^-$) of TNB-NE (spectrum not shown) suggests that the formation of the quinone FMOc-DA structure (due to an ion at m/z 374 in Fig. 3) is not related to the FMOc derivatization procedure. The peak at m/z 152, the most abundant ion in the spectrum, is formed by the loss of an FMOc group from the ion at m/z 374, which looks like quinone DA. Another fragment at m/z 196, formed by the loss of C_4H_{10} in the FMOc residue from the ion at m/z 374, supports the proposed quinone DA ion structure. Similar product ions, proposed to be quinone methides, were also observed in the CID spectra of oxidized DA and Dopa^{14,15}

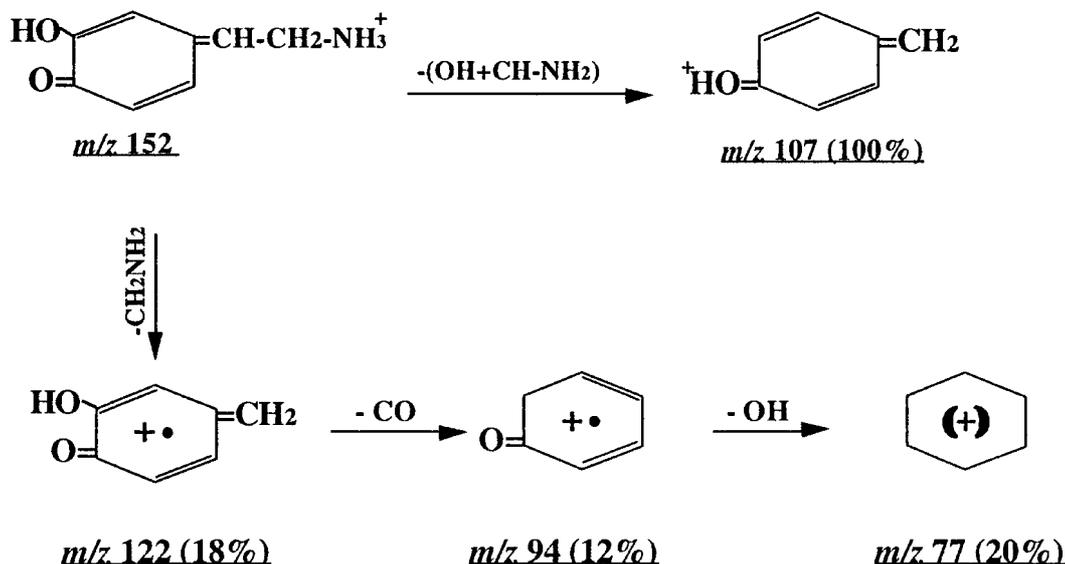
as well as histidyl-dopamine.¹⁶ Ions at m/z 237 and 179 are due to the FMOc group. An identical result was obtained by conventional ES-MS. Structurally diagnostic product ions, m/z 196, 179 and 152, were observed using 100 femtomoles of this derivative (spectrum not shown).

Informative fragments at m/z 237, 196, 179 and 152 were also obtained by CID (product ion spectrum not shown) of m/z 374 from ES-MS of FMOc-NE and confirmed the structural assignments of the ions in the spectrum obtained by LC/APCI-MS. Precursor-ion scanning using m/z 152 (likely quinone DA), generated by conventional ES-MS, resulted in product ions (Scheme 1; spectrum not shown) at m/z 122 [$152 - CH_2NH_2$], m/z 107 [$152 - (OH + CHNH_2)$], m/z 94 [$122 - CO$] and m/z 77 [$94 - OH$], which further supported the proposed structure of the ion at m/z 152.²¹⁻²³

FMOc-DHBA. DHBA, an analogue of DA (see Fig. 1), is a common internal standard in catecholamine assays using HPLC/electrochemical or HPLC/fluorometric detection because of its absence in biological materials. Figure 4 shows the mass spectrum of FMOc-DHBA from LC/APCI-MS (peak 2, Fig. 2). The protonated molecule of FMOc-DHBA appears at m/z 362. The peak at m/z 123 corresponds to the structure of the catechol group. A couple of complementary ions at m/z 184 and 179 support the structural assignment. An identical result was obtained by conventional ES-MS. Structurally diagnostic ions, at m/z 179 and 123, were observed using a total of 320 femtomoles of this derivative (spectrum not shown).

Fragmentation of FMOc-DHBA, obtained by CID (product ion spectrum not shown) of the ES-generated $[M + H]^+$ ion at m/z 362, is shown in Scheme 2. The peak at m/z 179 indicates the presence of the FMOc residue in the derivative. A fragment at m/z 77, formed by the losses of $(OH)_2$ and CH_2 from the ion at m/z 123 [$362 - C_{15}H_{12}O_2N$], is due to the benzyl ring in DHBA.

FMOc-DA. Figure 5 shows the mass spectrum of FMOc-DA from LC/APCI-MS (peak 3, Fig. 2). The $[M + H]^+$ ion of FMOc-DA was found at m/z 376. Peaks at m/z 153 and



Scheme 1. Diagnostic product ions for FMOc-NE.

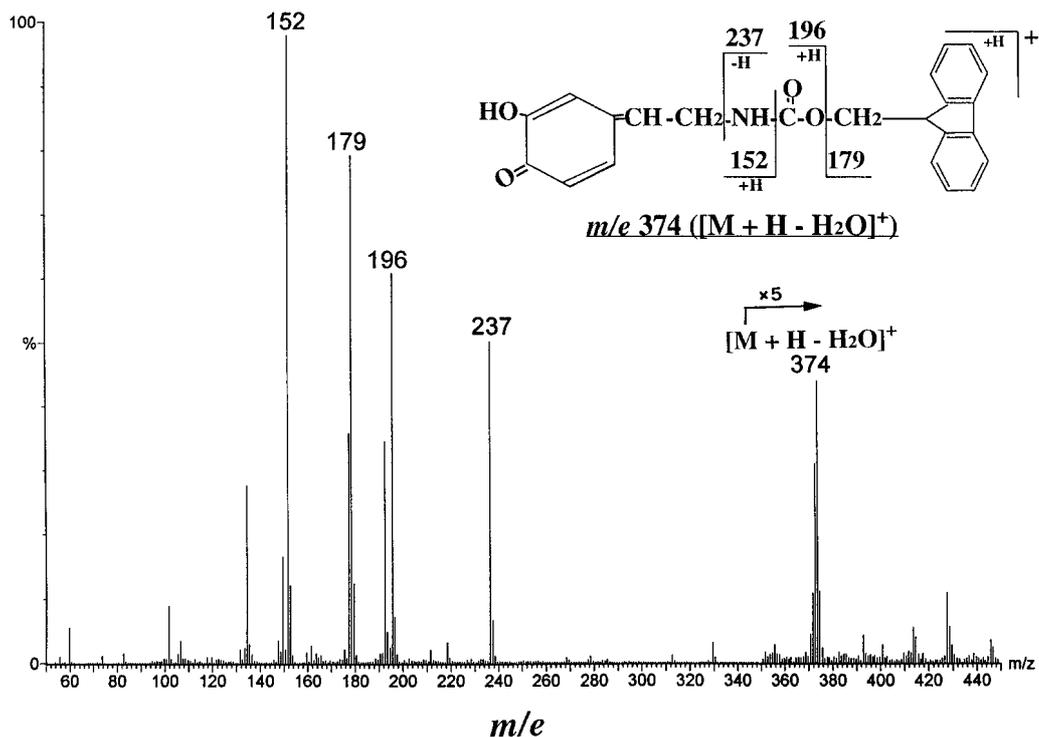


Figure 3. LC/APCI mass spectrum of Fmoc-NE.

137 as well as m/z 239 and 179 are diagnostic of both catechol and Fmoc groups in the derivative. An identical result was obtained by conventional ES-MS. Structurally diagnostic ions, at m/z 239, 198, 179 and 137, were observed using a total of 160 femtomoles of Fmoc-DA (spectrum not shown).

Fragmentation of Fmoc-DA, obtained by ES-MS/MS (product ion spectrum not shown) using m/z 376 as the

precursor ion, furnished information to support the structural determination of Fmoc-DA (Scheme 3). Product ions at m/z 137 and 91, formed by the losses of NH₂ and (OH)₂ from the m/z 153 precursor, confirmed the DA structure.¹³

Fmoc-EPI. Fragmentation of Fmoc-EPI was similar to Fmoc-NE. Figure 6 shows the mass spectrum of Fmoc-EPI from LC/APCI-MS (peak 4, Fig. 2). The $[M + H]^+$ ion

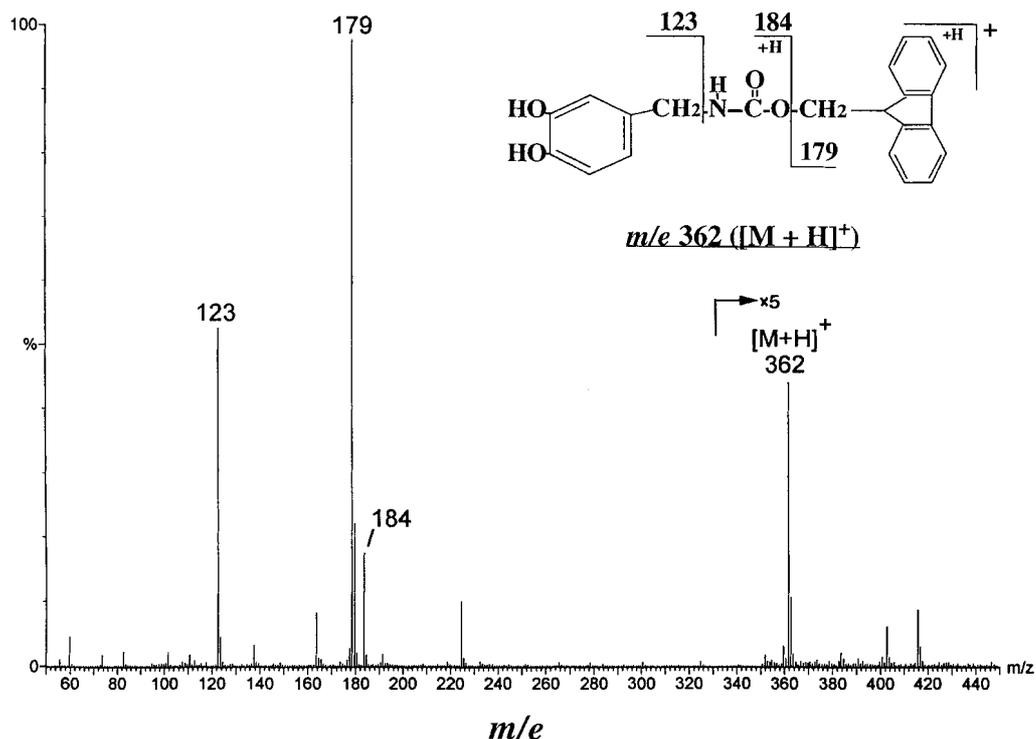
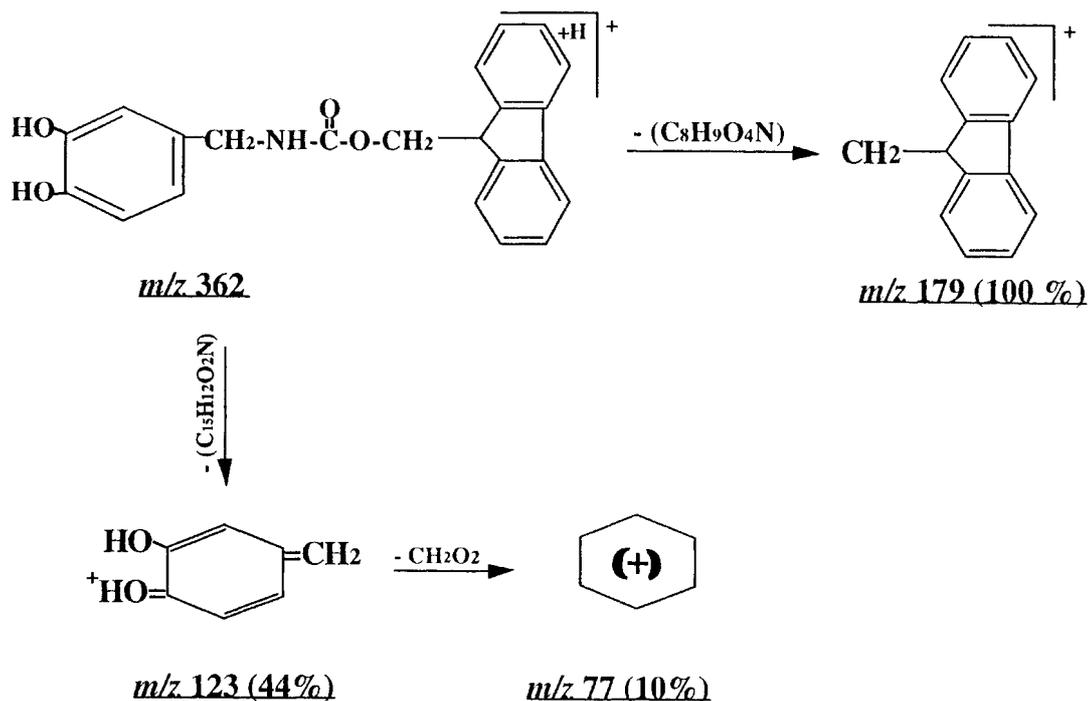


Figure 4. LC/APCI mass spectrum of Fmoc-DHBA.



Scheme 2. Diagnostic product ions for Fmoc-DHBA.

of Fmoc-EPI is absent. The loss of H_2O from the protonated molecule produced a product ion at m/z 388, probably due to a quinone Fmoc-EPI structure. The trinitrobenzyl (TNB) derivative of EPI was also prepared^{21,22} at pH 9 and detected by negative ion ES-MS. The presence of the deprotonated molecule ($[\text{M} - \text{H}]^-$) of TNB-EPI (spectrum not shown) suggests that the formation of the

quinone Fmoc-EPI structure (due to an ion at m/z 388 in Fig. 6) is not related to the Fmoc derivatization procedure. Product ions at m/z 251 $[388 - \text{Fmoc} - \text{NCH}_3]^+$, m/z 210 $[388 - \text{C}_{14}\text{H}_{10}]^+$, m/z 179 $[388 - \text{C}_{10}\text{H}_{11}\text{O}_4\text{N}]^+$, and m/z 166 $[388 - \text{Fmoc}]^+$ were present in the spectrum. A nearly identical spectrum was obtained using ES-MS. Structurally diagnostic ions, at m/z 251, 179 and 166, were observed

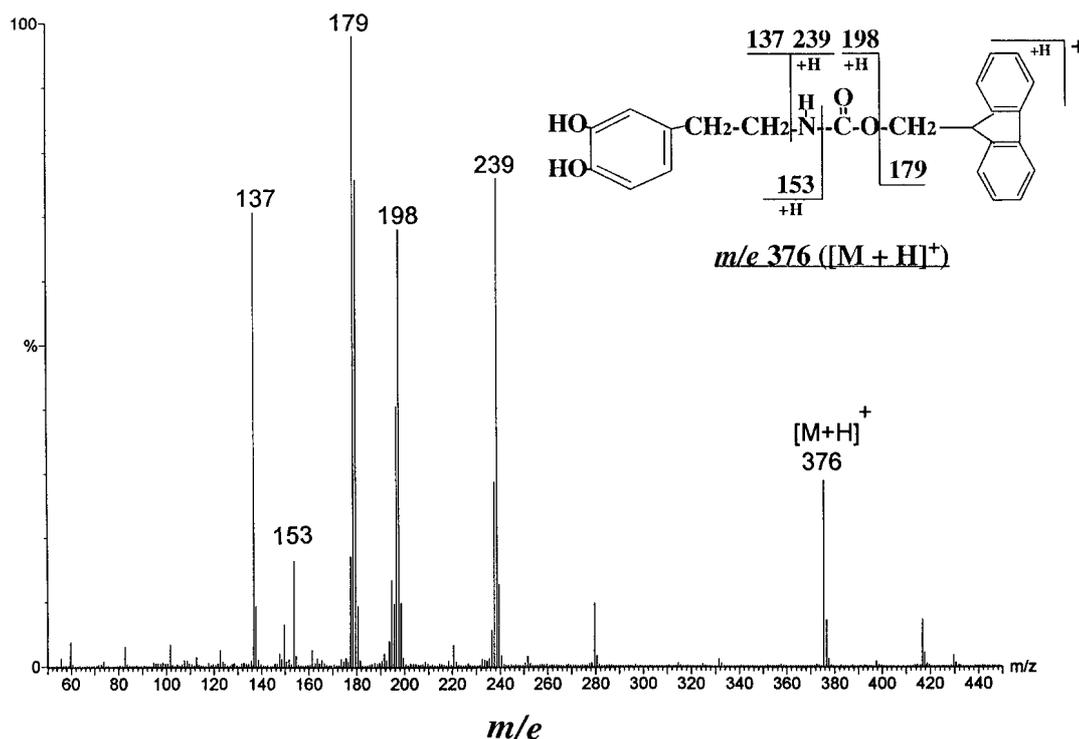
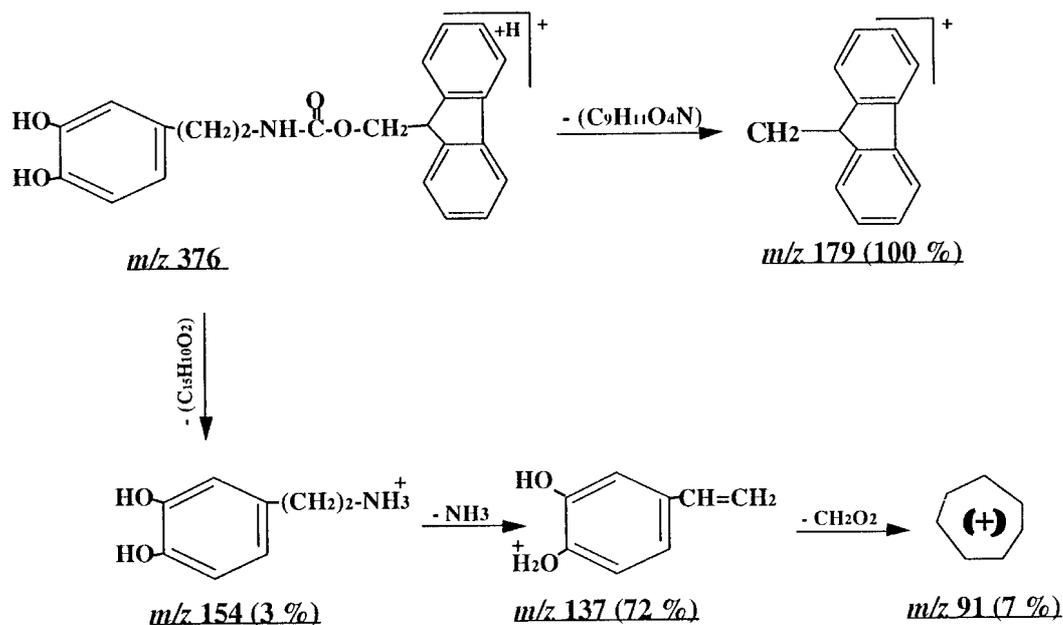


Figure 5. LC/APCI mass spectrum of Fmoc-DA.



Scheme 3. Diagnostic product ions for FMOc-DA.

using 260 femtomoles of this derivative (spectrum not shown).

Fragments at m/z 251, 210, 179 and 166, obtained by EM-MS/MS of FMOc-EPI (product ion spectrum not shown) of the m/z 388 precursor, confirmed the structural assignments of the ions in the spectrum from LC/APCI-MS. Precursor-ion scanning using m/z 166 (likely the quinone EPI structure) generated by conventional ES-MS, resulted in product ions at m/z 122 [$166 - \text{CH}_2\text{CH}_3\text{NH}_2$], m/z 107 [$166 - (\text{OH} + \text{CHNH}_2\text{CH}_3)$], m/z 94 [$122 - \text{CO}$] and m/z 77 [$94 - \text{OH}$] (Scheme 4; spectrum not shown), which further

supported the assignment of the proposed structure of the ion at m/z 166.²³⁻²⁵

Quantification of the derivatives by HPLC/fluorometric detection and LC/MS-SIR

The detection levels of the FMOc catecholamines under HPLC/fluorometric detection and selected ion recording (SIR) by LC/APCI-MS were obtained by calculating the peak areas of the derivatives based on the linear response of

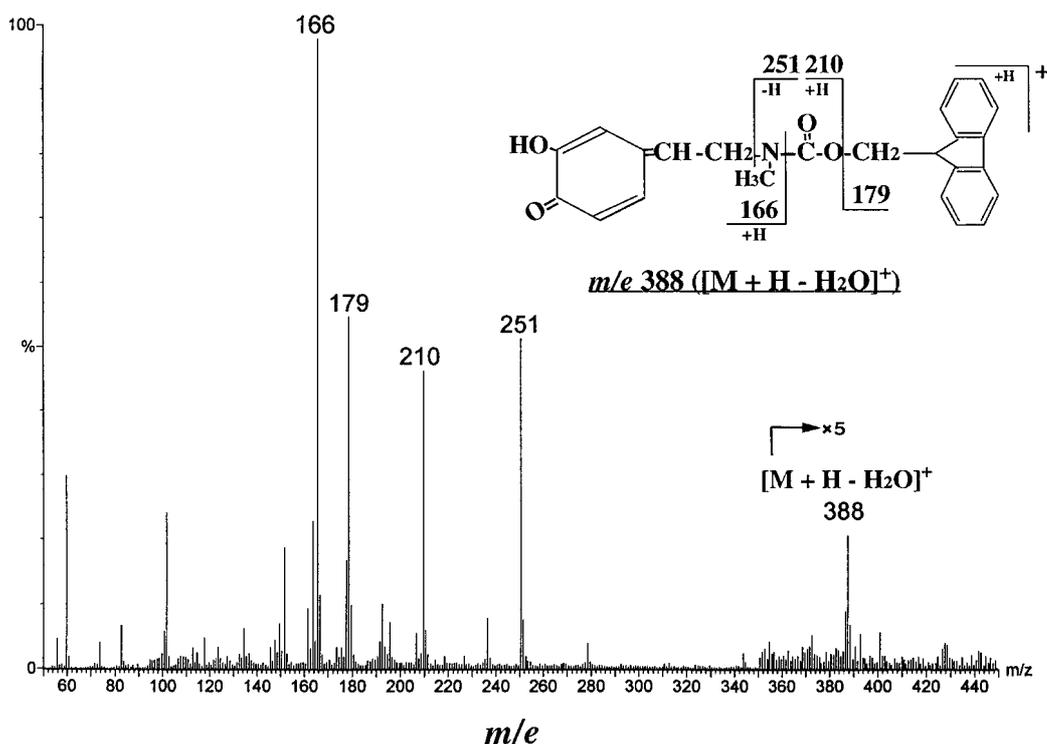
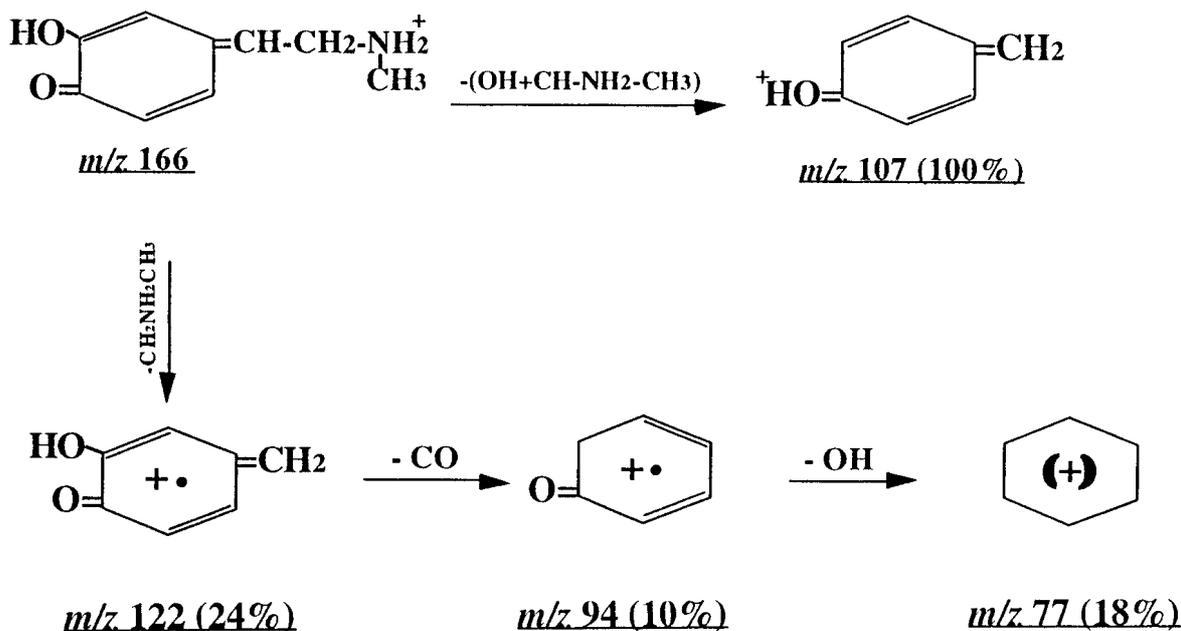


Figure 6. LC/APCI mass spectrum of FMOc-EPI.



Scheme 4. Diagnostic product ions for FMOC-EPI.

various concentrations of FMOC-DA against the peak areas in the range of 20 to 5000 fmol.

The lower detection levels (signal-to-noise (S/N) >5) of the FMOC derivatives (Fig. 7) measured by HPLC/fluorometric detection were at 25 (FMOC-NE), 80 (FMOC-DHBA), 40 (FMOC-DA) and 65 (FMOC-EPI) femtomoles. Quantification of FMOC catecholamines by LC/APCI-MS was done by SIR (Fig. 8) of the ions at m/z 152 (due to the quinone DA ion; see Fig. 2), m/z 123 (see Fig. 3), m/z 137 (Fig. 4) and m/z 166 (due to the quinone EPI ion; see Fig. 5) at various concentrations, and the lower quantitative levels (S/N >10) were at 100 (FMOC-NE), 320

(FMOC-DHBA), 160 (FMOC-DA) and 260 (FMOC-EPI) femtomoles because the SIR currents of the four ions with constant ratio (not shown) can be obtained above detection lines.

DISCUSSION

N-(9-Fluorenylmethyloxycarbonyloxy)succinimide and 9-fluorenylmethyl chloroformate are common reagents used for derivatizing primary or secondary amine-containing analytes, including amino acids^{26–28} and aminophospholipids,²⁹ to derivatives which are readily detected by both

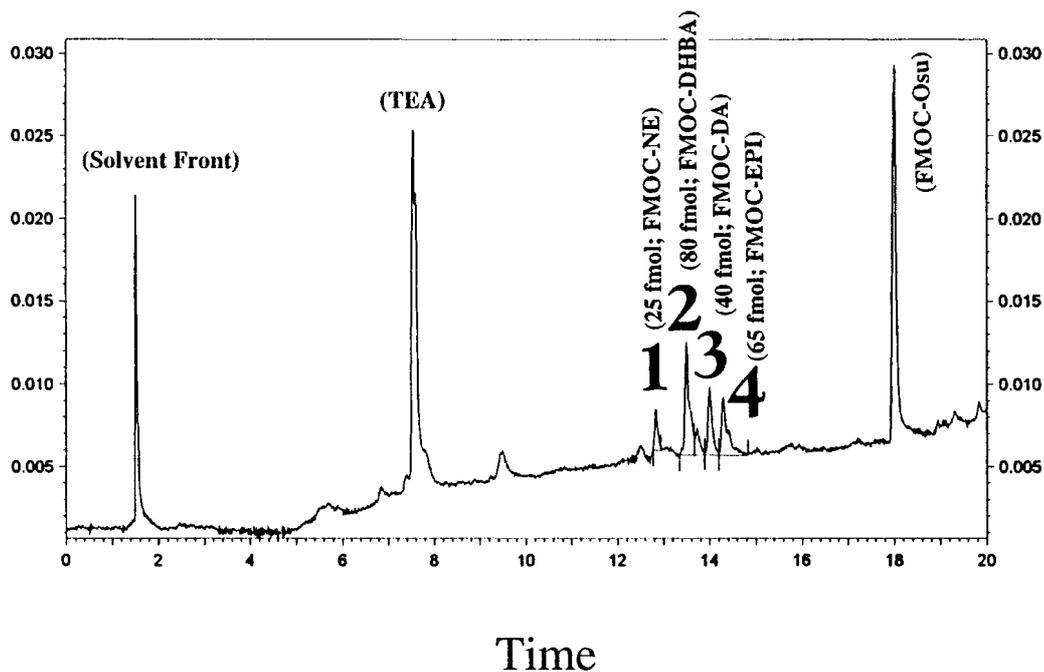


Figure 7. Lower detection levels of FMOC catecholamines and FMOC-DHBA measured by HPLC/fluorometric detection.

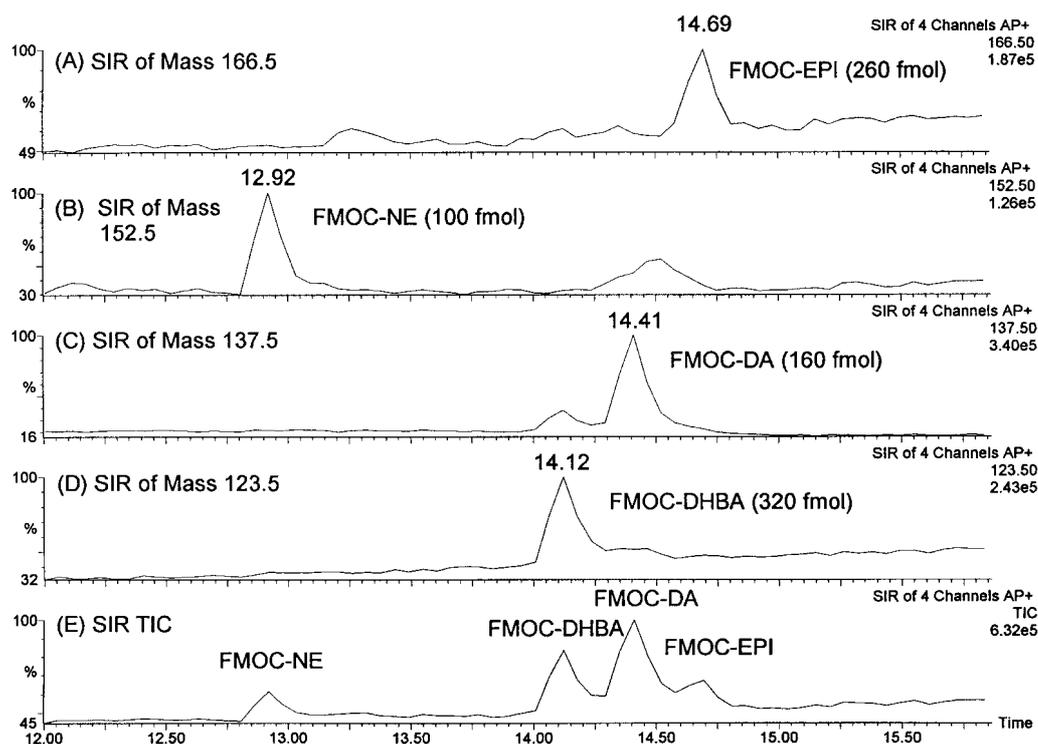


Figure 8. LC/MS-SIR of the FMO derivatives of catecholamines and DHBA.

HPLC/fluorometric detection and mass spectrometry. The amino group is widely distributed in biological materials and is an important functional group in biological systems. Both small biomolecules, such as catecholamines, and large biomolecules, such as peptides, contain amines. A number of compounds which have amino groups are also used in medicine. The determination of amino-containing compounds in biological materials is of vital importance for the diagnosis of some diseases and monitoring pharmacokinetics of drugs having amino group(s).

HPLC, LC/APCI-MS and ES/CID tandem mass spectrometry of the FMO derivatives of DA, NE, EPI and DHBA have not been previously documented. Oxidation^{30,31} of the catechol ring to form a quinone catecholamine structure at pH 8–9 does not occur in the FMO derivatization procedure used in this study. This may be due to the presence of acetonitrile in the reaction mixture. Neither LC/APCI-MS nor ES-MS generated structurally informative negative ion mass spectra of FMO catecholamines or FMO-DHBA. Although FMO acid, a major by-product in the FMO derivatization, is formed and may overlap with FMO-DHBA, it does not interfere with the LC/MS-SIR analysis due to the diversity of the selected ions.

In conclusion, we have introduced *N*-(9-fluorenylmethyl-oxycarbonyloxy)succinimide as a novel derivatizing reagent for the analysis of catecholamines, and methods for HPLC, LC/APCI-MS and ES/CID tandem mass spectrometric analysis of the FMO derivatives of DA, NE, EPI and DHBA have been described. The results presented here suggest that it is possible to analyze catecholamines as their FMO derivatives at low femtomole levels by LC/APCI-MS and HPLC/fluorometric detection. Investigation of the effective extraction of catecholamines from biological samples, optimization of the procedure for FMO derivatization and clean-up, as well as the further development of

methods for qualitatively and quantitatively analyzing catecholamines from biological materials are in progress.

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