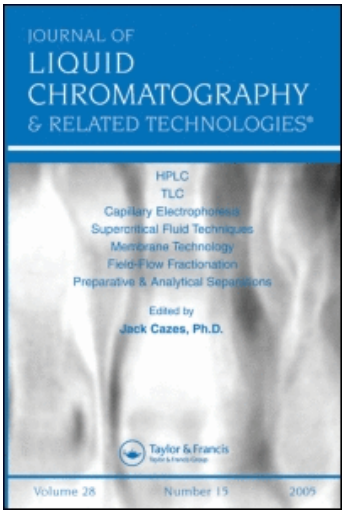

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Pre-Column Fluorescent Derivatization for High Pressure Liquid Chromatography with o-Phthalaldehyde: Separation of Urinary Catecholamines

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PRE-COLUMN FLUORESCENT DERIVATIZATION FOR HIGH PRESSURE LIQUID CHROMATOGRAPHY WITH o-PHTHALALDEHYDE: SEPARATION OF URINARY CATECHOLAMINES

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ABSTRACT

High-pressure liquid chromatography was used to separate the flourescent adducts formed from the reaction of o-phthalaldehyde with primary biogenic amines. Using pre-column derivatization and isocratic elution techniques fluorescent o-phthalaldehyde adducts can be detected and quantified with fluorometry in the low picogram range. The relative retention values of the o-phthalaldehyde adducts strongly depend on the pH of the eluent and percent organic solvent in the mobile phase. The method was applied to the analysis of free norepinephrine in pooled urine samples and in small-volume (2-h) urine collections obtained from thermally stressed subjects. Samples were treated with alumina, and the catecholamines, including internal standard 3,4-dihydroxybenzylamine, eluted from it were reacted with o-phthalaldehyde prior to injection onto a reverse-phase column (octadecylsilica stationary phase) with methanol/0.08 mol/liter acetic acid (50/50 by vol) as the mobile phase. Assay of pooled urine specimens (n = 10) for norepinephrine gave within-run and day-to-day coefficients of variation of 4.3 and 5.6% respectively. The use of o-phthalaldehyde as a pre-column derivatization agent for fluorometric determination of primary amines is rapid, sensitive and specific and applicable to many important biogenic amines.

INTRODUCTION

The lack of sensitive detection in high-performance liquid chromatography is frequently a deterrent to many potential applications of this powerful analytical technique. Although many applications for liquid chromatography can be solved using small-volume ultraviolet detectors, fluorescence detection is superior to most other measurement methods in terms of sensitivity and availability of commercial liquid chromatographic instrumentation.

The need for rapid analysis of biogenic amines and their derivatives arises from their important biological functions in both health and disease conditions (1-3). This laboratory is interested in determining the relationship of catecholamine metabolism to thermal regulation and cardiovascular function in heat acclimatization. As such, the measurement of primary amines, e.g. norepinephrine and dopamine, in small-volumes of biological fluids is necessary in order to relate catecholamine levels with varying degrees of human performance in hot environments.

For primary amines o-phthalaldehyde (4,5) has been shown to be superior to fluorescamine as a fluorogenic agent (6-8). In the presence of a strong reducing agent such as 2-mercaptoethanol, o-phthalaldehyde reacts with primary amines to produce highly fluorescent compounds. o-Phthalaldehyde is particularly advantageous for liquid chromatography since derivatization occurs rapidly at room temperature and in aqueous solutions. As such, o-phthalaldehyde has been used for non-chromatographic (9-11) and post-column (12-14) chromatographic detection of biogenic amines. The latter, however, necessitates the use of complex post-column derivatization components. Recently, Simons and Johnson (15,16)) presented evidence that the fluorescent o-phthalaldehyde reaction products are relatively small thio-substituted isoindoles.

The purpose of this study is to demonstrate that o-phthalaldehyde can be used with relative ease as a sen... pre-column fluorescent derivatization agent for biogenic amines. The technique involves derivatization of biogenic amine with o-phthalaldehyde immediately prior to column injection, separation by reversed-phase liquid chromatography with isocratic elution, and measurement of the fluorescent o-phthalaldehyde-biogenic amine adduct. The method will be applied to the determination of free norepinephrine in small-volume urine collections obtained from volunteers subjected to hyperthermal stress.

MATERIALS

A Model 204 Liquid Chromatograph with Models 6000A Solvent Delivery System and U6K Universal Injector (Waters Associates Inc., Milford, Mass., 01757) was used for chromatography. An isocratic mode was employed and the eluent was monitored at 280 nm with a Waters Model 440 ultraviolet absorbance detector in series with a Model SF 770 Spectroflow Monitor (Schoeffel Instrument Corp., Westwood, New Jersey, 07675) with excitation maximum at 340 nm and a 418 nm secondary filter. Ultraviolet and fluorescent chromatograms were recorded with two Model A-5211-15 strip chart recorders (Houston Instrument, Austin, Texas, 78753). Peak height, area and retention time were determined by a programmable computing integrator (Supergrator-2, Columbia Scientific Industries, Austin, Texas, 78766). Chromatographic conditions are described in the figure legends. A Waters μBondapak C_{18} column (10 μm av particle size, 4 mm i.d. x 30 cm) used for all analyses, was flushed daily with methanol (Burdick and Jackson Laboratories, Inc., Muskegon, Michigan 49442).

The solvent used for quantitative determination of catecholamines and most chromatographic experiments was a methanol/0.08 mol/liter acetic acid mixture (50/50 by vol),

pH 2.9. In certain experiments the pH of the eluent was varied by adjusting the acetic acid component of the mobile phase with 0.1 mol/liter NaOH. In studies where the methanol composition of the eluent was varied, the 0.08 mol/liter acetic acid was maintained at pH 2.9 All solutions were made with de-ionized, glass-distilled water. The mobile phase was filtered through a 0.45-μm filter and degassed immediately before use.

o-Phthalaldehyde, 3,4-dihydroxybenzylamine hydrogen bromide, and 3-O-methyldopamine were obtained from Aldrich Chemical Co., Milwaukee, Wis. 53233. All other catecholamines and related metabolites were obtained from Calbiochem, La Jolla, Calif. 92037. Mercaptoethanol was obtained from Sigma Chemical Co., St. Louis, MO 63178. Standard solutions (100 mg/liter) of each biogenic amine including internal standard, 3,4-dihydroxybenzylamine, were prepared by dissolving these compounds in methanol. All solutions were filtered through a 0.45-μm filter before use. Alumina (neutral, Brockman activity grade 1) was obtained from Sigma Chemical Co.; before use it was acid washed and stored in a desiccator.

In vitro samples were prepared by adding aliquots of cathecholamine standard to pooled urine specimens collected from randomly selected and apparently healthy males. Urine specimens for in vivo analysis were collected from healthy male volunteers undergoing hyperthermal work stress tests of 120 min duration. These specimens were 2-h collections obtained before (PRE), immediately after (POST), and two hours after (RECOVERY) thermal stress. Urine samples were collected with concentrated HCl as preservative, and those not immediately analyzed were stored at -35°C for no longer than five days.

o-Phthalaldehyde derivatizing reagent was prepared fresh daily by dissolving 160 mg of o-phthalaldehyde in 3 ml ethanol and 0.2 ml 2-mercaptoethanol. This solution was

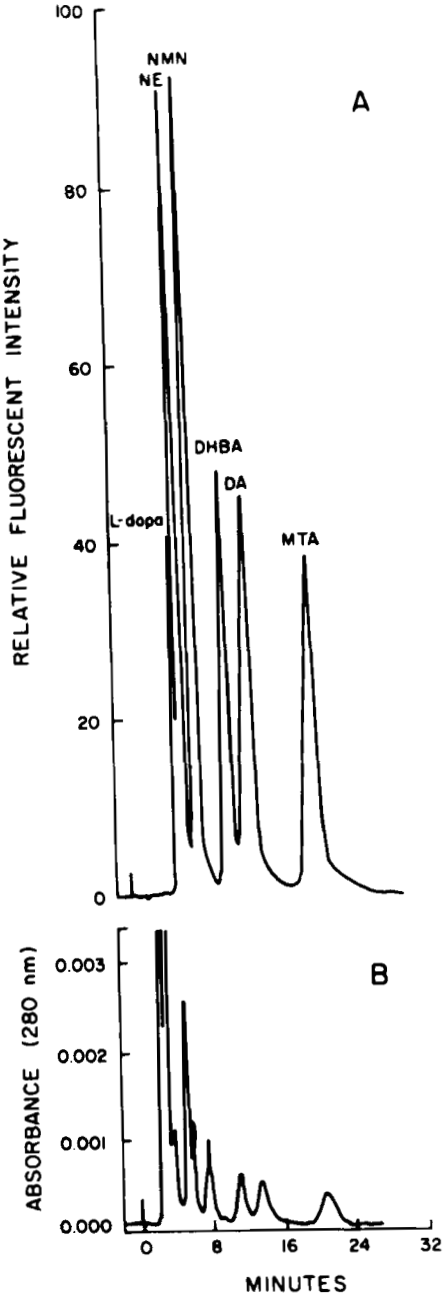
added to 100 ml of 0.4 mol/liter boric acid which was adjusted to pH 9.5 with potassium hydroxide.

METHODS

Chromatograms of biogenic amine standards were obtained by reacting 100 µl of o-phthalaldehyde derivatizing reagent with various amounts (usually 100-500 ng) of standard. After two minutes incubation at room temperature, various amounts of distilled water e.g. 100 µl, were added for purposes of dilution and aliquots of this mixture injected onto the column.

The procedure for extraction of biogenic amines from urine with alumina, and methodology for urinary norepinephrine and dopamine determination using reversed-phase liquid chromatography with ultraviolet detection has been described previously (17). For fluorescent detection with o-phthalaldehyde, 50 ml of urine was extracted with alumina. Ten microliters of internal standard solution containing 1 µg of 3,4-dihydroxybenzylamine and sufficient distilled water to bring small-volume urine specimens to 50 ml total volume were added to the urine aliquots prior to extraction with alumina. One hundred microliters of the effluent from the alumina extraction were reacted with 100 µl aliquots of the o-phthalaldehyde derivatization reagent and aliquots of this mixture injected onto the reversed-phase column for liquid chromatographic determination. Catecholamine content was calculated from peak area ratios by the following equation:

$$\text{ng/ml} = \frac{(\text{Peak area amine/Peak area Int. Std})_{\text{sample}}}{(\text{Peak area amine/Peak area Int. Std})_{\text{standard}}} \times \frac{\text{ng amine standard}}{\text{Sample volume (ml)}}$$



RESULTS

Figure 1 shows the fluorescent response (curve A) and the ultraviolet response (curve B, obtained simultaneously) for 82 ng each of selected biogenic amines and internal standard following derivatization with excess o-phthalaldehyde. No fluorescent response is observed if distilled water is substituted for biogenic amine. Of particular interest is the fact that only one peak is observed for each amine derivatized. Using the derivatization procedure previously outlined, the limit of detection is 55 picograms for norepinephrine and normetanephrine and 100 picograms for the other biogenic amines shown in Figure 1. For 50 ml pooled urine samples containing added catecholamines, sample concentration for morepinephrine and dopamine are linearly related from 1 µg/liter to at least 500 µg/liter. Data on within-run precision were determined by processing 10 aliquots of pooled urine through the complete procedure during a single day. The norepinephrine concentration was determined to be 46 ± 2 (SD) µg/liter (CV, 4.3%). Day-to-day precision data for another pooled urine sample gave a concentration of 54 ± 3 (SD) µg/liter, n = 10 (CV, 5.6%).

The effect of percent organic solvent on capacity factor, k', values for the fluorescent adduct of four biogenic

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Figure 1. Fluorescent (curve A) and ultraviolet (curve B) chromatograms of o-phthalaldehyde adduct for selected biogenic amines.

Eighty-two nanograms of each biogenic amine were derivatized with excess o-phthalaldehyde derivatizing reagent, pH 9.5. Column, µBondapak C_{18}; eluent, methanol/0.08 mol/liter acetic acid (50/50 by volume, pH 2.9); flow rate, 1.5 ml/min; temperature, 23°C; sample volume, 20 µl; fluorescence detection, excitation maximum 340 nm, 418 nm secondary filter, atten = 1 µA full scale; NE, norepinephrine; NMN, normetanephrine; DHBA, 3,4-dihydroxybenzylamine; DA, dopamine; MTA, 3-methyldopamine.

amines is illustrated in Figure 2. The observed reduction of capacity factor with increasing methanol concentration of the mobile phase is expected and can be predicted from solvophobic theory (18,19). The o-phthalaldehyde-amine adduct possesses sufficient hydrocarbonaceous moiety (15) than can enter into hydrophobic interactions with the octadecyl portion of the stationary phase. For such interaction, lower effective solvent surface tension, achieved by increasing organic solvent concentration of the mobile phase, results in decreased solute retention for mixed water-methanol solvents. Optimum separation of catecholamine o-phthalaldehyde adducts was achieved using a 50/50 (by volume) methanol/0.08 mol/liter (pH 2.9) acetic acid mobile phase.

In a similar manner, the effect of eluent pH on retention of the fluorescent adduct is illustrated in Figures 3 and 4. With octadecylsilica columns optimum separation is obtained when the aqueous-organic eluent is buffered in the neighborhood of pH 2 (19). This is observed in Figure 3 which shows decreasing resolution of norepinephrine and dopamine with only small increases from pH 2.9 in the mobile phase. The effect of pH on retention of the fluorescent o-phthalaldehyde adduct is summarized for four biogenic amines in Figure 4 which relates capacity factor k' to pH. Accurate retention values of norepinephrine and dopamine for pH 5 and above could not be determined due to excessive peak broadening. Although pH 9 is optimum for the o-phthalaldehyde-amine reaction, the use of alkaline-buffered mobile phases is not feasible for resolution of the fluorescent adduct. Siliceous column materials are not resistant to eluents having pH values higher than 7 (20,21), and excessive peak broadening and skewing is observed for mobile phases greater than pH 3.5 For the o-phthalaldehyde adducts of the biogenic amines shown in Figure 4, the pH dependence of the capacity factor

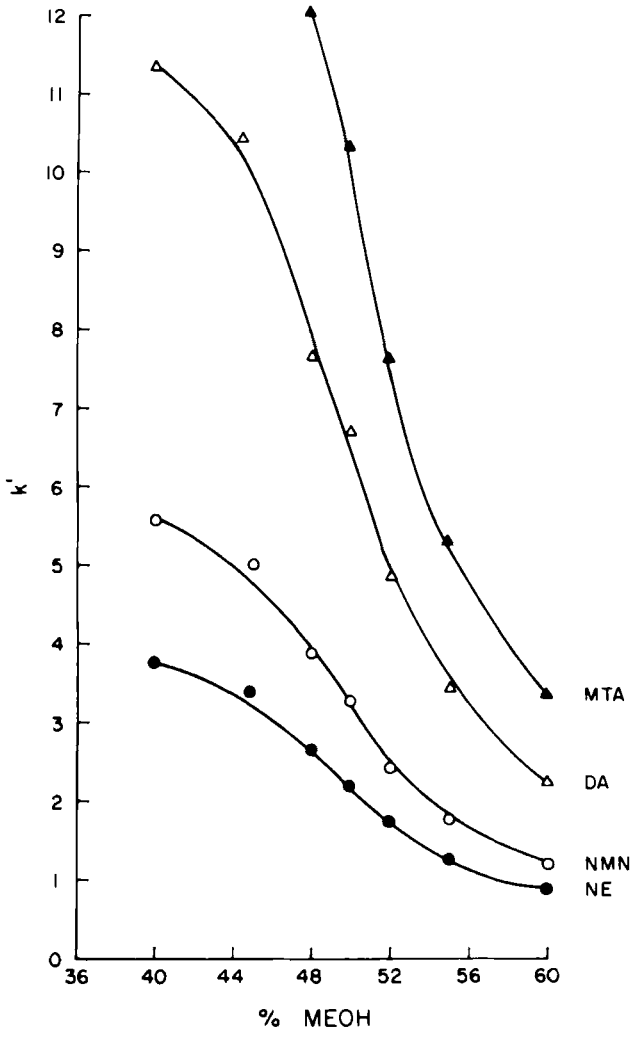

Figure 2. Effect of percent organic solvent on capacity factor k' values for selected amine o-phthalaldehyde derivatives.

One hundred nanograms of each biogenic amine were derivatized with excess o-phthalaldehyde. Column, μBondapak C_{18}; eluent, methanol/ 0.08 mol/liter acetic acid (by volume), aqueous phase at constant pH 2.9; sample volume, 20 μl; flow rate, 1.0 ml/min; See Figure 1 for symbols.

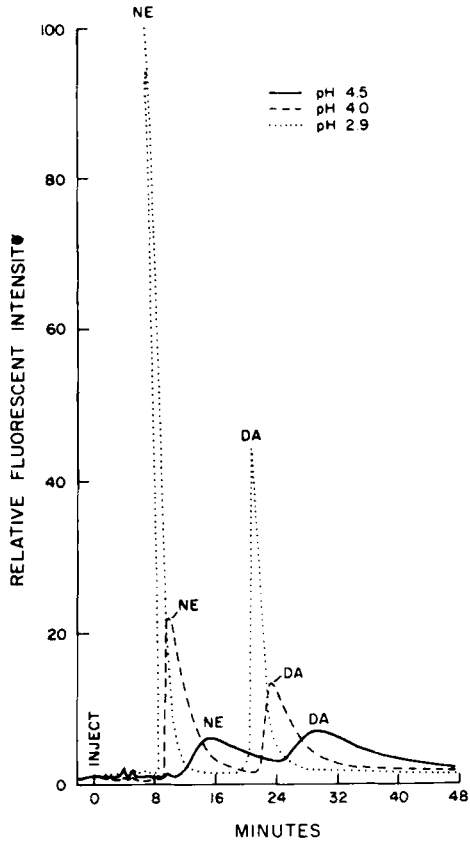

Figure 3. Effect of eluent pH on retention of norepinephrine (NE) and dopamine (DA) adducts.

One hundred nanograms of each biogenic amine were derivatized with excess o-phthalaldehyde. Column, μBondapak C_{18}; sample volume, 20 μl; flow rate, 1.0 ml/min; eluent, methanol/0.08 mol/liter acetic acid (50/50 by volume), pH of aqueous phase varied as shown.

is similar to theoretical curves of eluent pH vs. capacity factor obtained by Horvath et al. (20) for complex iogenic solutes.

Although the o-phthalaldehyde-amine reaction is rapid at room temperature, a time-dependent decay of adduct fluorescence

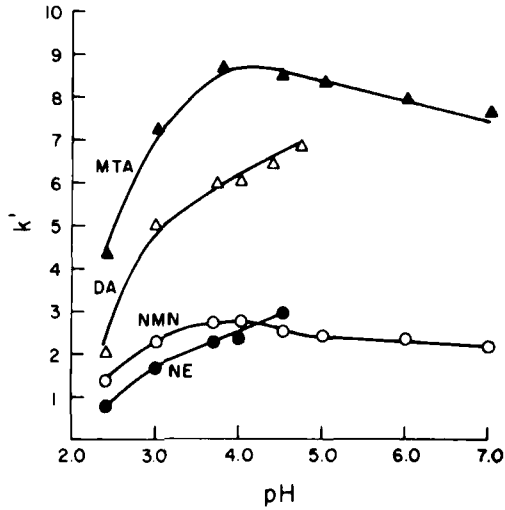

Figure 4. Effect of eluent pH on capacity factor k' values for selected amine o-phthalaldehyde derivatives.

Chromatographic conditions as in Figure 3. See Figure 1 for symbols.

(4,15) is observed in aqueous, alkaline buffers. This effect does not interfere with quantatative measurements if column injection occurs after a 2-3 minute delay following addition of biogenic amine to o-phthalaldehyde derivatizing reagent. Figure 5 illustrates that maximum adduct fluorescence is observed if column injection occurs after this short incubation period and supports previous evidence that fluorophor formation with o-phthalaldehyde occurs within five minutes of reaction time (references 4 and 6; column void time is approximately 3 minutes). Of additional interest is the fact that strong fluorescence is observed for the o-phthalaldehyde adducts of these amines when solvent pH is changed abruptly from 9.5 to 2.9, i.e. upon injection of an aliquot of derivatizing reagent mixture containing fluorescent adducts into the reversed-phase column. Evidence has been suggested that the

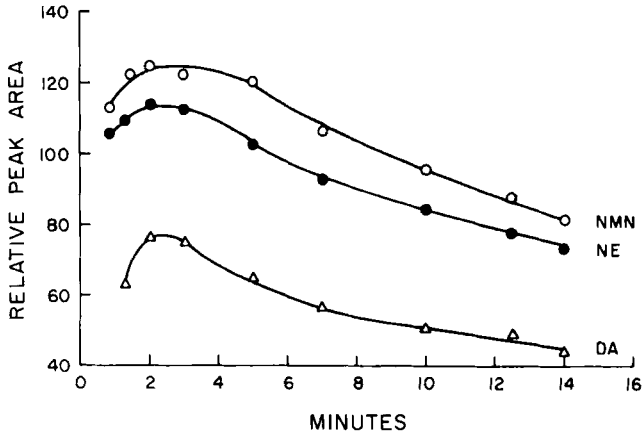

Figure 5. Fluorescent response for o-phthalaldehyde derivatives of normetanephrine (NMN), norepinephrine (NE), and dopamine (DA) as a function of reaction time prior to column injection.

Aliquots of standard amine were reacted with o-phthalaldehyde derivatizing reagent for various times prior to column injection. Column, μBondapak C_{18}; eluent, methanol/0.08 mol/liter acetic acid (50/50 by volume, pH 2.9); flow rate, 1.0 ml/min; temperature, 23°C; sample volume, 20 μl; fluorescence detection, excitation maximum at 340 nm, 418 nm secondary filter, atten = 1 μA full scale.

fluorescent intensity of the adduct is pH independent from 6.0 to 11.5 (16). Although little is known about the chemical properties of the adduct at low pH (Summers, Jr., S. S., personnel communication), this study describes conditions which extend the useful pH range under which these fluorescent adducts can be observed.

This method has been applied to the determination of the excretion of urinary-free norepinephrine from small-volume urine collections. Although there are differences among urine specimens, the chromatogram depicted in Figure 6 is typical of those obtained after extraction with alumina. For the specimen shown in Figure 6, the 2-h urinary excretions

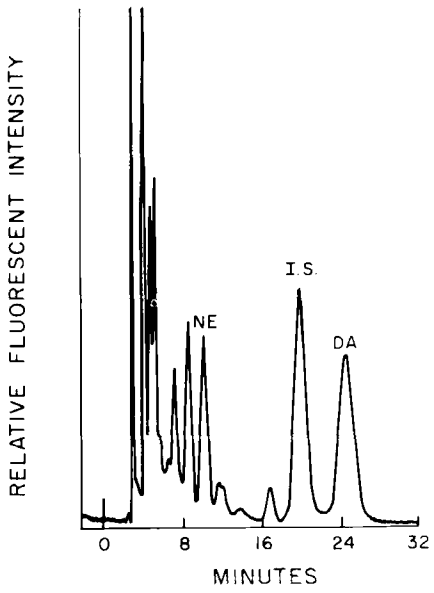

Figure 6. Chromatogram obtained after alumina extraction of a 2-h urine.

Column, μBondapak C_{18}; eluent, methanol/0.08 mol/liter acetic acid (50/50 by volume, pH 2.9); flow rate, 1.0 ml/min; temperature, 23°C; sample volume, 100 μl; fluorescence detection, excitation maximum at 340 nm, 418 nm secondary filter, atten = 1 μA full scale; NE, norepinephrine; IS, internal standard; DA, dopamine.

for norepinephrine and dopamine were 6 μg and 32 μg respectively. Norepinephrine excretion has been determined for five healthy subjects taking no medications, who have undergone standardized work regimens at elevated temperatures immediately after and prior to 2-h rest periods. In addition to the measurement of various physiological parameters such as EKG, blood pressure, tympanic temperature, etc., norepinephrine excretion was determined for the controlled periods of rest and activity. These results are summarized in Table 1. Norepinephrine excretion was determined in duplicate when sufficient urine volume was collected. In these cases both results are

TABLE 1

Norepinephrine Excretion (ng/min) for Five Male Volunteers 2-h Prior to (PRE), Immediately After (POST), and 2-h Following (RECOVERY) Thermal Stress

PRE			POST			RECOVERY		
Fluoro-Metric[a]	UV[b]	Sample Volume(ml)	Fluoro-Metric	UV	Sample Volume	Fluoro-Metric	UV	Sample Volume
35		90	152 156	160	440	26 27		100
43 40		200	81 85		100	98 100	105	400
42 45	52	395	63		75	44		80
70 69	77	260	65		66	138		75
52 50	52	250	44 46		100	166		87

[a]For duplicate analysis, both results are given.
[b]Norepinephrine excretion determined with UV detection (17).

given. For five urine collections adequate volume was obtained so that norepinephrine excretion could be accurately determined using ultraviolet detection (17), and these results are also given. When these values (mean for duplicate determinations) were regressed against those obtained by fluorometric analysis, the slope of the least-squares line was 1.003, the intercept was 5.517, and the coefficient of correlation was 0.997. Preliminary studies with standard solutions show no interference from other catecholamine metabolites or from the following common antihypertensive or diuretic drugs: methyldopa, triamterene, hydrochlorothiazide, indomethecin or propranolol HCl.

DISCUSSION

The justification for this work was the improved sensitivity and selectivity, needed for norepinephrine analysis from small-volume urine collections, which could be expected using pre-column derivatization with o-phthalaldehyde and fluorescent detection. This is evident from comparison of Figure 1 with a similar figure reported previously (17) for biogenic amine determination using reversed-phase techniques with ultraviolet detection. Prior extraction with alumina which allows small urine volumes to be analyzed and rapid fluorescent derivatization at room temperature with o-phthalaldehyde make this procedure readily adaptable to routine clinical use.

The application of this technique to other biologically active primary amines shows great promise. A recent report (22) described a similar procedure for gentamicin determination but only reported detection limits of 0.5 mg of gentamicin per liter and did not discuss the chromatographic response of the o-phthalaldehyde adduct in detail. The analysis of octopamine, a biogenic amine that, in invertebrate nervous systems, may function as a neurotransmitter (23), is currently being investigated by this laboratory. Of particular interest is the reversible loss of fluorescence that occurs upon solvent evaporation from organic solutions of the fluorescent adduct (16). Preliminary studies indicate that this phenomenon may be useful in obtaining further increases in sensitivity for biogenic amine analysis.

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REFERENCES

1. Iversen, L. L., Iversen, S.D. and Snyder, S.H., Eds. Biochemistry of Biogenic Amines, Plenum Press, New York, N. Y., 1975.
2. Axelrod. J., Sci. Am. 230, 59, 1974.
3. Hoeldtke, R., Metabolism 23, 663, 1974.
4. Roth, M., Anal. Chem. 43, 880, 1971.
5. Morton, L. and Lee, P. L. Y., Clin. Chem. 21, 1721, 1975.
6. Benson, J. R. and Hare, P.E., Proc. Nat. Acad. Sci. USA 72, 619, 1975.
7. Hakanson, R., Ronnberg, A.L. and Sjolund, K., Anal Chem. 59, 98, 1974.
8. Lindeberg, E.G.B., J. Chromatogr. 117, 439, 1976.
9. Komesu, N. N. and Thompson, J.H., Eur. J. Pharmacol. 16, 248, 1971.
10. Somerville, B. and Hinterberger, H., Clin. Chim. Acta 65, 399, 1975.
11. Butterworth, R. F., Landreville, F., Guitard, M., and Barbeau, A., Clin. Biochem. 8, 298, 1975.
12. Fujita, K., Nagatsu, T., Maruta, K., Teradaira, R., Beppu, I. T., Tsuji, Y. and Kato, T., Anal. Biochem. 82, 130, 1977.
13. Mays., D.L., Van Apeldoorn, R.J. and Lauback, K.G., J. Chromatogr. 120, 93, 1976.
14. Roth, M. and Hampai, A., J. Chromatogr. 83, 353, 1976.
15. Simons, Jr., S. S. and Jonhson, D. F., J. Am. Chem. Soc. 98, 7898, 1976.

16. Simons, Jr., S. S. and Johnson, D.F., Anal. Biochem. 82, 250, 1977.
17. Mell, L. D. and Gustafson, A.B., Clin. Chem. 23, 473, 1977.
18. Horvath, C., Melander, W. and Molanr, I., J. Chromatogr. 125, 129, 1976.
19. Molnar, I. and Horvath,C., J. Chromatogr. 143, 391, 1977.
20. Horvath, C., Melander, E. and Molnar, I., Anal. Chem. 49, 143, 1977.
21. Molnar, I. and Horvath, C., Clin. Chem. 22, 1497, 1976.
22. Maitra, S. K., Yoshikawa, T.T., Hansen, J.L., Nilsson-Ehle, I., Palin, W.J., Schotz, M.C.,and Guze, L.B., Clin. Chem. 23, 2275, 1977.
23. Saavedra, J. M., Brownstein, M.J., Carpenter, D.U. and Axelrod, J., Science 185, 364, 1974.