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Note

Separation of norepinephrine and metabolites as Dns derivatives by two-dimensional thin-layer chromatography

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As part of a continuing investigation of age-related changes in adrenergic chemical transmission at the cardiac neuroeffector system[1,2] we desired to evaluate alterations in the metabolic fate of norepinephrine (NE). The isolated rat heart preparation perfused according to the method of Langendorff preloaded with exogenous tritium-labelled NE would provide the model system to assess metabolism both under basal conditions and during electrical stimulation of the isolated right cardiac sympathetic nerve. A reliable method to separate and isolate relatively low levels of [^{3}H]NE and metabolites was necessary for these studies. Several previous studies[3,4] employed a multiple-column chromatographic technique; others developed a thin-layer chromatographic (TLC) method employing isotopic dilution with unlabelled compounds separated on cellulose plates with colorimetric detection[5]. The latter technique fails to resolve the two acid metabolites [3,4-dihydroxymandelic acid (DOMA) and 3-methoxy-4-hydroxymandelic acid (VMA)] or the two glycols [3,4-dihydroxyphenylethylene glycol (DOPEG) and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG)]. The separation of the three methoxy derivatives of NE, epinephrine and dopamine as their Dns derivatives via TLC[6] led us to attempt a similar separation of NE and its five metabolites as Dns derivatives. The impressive work of Seiler and Wiechmann[7] on separation of Dns derivatives of biogenic amines and selected metabolites provided a useful starting place for the assay described in this report.

EXPERIMENTAL

Materials

NE, normetaphrine (NMN), DOMA, VMA, DOPEG, MHPG, 5-N,N-dimethylamino naphthalenesulfonyl chloride (Dns-Cl), and high-performance silica gel on glass TLC plates (200 μm layer thickness, 20 × 20 cm), were from Sigma (St. Louis, MO, U.S.A.).

TLC development chambers, solvents and other chemicals were from Fisher Scientific (King of Prussia, PA, U.S.A.); the latter were used as supplied. Fluorescent spots were visualized by long-wavelength ultraviolet (UV) light (365 nm) (Mineral light lamp UVS1-25, Ultraviolet Products, San Gabriel, CA, U.S.A.).

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Methods

Formation of Dns derivatives. NE and the five metabolites were added singly and in combination to 10 ml deionized water (50–500 μg each/10 ml). Dns derivatization was accomplished basically by the method of ref. 5 using saturating Na_2CO_3 and adding 400 μl of a solution of Dns-Cl in acetone (400 μg/ml). Samples were left in the dark at room temperature overnight.

Extraction of Dns derivatives. Acetone was removed from the samples via a dry air stream, and the pH was lowered to 5–5.5 using concentrated hydrochloric acid. A 2-ml volume of ethyl acetate were added and the mixture shaken. The ethyl acetate layer was removed and dried using an air stream. The residue was redissolved in 200 μl acetonitrile.

TLC conditions. A 20-μl volume of the acetonitrile solution was spotted in one corner of a silica TLC plate. After drying, the plate was developed in the first direction with ethyl acetate–chloroform (2:1, v/v), dried and developed in the second direction with benzene–chloroform–methanol–5 *M* acetic acid (70:20:20:1, v/v). Alternatively the plate was irradiated with long-wavelength UV light (365 nm) for 3 min before development. For both, fluorescent spots were localized under UV light and circled.

RESULTS AND DISCUSSION

Table I contains the R_F values for NE and its metabolites in the TLC system described. It was found that lowering the pH of the reaction mixture before extraction improved the extraction efficiency for the two acid metabolites. With the concentration examined (50–500 μg of each compound), all six derivatives were easily visualized after spotting 20 μl of the acetonitrile solution in samples containing 250 μg/10 ml or greater. In the 100 μg/ml and lesser samples, the mono-Dns compounds (VMA, MHPG) were not discernable. Blank samples treated in the same fashion showed no fluorescent spots other than at the origin and at the solvent front.

TABLE I

SEPARATION OF Dns DERIVATIVES BY TWO-DIMENSIONAL TLC

Solvent 1: ethyl acetate–chloroform (2:1, v/v); solvent 2: benzene-chloroform–methanol–5 *M* acetic acid (70:20:20:1, v/v).

Compound	R_F *value*			
	Before irradiation		*After irradiation*	
	Solvent 1	*Solvent 2*	*Solvent 1*	*Solvent 2*
Dns-Norepinephrine	0.81	0.72	0.80	0.57
Dns-Normetaphrine	0.81	0.65	0.80	0.65
Dns-3,4-Dihydroxyphenylethylene glycol	0.41	0.43	0.40	0.74
Dns-3-Methoxy-4-hydroxyphenylethylene glycol	0.33	0.48	0.33	0.48
Dns-3,4-Dihydroxymandelic acid	0.03	0.19	0.05	0.31
Dns-3-Methoxy-4-hydroxymandelic acid	0.00	0.31	0.00	0.31

Frei *et al.*[8] have described the photochemical degradation of tri-Dns-epinephrine (adrenaline) and ascribed the reactivity to steric strain between the 3,4-bis-Dns-phenolic groups. As phenolic di-Dns derivatization could occur in NE, DOMA and DOPEG we examined the effect of UV light on R_F values by reacting each of the six compounds individually. After spotting, the TLC plate was irradiated for 3 min by long-wavelengths UV light to allow ample time for the photochemical reaction to go to completion. Development of the plates and visualization showed that the R_F values of the dihydroxy compounds were altered while those with the 3-methoxy-4-hydroxy nucleus were unchanged (Table I). Performing the reaction in the dark and working up the samples with minimal light exposure is thus important for these compounds.

The foregoing describes a straightforward method for separating norepinephrine and its metabolites. The method is currently being employed to assess age-related changes in rat heart NE metabolism. The results of these studies will be reported subsequently.

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