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# High-performance liquid chromatographic assay for catecholamines and metanephrines using fluorimetric detection with pre-column 9-fluorenylmethyloxycarbonyl chloride derivatization

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**Abstract**

A convenient HPLC-fluorescent assay of norepinephrine (NE), epinephrine (E), dopamine (DA) and their 3-*O*-methylated metabolites, normetanephrine (NM) and metanephrine (MN) was developed. These analytes were coupled to 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) before assays. Results showed that using a linear gradient elution, peaks of FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM, FMOC-MN and FMOC-DHBA (3,4-dihydroxybenzylamine, internal standard) were simultaneously resolved within 40 min. Optimization of the chromatographic and derivatization conditions, and validation of the assay were further discussed in the paper. The structures of these derivatives were confirmed by atmospheric pressure chemical ionization mass spectrometry (APCI-MS). The molecular ions $[M+H]^+$ of FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM and FMOC-MN were $m/z$ 836, 850, 820, 628 and 642, respectively. Based on these findings, the FMOC-derivatives of metanephrines and catecholamines were confirmed to be bi-substituted and tri-substituted respectively at the amino and catechol functional groups. Finally, the assay was successfully applied to the measurement of urinary E, DA, NM and MN after direct derivatization and simple cleaning. 

*Keywords:* Derivatization, LC; Catecholamines; Metanephrines; 9-fluorenylmethyloxycarbonyl chloride

## 1. Introduction

Levels of catecholamines and their metabolites in biological fluids are of clinical interest in fields such as diagnosis of altered function of catecholamine-synthesizing tissues, assessment of disease severity and prognosis and study of adaptive responses to stress and pharmacotherapy [1]. Quantification of catecholamines (norepinephrine (NE), epinephrine (E)) and their 3-*O*-methylated metabolites (metanephrine (MN), normetanephrine (NM)) (Fig. 1) in biological fluids is routinely performed in the diagnosis of suspected pheochromocytoma [2–4], a tumor of chromaffin cells. If neuroblastoma is suspected, dopamine (DA) is also measured. In pharmacotherapy, patients in clinical shock are usually treated with intravenously administered pressor amine, including isoproterenol, E, NE, DA and dobutamine [1]. Clearance of these drugs is characterized by large individual differences, which are

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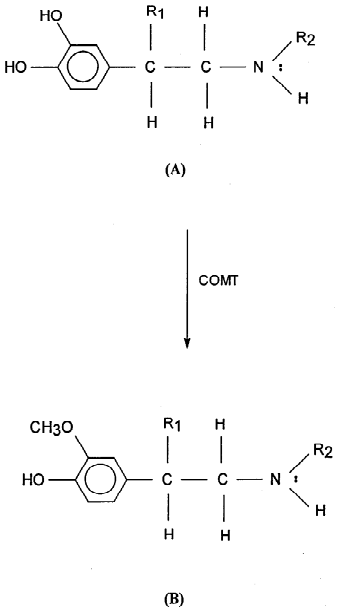



| | Compounds | $R_1$ | $R_2$ |
|---|---|---|---|
| (A) | NE | OH | H |
| | E | OH | $CH_3$ |
| | DA | H | H |
| (B) | NM | OH | H |
| | MN | OH | $CH_3$ |

Fig. 1. Metabolic path of catecholamines.

augmented in patients with hepato-renal failure. Thus, to aid the treatment of these patients, the therapeutic levels of these amines should be accurately and rapidly monitored.

Until now, the most common analytical technique used for the determination of catecholamines and their metabolites in biological samples is high-performance liquid chromatography (HPLC). Because of the minute amount of material present in the sample, methods of detection generally have to be very sensitive. The detection methods prevalently found in most research works are electrochemical [5–8] and fluorometric [9–12] detections. Recently, chemiluminescence was also explored as a detection method for catecholamines and their 3-*O*-methylated metabolites in rat plasma [13].

In fluorometric detection, the amines may be monitored either by natural fluorescence [9,10], or after derivatization reaction with either 1,2-diphenylethylenediamine (DPE) [11,12], trihydroxyindole (THI) [14–16], *O*-phthalaldehyde (OPA) [17] or fluorescamine. The natural fluorescence methods show low sensitivity of the catecholamines. DPE increases the sensitivity of all three catecholamines, however, the reaction products are only stable for 30 min after the reaction. When THI is used, the HPLC requires rather complicated instrumentation for the postcolumn derivatization and cannot be applied to DA. Derivatization with OPA and fluorescamine increases the sensitivity of NE and DA, but E is not measured because only primary amines are derivatized by this reaction. Furthermore, it was noted that most of the reported assays of urinary catecholamines and metanephrines required tedious sample extraction procedures [7–12].

A 9-fluorenylmethyloxycarbonyl chloride (FMOC-Cl) is used commonly as an amino group protector in organic synthesis and a derivatizing agent for amino acid analysis (Fig. 2). Recently, FMOC-Cl was successfully used for the sensitive determination of gentamicin sulfate [18] and aminoglycosides [19] in biological matrices. The advantages of using FMOC-Cl are the reaction is straightforward, rapid and can be performed at ambient temperature; the reaction products are stable at room temperature and more importantly, FMOC-Cl can react with both primary and secondary amines. Recently, Descombes et al. [20] reported a method for measuring NE, E and DA in urine after solid-phase extraction with Fe(III)-

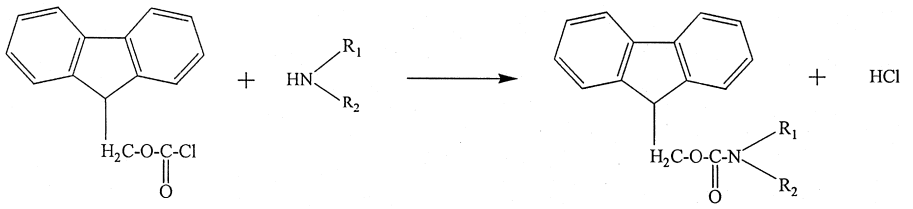



Fig. 2. Reaction between FMOC-Cl and an amine.

loaded silica (SQ-Fe) and derivatization with FMOC-Cl. However, analysis of MN and NM was not investigated in their study. Moreover, there is no study found in the literature that characterizes the chemical structures of the FMOC-derivatives of catecholamines and metanephrines.

This paper describes a simple HPLC-fluorescent assay for the simultaneous determination of NE, E, DA, MN and NM after pre-column derivatization with FMOC-Cl. This assay was shown in our preliminary study to be suitable for the detection of E, DA, NM and MN in human urine sample without prior solid-phase extraction or preconcentration of these analytes. Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) was also used in this study to ascertain the chemical structures of the FMOC-catecholamine and FMOC-metanephrine derivatives.

## 2. Experimental

### *2.1. Chemicals*

All reagents were of analytical grade and Milli-Q reagent water was used throughout the experiment. NE, E, DA, NM, MN and chloroform were purchased from Sigma (St. Louis, MO, USA). Hydrochloric acid and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Orthoboric acid and FMOC-Cl were purchased from BDH Laboratories (Poole, UK) and Tokyo Kasei Organic Chemicals (Tokyo, Japan), respectively. Methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). 3,4-dihydroxybenzylamine (DHBA) was used as the internal standard (I.S.) in this study and it was obtained from Sigma (St. Louis, MO, USA).

### *2.2. Instrumentation*

#### *2.2.1. High-performance liquid chromatography*

The liquid chromatographic system comprised of a solvent delivery system (model LC-10AT), a low-pressure gradient flow control valve, an on-line degasser and a fluorescence detector (model RF-10AXL) (Shimadzu Co., Kyoto, Japan). Detection was performed at excitation and emission wavelengths of 263 nm ($\lambda_{ex}$) and 313 nm ($\lambda_{em}$), respectively. A $C_{18}$ guard column (4×3.0 mm, Phenomenex Inc., CA, USA) connected to a Spherisorb $C_8$ 5-μm reversed-phase column (150×4.6 mm, Phase Separations Inc., CT, USA) was used for separation. The injector was fitted with a 100 μl loop. During the method development, 32.0% of acetic acid solution (3.0 g/l) (Solvent A) was mixed on-line with 68.0% of acetonitrile (solvent B), unless otherwise stated. The flow-rate used was 1.0 ml/min throughout the experiment.

#### *2.2.2. Mass spectrometry*

Atmospheric pressure chemical ionization mass spectrometry (APCI-MS) measurements were carried out in the positive ionization mode and were performed using the LCQ MS detector from Finnigan Corporation (San Jose, CA, USA). Both the auxiliary gas and sheath gas used in this study was nitrogen gas. The APCI vaporizer temperature and the capillary temperature were kept at 450 and 150°C, respectively. The discharge current used was 5.00 μA. MS detection was carried out in the full scan mode for the positive molecular ion. The liquid chromatographic pump was programmed to deliver a solvent (methanol–water; 50:50) at a flow-rate of 1.0 ml/min.

### *2.3. Stock solutions of catecholamines, metanephrines and DHBA*

Stock solutions of NE, E, DA, NM, MN and DHBA (I.S.) were prepared in 0.1 *M* HCl at 10 μmol/ml and were kept in the dark at −20°C. These frozen solutions were thawed; each mixed thoroughly and diluted with 0.1 *M* HCl to obtain the concentrations required for the experiments.

### *2.4. Optimization of derivatization*

In this part of the study, FMOC-Cl concentration in the final reaction mixture was varied from 0 to 7.5 mg/ml and the reaction time from 0 to 18 min. These two parameters were independently varied in turn. Generally, 100 μl of the diluted standard solution containing 1000 pmol/ml each of NE, E, DA, NM, MN and DHBA in 0.1 *M* HCl was added to 100 μl of 1.0 *M* borate buffer (pH 8.0) and mixed thoroughly. A 200 μl FMOC-Cl solution in acetoni-

trile was immediately added and the mixture was vortexed at ambient temperature (24°C) for a prestipulated length of time. The final mixture was centrifuged at 500 *g* for 2 min and 100 μl of the supernatant was injected into the system.

### 2.5. Stability of the FMOC-derivatives

Triplicate samples containing 1000 pmol/ml each of NE, E, DA, NM, MN and DHBA were reacted with FMOC-Cl (3.75 mg/ml in the final reaction mixture) for 15 min. A measure of 100 μl of each of the triplicate samples was injected at 0, 20, 40, 60 min, day 1 and day 3 after the reaction. These samples were stored in the light at ambient temperature (24°C) throughout the study.

### 2.6. Urine sample

A spot urine sample was collected from a healthy subject (male, 27-years-old). A measure of 100 μl of the fresh urine sample was mixed immediately with 100 μl of 1.0 *M* borate buffer (pH 8.0). The mixture was further reacted with a 200 μl aliquot of FMOC-Cl solution for 15 min. Four aliquots of urine samples were individually reacted with 3.75, 6.00, 7.50 and 9.00 mg/ml of FMOC-Cl in the final reaction mixture to determine its optimal amount for derivatization. 800 μl of chloroform was added, the mixture was shaken on a horizontal shaker at 200 rpm for 5 min and thereafter, centrifuged at 500 *g* for 2 min. The top aqueous layer was removed and was further extracted with another 800 μl of chloroform. The pooled chloroform layer (1600 μl) was blown dry under a stream of nitrogen gas. After reconstituting the sample with 400 μl of the reaction mixture (100 μl of water, 100 μl of borate buffer and 200 μl of acetonitrile), 100 μl of the solution was injected. For peak identification, a standard solution containing 1000 pmol/ml each of the amines and a spiked urine sample were similarly reacted, injected and compared. The urine sample was spiked with 1000 pmol/ml each of the amines. A linear gradient from 50 to 78% solvent B in 40 min was used in this part of the study. These linear gradient conditions were also used for the subsequent calibration study and validation of the stability-indicating capacity of this assay.

### 2.7. Validation of stability-indicating capacity of the HPLC assay

In clinical laboratory analysis, an assay is required to be proven beyond doubt to be stability-indicating. A stability-indicating assay is one that can accurately and selectively differentiate an intact analyte from its potential decomposition products and this can effectively reflect the status of degradation. To assess the stability-indicating capacity of our developed assay, the validation procedures as demonstrated in our recent study [21] were adopted. Briefly, samples containing the various amines were subjected to acidic, alkaline and oxygen stress conditions. Each 100 μl portion of a standard solution, consisting of 1000 nmol/ml each of the NM, MN, NE, E, DA and DHBA in 0.1 *M* HCl, was added to 2400 μl of Milli-Q reagent water (A), 1 *M* HCl (B), 1 *M* NaOH (C) and 30% hydrogen peroxide ($H_2O_2$) (D). These solutions were thoroughly mixed and heated for 24 h at 90°C. After heating, solutions A and D were added with 2500 μl of Milli-Q reagent water while solution B and C were added with 2500 μl of 1 *M* NaOH and 1 *M* HCl, respectively, for neutralization. A measure of 20 μl of each solution was further diluted with 180 μl of 0.1 *M* HCl to give a final concentration of 2000 pmol/ml of each of the amines, if not degraded, and 100 μl of this final solution was derivatized with 200 μl of FMOC-Cl solution (3.75 mg/ml in the final reaction mixture) in the presence of 100 μl of borate buffer (pH 8.0). The mixture was vortexed for 15 min and centrifuged at 500 *g* for 2 min and 100 μl of the supernatant was injected. The chromatograms obtained were compared with that of an undegraded sample.

### 2.8. Reproducibility and linearity of the HPLC assay

A set of calibrators, each containing 50, 200, 500 and 1000 pmol/ml of the amines, was prepared in 0.1 *M* HCl. A measure of 100 μl of each of the calibrators, spiked with 5 μl of internal standard solution (100 nmol/ml of DHBA) was derivatized and injected as previously described in Section 2.7. Reproducibility of the HPLC assay was assessed: intra-day variations were monitored by analyzing triplicates of the calibrators; and inter-day variations

were assessed by analyzing fresh calibrators over 3 days. Calculations for the calibration graphs and regression equations were based on the peak-area ratios between the amines and the internal standard.

### 2.9. Structural characterization by APCI-MS

A 100 μl of NE, E, DA, NM or MN in 0.1 *M* HCl (10 μmol/ml) was individually reacted with FMOC-Cl (7.5 mg/ml in the final reaction mixture). The reaction mixture was extracted twice with chloroform and the pooled organic layer was evaporated as described in Section 2.6. The sample was reconstituted with 400 μl of methanol containing 1.0% acetic acid and 10 μl was injected directly into the MS.

## 3. Results and discussion

### 3.1. Optimization of derivatization

Results of the optimization of the derivatization of catecholamines, metanephrines and DHBA are presented in Fig. 3. As shown in Fig. 3A, the optimum amount of FMOC-Cl for the reaction with 1000 pmol/ml each of the analytes was found to be 3.75 mg/ml in the final reaction mixture. This high concentration of FMOC-Cl was required for the reaction to proceed efficiently. Concentrations of FMOC-Cl above this level were found to decrease the signal intensities of all analytes (Fig. 3A). At the optimum concentration of FMOC-Cl used in our studies, all the reactive functional groups of the biogenic amines were derivatized as subsequently confirmed by our MS findings. With more FMOC-Cl added, the excessive reagent could co-precipitate with the FMOC-derivatized analytes in the reaction mixture. This phenomenon is similar to the ''salting-out'' effect of electrolytes, leading to the decreased signals of the analytes in our assays. This explanation was consistent with our observation that the FMOC-Cl reagent, at very high concentration, precipitated in the reaction mixture. The levels of amines in different biological matrices vary considerably. Other metabolites of catecholamines such as, homovanillic acid, vanillylmandelic acid, hydroxymethoxyphenylglycol, 3-methoxy-tyramine, hydroxymethoxyphenylethanol and 3,4-dihydroxyphenylacetic acid present in biological samples may also react with FMOC reagent. Therefore, optimization of FMOC-Cl concentration should be performed for each type of biological samples to be analyzed.

Results of the optimization of reaction time are presented in Fig. 3B. It was apparent that the reaction was fully completed after 15 min for NE, E, DA, NM, MN and DHBA. This confirmed that the reaction between FMOC-Cl and these compounds is rapid and can be conveniently performed at ambient temperature (24°C). A 15-min reaction time was subsequently used in our experiments.

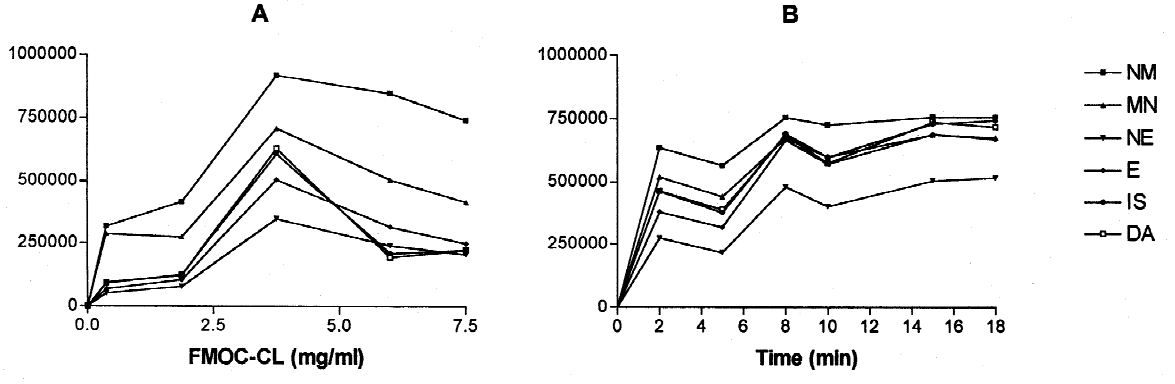



Fig. 3. Effect of (A) FMOC-Cl concentration in reaction mixture (reaction time was fixed at 5 min) and (B) reaction time (amount of FMOC-Cl was fixed at 3.75 mg/ml in reaction mixture) on the derivatization of NE, E, DA, NM, MN and DHBA (I.S.) with FMOC-Cl. Results are plotted as peak areas. Conditions as in Section 2.4.

### 3.2. Stability of the FMOC-derivatives

Initially, samples of the FMOC-derivatives were kept in ice before analysis. However, when these samples were assayed at 0, 40 and 60 min after the reaction, inconsistent reduction in peak areas and fluctuations in their peak area ratios were observed (Data not shown). One possible explanation for this observation is the precipitation of these FMOC-derivatives at lower temperatures. Therefore, subsequently, the FMOC-derivatives were kept at ambient temperature. Results of the stability of the FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM and FMOC-MN derivatives at ambient temperature ($n=3$) are presented in Fig. 4. It is shown that all of these derivatives displayed stable profile over 3 days. The coefficients of variation of the peak area ratios of FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM and FMOC-MN to FMOC-DHBA were 4.97, 4.20, 3.76, 7.30 and 4.28%, respectively. From these results, we confirmed that these FMOC-derivatives are sufficiently stable at ambient temperature for routine analysis in clinical laboratories.

### 3.3. Urine sample

Results of direct derivatization of aqueous, urine and spiked urine samples with FMOC-Cl are presented in Fig. 5. It was found that the peaks of interest were poorly resolved in the urine samples when the previously established isocratic condition (68% solvent B) was adopted. Hence, a linear gradient from 50 to 78% solvent B in 40 min was developed in this part of the study for the optimal resolution of these peaks of interest. From Fig. 6, it was observed that peaks of FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM and FMOC-MN were all detected within 40 min.

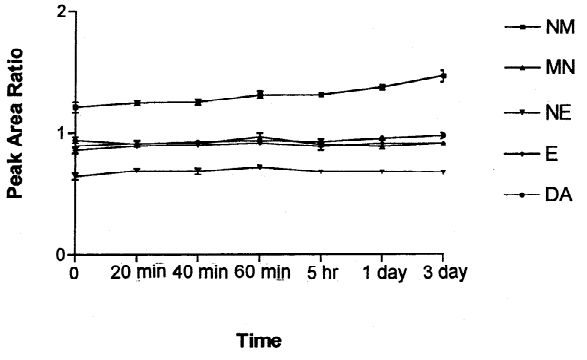



Fig. 4. Stability profiles of FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM and FMOC-MN. Results are plotted as peak area ratios of these derivatives to FMOC-DHBA.

After four aliquots of urine samples were reacted with 3.75, 6.00, 7.50 and 9.00 mg/ml of FMOC-Cl in the reaction mixture, it was found that the 7.50 mg/ml of FMOC-Cl resulted in the highest signal intensities of the FMOC-derivatives (data not presented). By comparing the three chromatograms in Fig. 5A–C, it was observed that peaks of NM, MN, E, DHBA (I.S.) and DA were all detectable in urine sample. However, it was suspected that the peak of NE co-eluted with other endogenous compounds in the urine sample. The peak area of this ''pseudo NE'' peak (32 min) was too outsized to account for the normal physiological level of NE in urine (normal level is less than 100 μg/day).

### 3.4. Stability-indicating validation

All amines degraded under the stress conditions. In alkaline medium, all the amines exhibited almost complete degradation (>98%). This was not unexpected since catecholamines and metanephrines were most unstable and were favorably converted to their respective quinones under basic conditions. Similarly, degradation of these amines in 30% $H_2O_2$ was also pronounced with mean percentages of degradation reaching more than 98% for all amines, except for NM, which showed a mean degradation of 28.99%. Degradation of these compounds in 1 *M* HCl was greater with E and DA (>75%) as compared to NM, MN and NE, which were 27.68, 27.04 and 57.55%, respectively. Degradation of these amines in Milli-Q reagent water was minimal for all (<15%) except for E, which reached up to a mean of 51.13%. Therefore, in general, these compounds were least stable in 1 *M* NaOH and 30% $H_2O_2$, followed by 1 *M* HCl and then Milli-Q reagent water. DHBA also degraded under these conditions. The findings that catecholamines and metanephrines are more resistant to degradation in Milli-Q reagent water than in acid were consistent with observations in our previous studies [21,22], in which the analytes were measured by a stability-indicating reversed-

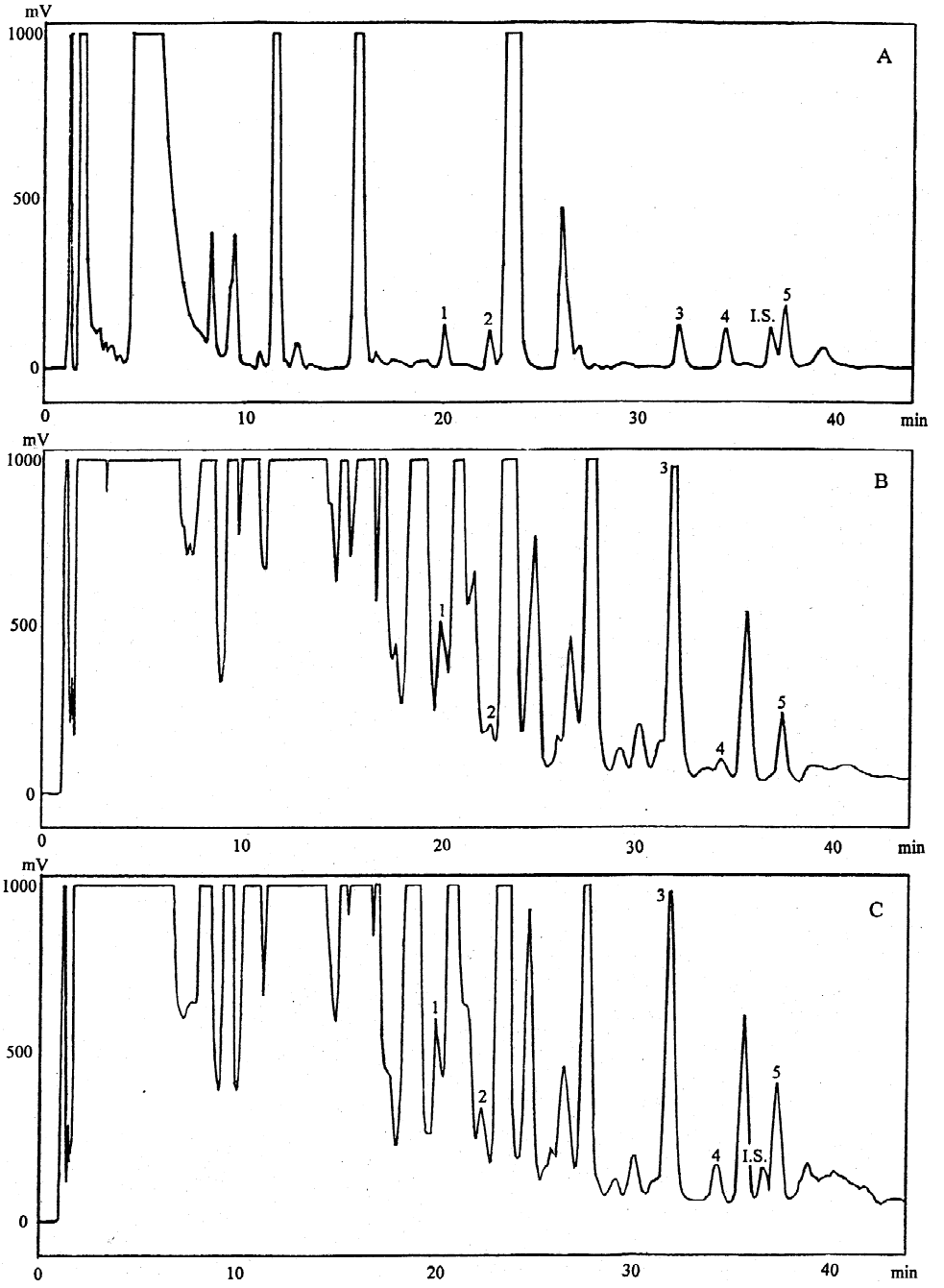
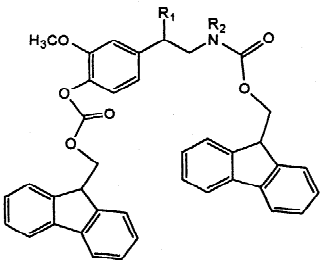
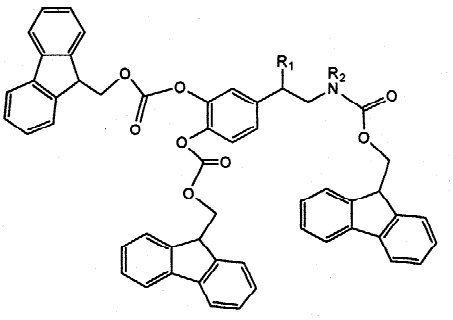



Fig. 5. Chromatograms of FMOC-Cl derivatization of (A) aqueous sample, (B) urine sample and (C) spiked urine sample. Peaks: 1 = NM, 2 = MN, 3 = NE, 4 = E, 5 = DA and I.S. = internal standard, DHBA. Peak at 32 min of (B) and (C) is a co-elution of FMOC-NE and other compounds. Conditions as in Section 2.6.




A




B

| Derivatives | Structures | $R_1$ | $R_2$ | Exact mass |
|---|---|---|---|---|
| FMOC-normetanephrine | A | OH | H | 627.68 |
| FMOC-metanephrine | A | OH | $CH_3$ | 641.24 |
| FMOC-norepinephrine | B | OH | H | 835.28 |
| FMOC-epinephrine | B | OH | $CH_3$ | 849.29 |
| FMOC-dopamine | B | H | H | 819.28 |

Fig. 6. Proposed chemical structures and exact molecular masses of the FMOC-derivatives of catecholamines and metanephrines.

phase ion-pair HPLC method with electrochemical detection. As no interfering peaks co-eluted with the peaks of interest and the retention times remained consistent throughout the entire study after subjecting the analytes to degradation, it was concluded that the present assay was also stability-indicating.

### *3.5. Reproducibility and linearity*

Using the developed linear gradient chromatographic conditions, peaks of NE, E, DA, NM, MN and DHBA were resolved in aqueous sample (Fig. 5A). The peaks of the excess and hydrolyzed FMOC-Cl did not interfere the assay. The elution order of these analytes was NM, MN, NE, E, DHBA and DA and their respective retention times ($t_r$) were 19.58, 21.95, 31.63, 33.92, 36.17 and 36.87 min. A linear relationship of peak area ratios over concentration range of 50–1000 pmol/ml for each of the five analytes were obtained with $r^2$-values of 0.9949, 0.9960, 0.9940, 0.9890 and 0.9800 for NM, MN, NE, E and DA, respectively. The intra-day and

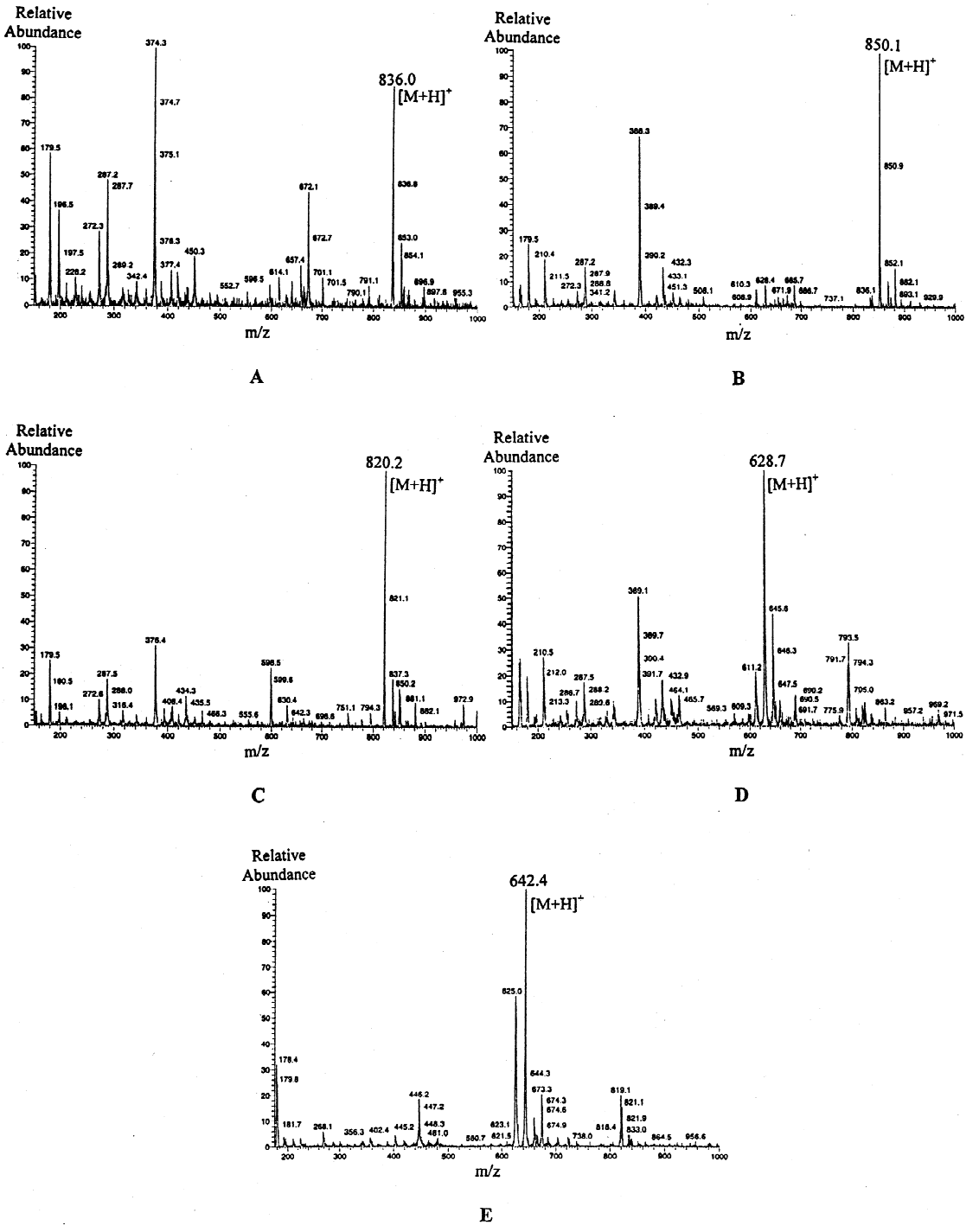



Fig. 7. Mass spectrums of FMOC-derivatives of (A) NE, (B) E, (C) DA, (D) NM and (E) MN.

inter-day percentage coefficients of variation for NM, MN, NE, E and DA were less than 10%. For all amines at a concentration of 500 pmol/ml, the intra-day variations were 0.87, 1.52, 2.77, 2.57 and 1.38%, respectively and the inter-day variations were 0.63, 1.48, 2.07, 2.42 and 1.26%, respectively. The LOQ and LOD of these analytes per injection (100 μl) were approximately 1250 fmol and 375 fmol, respectively. These values were lower compared to the LOQ and LOD values (4000 and 1200 fmol, respectively) obtained in our previous study using a reversed-phase ion-pair HPLC method with amperometric detection [22]. However, the analysis time of the present method (40 min) is longer than that obtained using the amperometric detection method (20 min) [22].

### *3.6. Structural characterization by APCI-MS*

Fig. 6 shows the proposed chemical structures and the expected exact mass of the FMOC-derivatives of catecholamines and metanephrines. This proposal was based on the observed elution order of these compounds (NM, MN, NE, E, DHBA and DA) under reversed-phase chromatography. If based on the chemical structures of these amines and the assumption that derivatization of FMOC-Cl was only restricted to the amino functional groups, the expected elution pattern would be very different from that observed in this study. The structures of these derivatives were confirmed by APCI-MS (Fig. 7). The molecular ions $[M+H]^+$ of FMOC-NE, FMOC-E, FMOC-DA, FMOC-NM and FMOC-MN were $m/z$ 836, 850, 820, 628 and 642, respectively. It was therefore confirmed that the derivatives of metanephrines are bi-substituted while those of catecholamines are tri-substituted at the amino and catechol or phenol functional groups. The catecholamines and metanephrines are highly unstable due to the catechol rings that are readily oxidized to quinones. Due to the derivatization of the catechol functional groups, it is not unexpected that the FMOC-derivatives are more stable than their native amines. In comparison to an alcohol, a phenolic group has relatively higher acidity. There will be an appreciable fraction of nucleophilic phenoxide ion at a given pH. However, due to the low acidity of a normal alcohol, there will be no substantial concentration of the alkoxide ion in any aqueous media. This is the reason why the phenolic groups, but not the secondary hydroxyl groups of NM, MN, NE and E are reactive towards FMOC-CL reagent.

## 4. Conclusion

This assay for the simultaneous determination of catecholamines and metanephrines is rapid and convenient for the simultaneous analysis of NM, MN, E and DA in human urine sample without prior extraction procedures. In this study, urine was directly derivatized and subjected to a simple cleaning step with chloroform prior to injection. With this assay, the requirement for time-consuming solid-phase or liquid–liquid extraction of these compounds in urine sample is therefore circumvented. The potential errors involved during extraction and recovery would also be reduced.

After derivatization, the samples were found to be stable at ambient temperature for at least 3 days. The enhanced stability of these FMOC-derivatives is imparted by the reaction of the unstable phenol or catechol functional groups with FMOC-Cl. The latter reaction was confirmed in this study using APCI-MS.

In summary, this assay could be used in the clinical laboratory for routine measurements of these compounds in the diagnosis of pheochromocytoma or in clinical trials for monitoring the efficacy of drugs indicated for diseases where catecholamines (E, DA) and/or metanephrines (MN, NM) are the surrogate markers. Although this present fluorescent assay does not measure urinary NE accurately, this shortcoming can be overcome by using mass spectrometric detection or solid-phase extraction of urine sample prior to FMOC-Cl derivatization.

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