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Note

Determination of O-methylated metabolites of cathecholamines using high-performance liquid chromatography and electrochemical detection

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The use of high-performance liquid chromatography (HPLC) with electrochemical detection (ED) for determining catecholamines in biological fluids has proved to be a simple, inexpensive and accurate analytical technique[1–5], involving separation of the catecholamines by chromatography on a cation-exchange resin and quantitative detection based on electrochemical reactivity[5].

As catecholamine metabolites, including those arising from oxidation (by monoamine oxidase), O-methylation (by catechol-O-methyltransferase), and sulphation (by phenol-sulphotransferase) are also electrochemically active, it should be feasible to utilize HPLC–ED to ascertain the levels of these metabolites in biological fluids and to develop assays for determining the activities of the corresponding metabolic enzymes. Such techniques were recently developed as assays for the biosynthetic enzymes tyrosine hydroxylase[1] and phenylethanolamine-N-methyltransferase[6].

Here, we describe a procedure for the separation of catecholamines* (*e.g.*, norepinephrine) and their O-methylated metabolites (*e.g.*, normetanephrine and norparanephrine) by liquid chromatography on a cation-exchange resin and their individual quantitation with use of an electrochemical detector. This technique has proved itself useful as an assay for catechol-O-methyltransferase (EC 2.1.1.6).

MATERIALS AND METHODS

Instrumentation

Detailed descriptions of the HPLC-system and the carbon-paste electrochemical detector have been reported[2,5,7]. A glass chromatographic column (50 cm × 2 mm) dry-packed with Zipax SCX cation-exchange resin (DuPont) was used as stationary phase, and the mobile phase was 0.1 *M* perchloric acid (flow-rate *ca.* 0.3 ml/min). The cell potential of 0.9 V was established on the potentiostat. Calibration

* The following abbreviations are used: norepinephrine, 1-(3,4-dihydroxyphenyl)-2-aminoethanol; normetanephrine, 1-(3-methoxy-4-hydroxyphenyl)-2-aminoethanol; norparanephrine, 1-(3-hydroxy-4-methoxyphenyl)-2-aminoethanol; dopamine, 1-(3,4-dihydroxyphenyl)-2-aminoethane; 3-methoxytyramine, 1-(3-methoxy-4-hydroxyphenyl)-2-aminoethane; epinephrine, 1-(3,4-dihydroxyphenyl)-2-(methylamino)ethanol; metanephrine, 1-(3-methoxy-4-hydroxyphenyl)-2-(methylamino ethanol.

graphs were constructed for norepinephrine and normetanephrine with use of dopamine as internal standard. The amount of methylated product (*e.g.*, normetanephrine) in an unknown sample could be calculated from the observed ratio (R) of peak heights for the methylated product (*e.g.*, normetanephrine) and a known amount of the internal standard (*e.g.*, dopamine) by using previously established calibration graphs. 3,4-Dihydroxybenzylamine was used as internal standard[5] when metanephrine or 3-methoxytyramine was being determined.

Assay conditions

For the HPLC–ED assay, a typical incubation mixture consisted of the following components: sufficient water to give a final volume of 0.25 ml; magnesium chloride (0.32 μmole); norepinephrine (0.25 μmol); S-adenosyl-L-methionine (0.25 μmole); phosphate buffer, pH 7.60 (40 μmoles) and the enzyme preparation (variable). The reactions were initiated by addition of enzyme, and incubations were carried out at 37°. Aliquots (10 μl) were removed from the incubation mixtures at various times and quenched with 200 μl of 0.01 M perchloric acid containing dopamine (6 μmoles) as internal standard; chromatographic measurements were made by injection of 10 μl of this quenched reaction mixture. At least two injections were made for each quenched aliquot, and the average of the ratios of normetanephrine and dopamine peak peak heights was taken. The normetanephrine concentrations were determined from

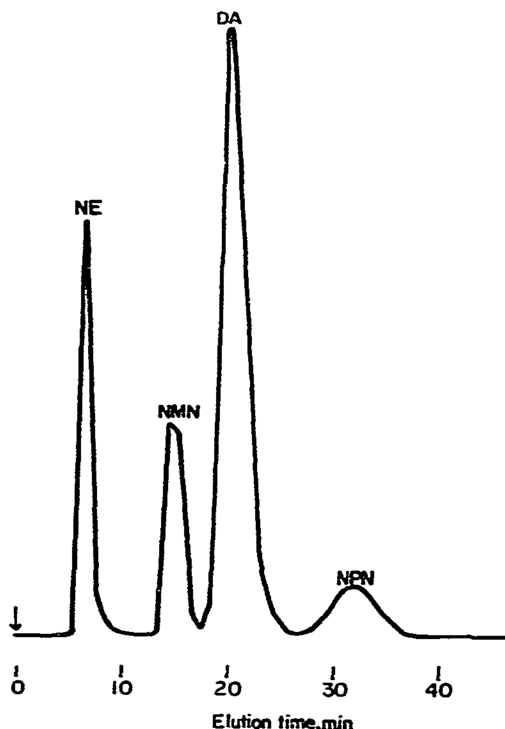

Fig. 1. A typical HPLC–ED elution pattern for norepinephrine (NE), normetanephrine (NMN) and norparanephrine (NPN), with dopamine (DA) as internal standard: NE = 1.25 ng; NMN = 3.82 ng; DA = 3.77 ng; NPN = 4.52 ng. The arrow denotes the time of injection. See text for details of conditions.

these average peak ratios and the calibration graphs. Catechol-O-methyltransferase was purified from rat liver by techniques previously described[8].

RESULTS AND DISCUSSION

In Fig. 1 is shown a typical elution pattern observed after an injection of a mixture containing norepinephrine, the corresponding O-methylated products (normetanephrine and norparanephrine) and the internal standard (dopamine). The baseline separation obtained on the Zipax SCX column with 0.1 *M* perchloric acid as mobile phase allows for individual quantitation of these catecholamines. In Fig. 2 is shown the linear relationship between the peak heights for normetanephrine and the amount of normetanephrine present per injection. The electrochemical detector used in this system is extremely sensitive, permitting accurate determination of as little as 0.25 ng (1 pmole) per injection of normetanephrine.

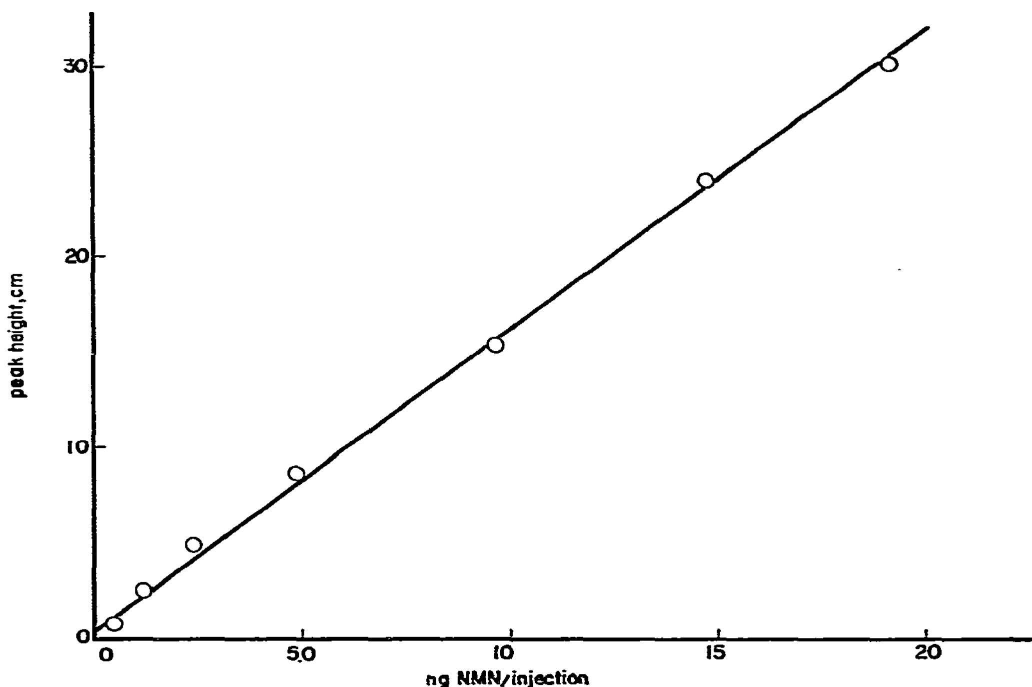

Fig. 2. Relationship between observed peak height and amount of normetanephrine injected (see text for details of conditions). Each point represents the average of at least three determinations.

This technique can also be used to separate dopamine from 3-methoxytyramine, and epinephrine from metanephrine. When metanephrine or 3-methoxytyramine is being determined, we use 3,4-dihydroxybenzylamine as internal standard rather than dopamine. Under the elution conditions of Fig. 1 and described in Materials and methods, the observed retention times are as follows: norepinephrine, 7 min; 3,4-dihydroxybenzylamine, 12 min; normetanephrine, 16 min; epinephrine, 18 min; dopamine, 21 min; metanephrine, 96 min; 3-methoxytyramine, 156 min.

In the development of an assay for catechol-O-methyltransferase, norepinephrine was chosen as the substrate because normetanephrine (the O-methylated product) has a short retention time. In Fig. 3 is shown the time course for formation of normetanephrine in enzyme incubation mixtures containing norepinephrine as acceptor substrate. Under these conditions, the reaction was observed to be linear for at least 30 min, permitting the accurate determination of initial enzyme velocities. The production of normetanephrine was also shown to be linear with increasing enzyme concentration.

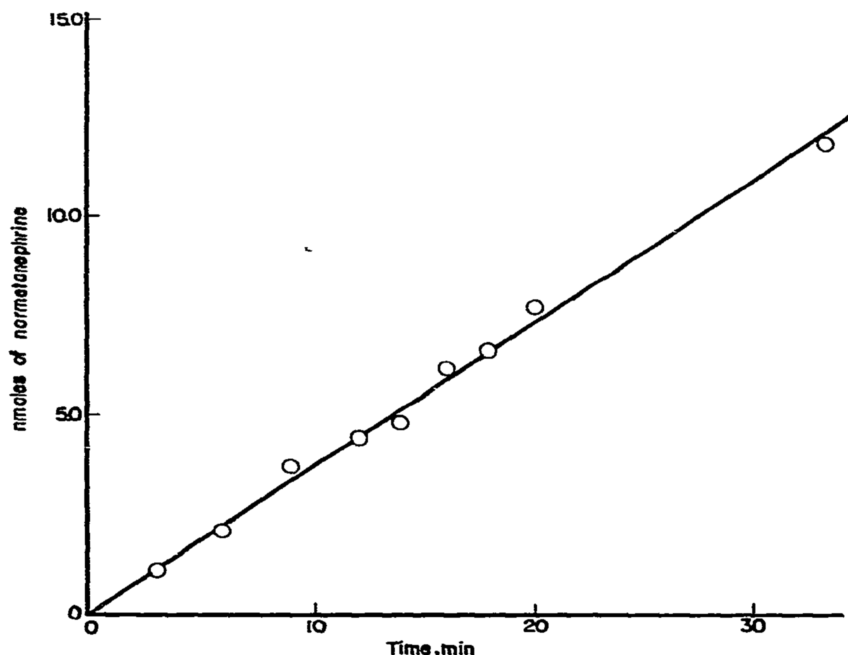

Fig. 3. Linearity of assay as a function of increasing incubation time. The incubation mixtures were prepared as described in the text, with use of catechol-O-methyltransferase purified from rat liver by affinity chromatography[8]. The quantity of normetanephrine (NMN) was calculated for the entire reaction mixture. Each point represents the average of at least two determinations.

To evaluate the utility of this assay for catechol-O-methyltransferase, we measured the enzyme activity present in both the soluble and microsomal fractions of rat brain, heart and liver using the LCEC assay and the standard radiochemical assay[9]; the results were if good agreement.

The assay described for catechol-O-methyltransferase has the advantage that it involves specific identification of the O-methylated products but includes no non-specific and time-consuming extraction procedures. The equipment used for this assay is also being used in our laboratory to determine catecholamines (norepinephrine, dopamine and epinephrine) in biological samples and in assaying for phenylethanolamine-N-methyltransferase activity[6]. Other laboratories have used the same or similar equipment to measure tyrosine hydroxylase activity[1] and the levels of catecholamines

and their metabolites in various biological fluids[2–5]. Thus, the technique appears to be of general utility in various aspects of catecholamine biochemistry.

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REFERENCES

1 C. L. Blank and R. Pike, *Life Sci.*, 18 (1976) 859.
2 R. Keller, A. Oke, I. Mefford and R. N. Adams, *Life Sci.*, 19 (1976) 995
3 P. T. Kissinger, L. J. Felice, R. M. Riggin, L. A. Pachla and D. C. Wenke, *Clin. Chem.*, 20 (1974) 992.
4 P. T. Kissinger, R. M. Riggin, R. L. Alcorn and L. D. Rau, *Biochem. Med.*, 13 (1975) 299.
5 C. Refshauge, P. T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R. N. Adams, *Life Sci.*, 14 (1974) 311.
6 R. T. Borchardt, W. C. Vincek and G. Grunewald, *Anal. Biochem.*, 82 (1977) 149.
7 P. T. Kissinger, C. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465.
8 R. T. Borchardt, C. F. Cheng and D. R. Thakker, *Biochem. Biophys. Res. Commun.*, 63 (1975) 69.
9 J. Axelrod and R. Tomchick, *J. Biol. Chem.*, 233 (1958) 702.