1,2-Diarylethylenediamines as Pre-column Fluorescence Derivatization Reagents in High-Performance Liquid Chromatographic Determination of Catecholamines in Urine and Plasma

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***meso-* and *dl*-1,2-diarylethylenediamines (14 species) were evaluated for pre-column fluorescence derivatization reagents in the high-performance liquid chromatographic determination of catecholamines (norepinephrine, epinephrine and dopamine) in human urine and plasma. Of the compounds, *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine was most preferable for all the catecholamines in terms of sensitivity and selectivity. The detection limit for each catecholamine is approximately 0.5 fmol in a 50-μl injection volume.**

Keywords catecholamine; pre-column fluorescence derivatization reagent; 1,2-diarylethylenediamine; HPLC; *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine

Many high-performance liquid chromatographic (HPLC) methods coupled with fluorescence detection have been introduced to determine the catecholamines [CAs; norepinephrine (NE), epinephrine (E) and dopamine (DA)] in biological materials. Recently, we have reported a simple and highly sensitive HPLC method for the determination of CAs in biological samples based on pre-column fluorescence derivatization with *meso*-1,2-diphenylethylenediamine (*meso*-DPE).[1–5] More recently, we found that many other *meso*- and *dl*-1,2-diarylethylenediamines (DAEs, 27 species) could also be fluorogenic reagents for CAs.[6] This study aims to search for more sensitive and selective reagents for the pre-column derivatization HPLC of CAs by using 14 DAEs which have spectrofluorimetrically been shown to be highly sensitive.[6] Their availabilities were examined by applying to the HPLC determination of CAs in human urine and plasma. Isoproterenol (IP) was used as an internal standard.

Experimental

Reagents and Solutions NE bitartrate and DA hydrochloride were purchased from Wako Pure Chemicals (Osaka, Japan). IP hydrochloride and E bitartrate were obtained from Nacalai Tesque (Kyoto, Japan) and Sigma (St. Louis, U.S.A.), respectively. Fourteen DAEs [*meso*-DPE, *meso*-1,2-bis(4-methoxyphenyl)ethylenediamine (*meso*-*p*-MOED), *meso*-1,2-bis(3,4-dimethoxyphenyl)ethylenediamine (*meso*-DMOED), 1,2-bis(4-methylphenyl)ethylenediamine, 1,2-bis(4-ethoxyphenyl)ethylenediamine, 1,2-bis(4-ethylphenyl)ethylenediamine, 1,2-bis(3,4-methylenedioxyphenyl)ethylenediamine, 1,2-bis(4-fluorophenyl)ethylenediamine, 1,2-bis(4-chlorophenyl)ethylenediamine and 1,2-bis(1- and 2-naphthyl)ethylenediamines, all in the *meso*-form; 1,2-diphenylethylenediamine (*dl*-DPE), 1,2-bis(4-methoxyphenyl)ethylenediamine (*dl*-*p*-MOED) and 1,2-bis(4-methylphenyl)ethylenediamine (*dl*-MED), all in the *dl*-form] were synthesized as described previously.[6] All other chemicals were of a reagent grade. Deionized and distilled water was used.

Each DAE solution (10 mM or 0.1 M, apparent pH 6.7) was prepared in acetonitrile–20 mM hydrochloric acid (1 : 1, v/v).

TABLE I. Retention Times (t_R), Fluorescence Excitation and Emission Maxima (λ_{ex}^{max}, λ_{em}^{max}), and *RPA*s of the DAE Derivatives of CAs and IP

1,2-Diaryl-ethylenediamine (aryl=)	Mobile phase[a]	NE t_R (min)	NE λ_{ex}^{max} (nm)	NE λ_{em}^{max} (nm)	NE *RPA*[b]	E t_R (min)	E λ_{ex}^{max} (nm)	E λ_{em}^{max} (nm)	E *RPA*[b]	DA t_R (min)	DA λ_{ex}^{max} (nm)	DA λ_{em}^{max} (nm)	DA *RPA*[b]	IP t_R (min)	IP λ_{ex}^{max} (nm)	IP λ_{em}^{max} (nm)	IP *RPA*[b]
meso-Form																	
Phenyl (*meso*-DPE)	A	2.9	350	470	100	4.6	360	480	61	6.6	355	470	41	7.8	370	485	120
4-Chlorophenyl	C	3.8	360	485	106	6.3	355	495	52	9.3	365	500	16	10.3	375	495	71
4-Fluorophenyl	A	3.2	350	470	94	5.1	360	475	61	7.5	355	475	31	8.6	370	470	120
4-Methylphenyl	B	2.6	355	470	108	4.4	360	475	73	5.6	355	475	52	7.2	370	475	141
4-Ethylphenyl	B	3.2	350	465	77	6.0	360	475	62	7.4	355	470	44	10.2	365	475	118
4-Methoxyphenyl (*meso*-*p*-MOED)	A	3.1	350	460	125	4.9	360	470	81	6.7	350	470	56	7.5	365	470	163
4-Ethoxyphenyl	A	3.9	350	460	119	6.6	360	470	81	9.5	355	470	61	11.6	370	470	155
3,4-Dimethoxyphenyl (*meso*-DMOED)	A	2.4	355	465	140	3.5	360	470	86	4.6	355	470	65	5.6	370	470	162
1-Naphthyl	D	3.2 3.8	360 370	475 515	22 20	5.4 7.0	365 375	475 510	19 17	7.8 8.4	365 365	460 505	6 9	8.9 11.8	375 380	465 525	46 26
2-Naphthyl	D	3.6	365	525	46	5.6	370	530	22	7.5	365	530	7	8.5	380	530	33
3,4-Methyl-enedioxyphenyl	A	2.9	355	475	132	5.2	370	490	79	7.4	360	490	40	8.8	370	500	137
dl-Form																	
Phenyl (*dl*-DPE)	A	2.9	350	470	112	4.6	360	480	66	6.6	355	470	52	7.8	370	480	131
4-Methoxyphenyl (*dl*-*p*-MOED)	A	3.1	350	460	137	4.9	360	470	85	6.7	350	470	74	7.5	365	470	171
4-Methylphenyl (*dl*-MED)	B	2.6	350	470	111	4.4	360	475	75	5.6	350	475	62	7.2	370	475	141

a) Mobile phase: CH_3CN–CH_3OH–Tris HCl buffer (pH 7.0) [A, 5:1:4; B, 11:3:6; C, 10:3:7; D, 2:1:1, v/v]. *b*) The peak area of the *meso*-DPE derivative of NE was taken as 100.

A Toyopak IC-SP S(strong cation-exchanger, sulfopropyl resin, Na^+ form, particle size 19—40 μm; Tosoh, Tokyo, Japan) cartridge was equilibrated with a 0.2 M lithium phosphate buffer (pH 5.8) before use. Temporary urine was stored at 4 °C. Heparinized blood (2 ml) was taken into a chilled polyethylene tube containing 5 mg of reduced glutathione and centrifuged at 1000 *g* at 4 °C for 15 min. The plasma was stored at −70 °C until assay.

Chromatograph and Its Operation Conditions A Hitachi (Tokyo, Japan) 635 A high-performance liquid chromatograph was used, which was equipped with a Rheodyne 7125 syringe-loading sample injector valve (50-μl loop), a TSK-gel ODS-120T column (150 mm × 4.6 mm i.d., Tosoh) and a Hitachi 650-10S spectrofluorometer fitted with an 18-μl flow cell; spectral bandwidths of 10 nm were used in both the excitation and emission monochromators. The mobil phase was mixtures of acetonitrile, methanol and 50 mM Tris·hydrochloric acid buffer (pH 7.0) at various ratios, depending on the DAE used (Table I). The flow rate was 1.0 ml/min. Relative peak areas (*RPA*s) were obtained by using a Hitachi 655-60 integrator.

Procedure for Screening DAEs To 20 μl of a standard mixture of CAs [10 nmol/ml each of NE, E, DA and IP], 10 μl of 60 mM potassium hexacyanoferrate (III) and 0.5 ml each of 10 mM DAE solution and acetonitrile–water (3 : 2, v/v) were added. The mixture was allowed to stand at 37 °C for 45 min. A 50-μl portion of the resulting mixture was injected into the chromatograph.

Procedure for Clean-up of Biological Samples and Fluorescence Derivatization Urine Sample: To 10 μl of urine sample, 10 μl of 0.5 nmol/ml IP as an internal standard, 0.5 ml each of 10 mM DAE solution and acetonitrile–0.6 M potassium chloride (3 : 7, v/v), and 10 μl of 60 mM potassium hexacyanoferrate (III) were successively added. The mixture was allowed to stand at 37 °C for 45 min, and the resulting mixture (50-μl) was subjected to HPLC.

Plasma Sample: To 0.5 ml of plasma were added 25 μl of 10 pmol/ml IP and 0.5 ml of 0.2 M lithium phosphate buffer (pH 5.8). The mixture was poured into a Toyopak IC-SP S cartridge. The cartridge was washed successively with 5 ml of water (twice) and 1 ml of aqueous 50% acetonitrile (once). The adsorbed amines were eluted with 500 μl of 0.6 M potassium chloride–acetonitrile (1 : 1, v/v) containing 0.6 mM potassium hexacyanoferrate (III). To the resulting elute, 50 μl of 0.1 M DAE solution was added and the mixture was allowed to stand at 37 °C for 40 min. A 50-μl aliquot of the mixture was used for HPLC.

Results and Discussion

The best conditions of the pre-column derivatization were essentially the same as reported for the spectrofluorimetric determination of CAs.[6]

Table I shows the retention times, fluorescence excitation and emission maxima, and *RPA*s of the DAE derivatives of CAs and IP, obtained under the recommended conditions of HPLC. The DAE derivatives were completely separated within 12 min in the same order (NE, E, DA and IP) in every case of DAEs. Every DAE afforded a single peak to each CA and IP in the chromatograms, except for *meso*-1,2-bis(1-naphthyl)ethylenediamine, of which the derivative of each CA and IP gave two peaks with different fluorescence excitation and emission maxima (Table I) for unknown reasons.

Of the DAEs, *meso*-DMOED and *meso-p*-MOED were most sensitive for all CAs. *dl*-DPE, *dl-p*-MOED and *dl*-MED were 1.3—1.8 times more sensitive to DA than the corresponding DAEs in the *meso*-form. Each retention time of the *dl*-DAE derivatives coincided with that of the corresponding *meso*-DAE derivatives. *dl*-DAEs were less soluble in aqueous acetonitrile than *meso*-DAEs, so *meso*-DAEs are preferable to *dl*-DAEs as derivatization reagents for CAs.

Determination of CAs in Urine and Plasma Figures 1 and 2 depict typical chromatograms obtained with human urine and plasma, respectively, according to the procedures using *meso*-DMOED and *meso-p*-MOED. The peaks for CAs were identified on the basis of the retention times and fluorescence excitation and emission spectra, in comparison with the standard compounds, and co-chromatography with the standards. The concentration values of CAs in urine and plasma obtained with *meso*-DMOED and *meso-p*-

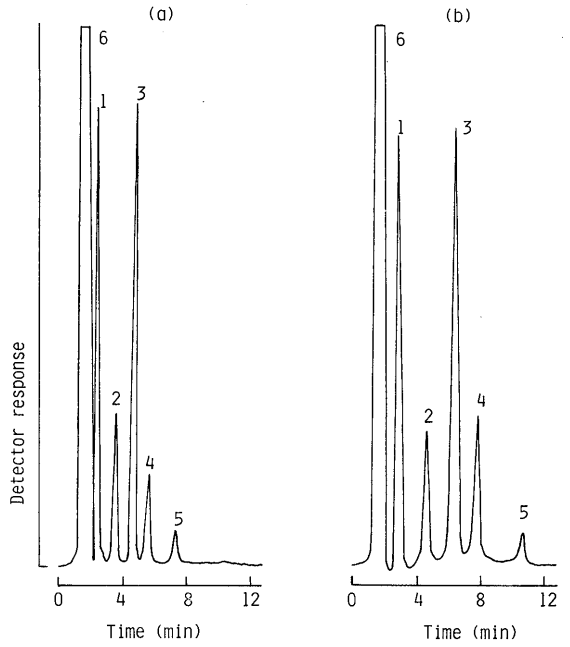

Fig. 1. Chromatograms of (a) *meso*-DMOED and (b) *meso-p*-MOED Derivatives of CAs and IP in Human Urine

A urine sample (10 μl) was treated according to the procedure. Peaks and concentrations (nmol/ml) in parentheses: 1, NE (1.63); 2, E (0.35); 3, DA (2.04); 4, IP (0.50); 5, DOPAC; 6, unidentified.

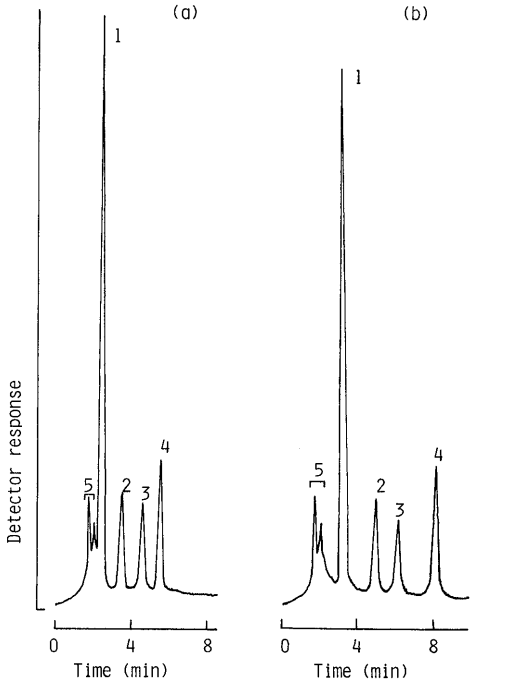

Fig. 2. Chromatograms of (a) *meso*-DMOED and (b) *meso-p*-MOED Derivatives of CAs and IP in Human Plasma

A plasma sample (0.5 ml) was treated according to the procedure. Peaks and concentrations (pmol/ml) in parentheses: 1, NE (1.82); 2, E (0.14); 3, DA (0.09); 4, IP (0.50); 5, unidentified.

MOED were coincided with each other. The peak for NE and an unidentified peak in the chromatogram of plasma obtained with *meso*-DMOED (peaks 1 and 5, respectively, in Fig. 2a) partially overlapped. Thus, *meso-p*-MOED was the best reagent in terms of sensitivity and selectivity.

Linear relationships were obtained by using *meso-p*-MOED between the ratios of the peak heights of CAs to that of IP and the amounts of CAs added in the range of 0.01—2.0 nmol each to 10 μl of urine and 0.02—10 pmol each to 500 μl of plasma. The limits of detection ($S/N=3$) for NE, E and DA were 1, 1 and 2 pmol/ml, respectively, in urine and 10, 10 and 20 fmol/ml, respectively, in plasma (these corresponded to 0.5, 0.5 and 1 fmol in a 50-μl injection volume in each sample).

The concentration values of CAs in urine and plasma from healthy persons obtained with *meso-p*-MOED agreed well with those obtained by the method using *meso*-DPE.[1-3] The relative standard deviations for NE, E and DA ($n=10$) were 2.9, 3.3 and 2.2% at mean concentrations of 518, 65 and 2055 nmol/ml of urine, and 2.1, 2.9 and 3.9% at mean concentrations of 1.76, 0.16 and 0.04 pmol/ml of plasma, respectively.

In conclusion, of the DAEs, *meso-p*-MOED is most preferable for the pre-column fluorescence derivatization in the HPLC determination of CAs in urine and plasma.

References

1) H. Nohta, A. Mitsui and Y. Ohkura, *Bunseki Kagaku*, **33**, E263 (1984).
2) A. Mitsui, H. Nohta and Y. Ohkura, *J Chromatogr.*, **344**, 61 (1985).
3) H. Nohta, A. Mitsui and Y. Ohkura, *J. Chromatogr.*, **380**, 229 (1986).
4) H. Nohta, A. Mitsui, Y. Umegae and Y. Ohkura, *Anal. Sci.*, **2**, 303 (1987).
5) H. Nohta, A. Mitsui, Y. Umegae and Y. Ohkura, *Biomed. Chromatogr.*, **2**, 9 (1987).
6) Y. Umegae, H. Nohta and Y. Ohkura, *Anal. Chim. Acta*, **208**, 59 (1988).