Modified Fluorometric Method for Determining Plasma Catecholamines

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Plasma catecholamines were fluorometrically determined by a modification of the standard alumina-triiodoacetamide method in which radioactive tracer catecholamines are used to follow the chromatographic behavior of the endogenous catecholamines on alumina. This modification permits appropriate fluorometry of that portion of the eluate with the highest concentration of catecholamines, because incomplete elution of the total endogenous catecholamines can be corrected by use of the isotope recovery data. Additionally, a low column blank was obtained; this method of blank preparation may have obviated some of the known difficulties associated with unoxidized and incomplete plasma blanks that have traditionally been used.

Additional Keyphrases  alumina-triiodoacetamide method  radioactive tracer catecholamines  column chromatography

Although modern fluorometry permits determination of catecholamines in urine and tissues, the application of fluorometric principles to the determination of plasma catecholamines has been hindered by two problems related to the low concentrations of plasma catecholamines.

The first is that, to extract the catecholamines from plasma, they are generally adsorbed onto alumina, from which they are subsequently eluted. Total elution from the alumina requires comparatively large volumes of acid; the resulting catecholamine concentration in an acid eluate is frequently very close to the sensitivity limit of the method.

Secondly, there has been some uncertainty as to the best method of preparing a sample blank for plasma. In some methods incomplete (i.e., "faded") blanks are prepared, but these are not true blanks, because they do not contain all of the materials added to the sample, and in some cases are not read at the same time as the sample. Alternatively, some workers make "unoxidized" blanks by adding all of the reagents in an atypical sequence. However, even when the reagents are added out of order, spontaneous oxidation may occur and so increased blank fluorescence may be produced.

Experiments designed to determine the amount of norepinephrine or epinephrine that would saturate 2.0 g of alumina yielded the incidental opportunity to observe the chromatographic behavior of radioactive epinephrine and norepinephrine (Figure 1). (In these initial experiments and in the method reported we assume that radioactive and nonradioactive catecholamines are chromatographically indistinguishable.) When the experiment was repeated with plasma, the data shown in Figure 2 were obtained.

We noted that some radioactivity appeared in the plasma filtrate, indicating that not all of the catecholamine was adsorbed to the alumina and (or) that some of it had been oxidized during the titration to pH 8.4. In addition, we noted that, although the acid eluate from the alumina contained most of the catecholamine in the early fractions, there was variable "tailing" of eluted catecholamine. These observations suggested that radioactive tracers could be used to improve plasma catecholamine methodology in the following ways: (a) to identify that portion of the acid eluate with the greatest concentration of catecholamine, and (b) to correct for the incomplete recovery that would result if only that portion of the eluate was used for the fluorometric determinations. In addition, it would be possible to identify a terminal portion of the acid eluate that contained no catecholamines, thus permitting the use of a column blank and so avoiding the difficulties attendant upon the use of incomplete or unoxidized plasma blanks.
Fig. 1. Chromatographic behavior of 200 µg of norepinephrine, 200 µg of epinephrine, and tracer quantities of both norepinephrine-4H and epinephrine-14C in 20 ml of urine.

After titration to pH 8.4 the mixture was passed through a column of alumina with a diameter of 1.5 cm. After a 10 ml water wash, elution was begun with 0.2M acetic acid. The ordinate indicates the dpm of each isotope in each 2 ml of column effluent.

Fig. 2. Results obtained with 20 ml of plasma (instead of urine) treated as in Figure 1, except that the tracer labels were reversed.

Materials

dL-Epinephrine-7-14C, dL-norepinephrine-7-14C, dL-epinephrine-7-3H, and dL-norepinephrine-7-3H were purchased from New England Nuclear Co., Boston, Mass. 02118. Initially, purification was attempted on a 0.1 X 15 cm column of 200-400 mesh Amberlite CG-50 buffered with 0.2M sodium acetate (pH 6.1) and developed with 0.1M acetic acid. However, because the 14C analogs were synthesized from veratic acid we were concerned that basic O-methylated radioactive byproducts might not be separated from the catecholamines. Initially, we attempted to purify them by absorption to alumina at pH 8.4 and elution with 0.1M acetic acid. However, the tritiated catecholamines were synthesized from adrenaline analogs, and were known to contain radioactive dopamine by-products as contaminants. These contaminants might be incompletely separated from the beta hydroxylated catecholamines on columns of alumina, because the dopamine analogs would also have neighboring hydroxyl radicals on the phenol nucleus. Accordingly, we finally purified all substances by using a 0.9 X 30 cm column of 200-400 mesh Amberlite CG-50 resin buffered in 0.2M sodium acetate (pH 6.1) and developed with 0.4M sodium acetate buffer (pH 5.0)—a method that did in fact reveal that the commercially obtained catecholamines frequently contained a number of contaminants (always less than 10% of the total radioactivity), some of which could not be resolved by either of the first two chromatographic separations. Furthermore, these latter contaminants were frequently unresolvable by paper chromatography from the authentic catecholamines when the customary 4:1 butanol-acetic acid or 4:1 isopropanol-ammonia solvent systems were used. Accordingly, all radioactive catecholamines were finally checked by ascending chromatography on Whatman no. 4 paper using 4:1 butanol in 1N hydrochloric acid and 85:15 phenol in 0.1N hydrochloric acid; the latter solvent system did separate the above-mentioned contaminants from the authentic catecholamines.

Method

To determine plasma catecholamine concentrations, we placed 10 to 50 ml of whole blood into a chilled round-bottom glass-stoppered 50-ml centrifuge tube containing 200 mg of ethylenediaminetetraacetic acid or 400 mg of disodium ethylenediaminetetraacetate. After the anticoagulant was thoroughly mixed in the tube, it was placed in an ice bath. After centrifugation for 10 min at 4°C and 1700 rpm (650 g), the plasma was aspirated, and its volume measured and recorded as "initial volume." Then 0.1 µCi of tritiated epinephrine (specific activity, 6-7 Ci/mm mol) and 0.01 µCi of norepinephrine-14C (specific activity, 40-50 mCi/mm mol) were added to the plasma.

A column of alumina was initially prepared by slurring 2 g of alumina (British Drug House) in 0.05M diethylbarbituric acid buffer (pH 8.4) into a chromatographic column (1.5 cm i.d.). (In later experiments, the alumina was slurried in distilled water, since comparison experiments showed no advantage to using the barbituric acid buffer.) The plasma was then poured onto the alumina and the effluent collected in a graduated cylinder. The alumina was washed with 10 ml of water, the effluent being collected in a second graduated cylinder. Most of the catecholamines were then eluted with 10 ml of 0.2M acetic acid, which was
collected in a third graduated cylinder. The remainder of the catecholamines were eluted with another 10 ml of 0.2M acetic acid, collected in a fourth graduated cylinder; material for the blanks was obtained by washing the columns with another 10 ml of 0.2M acetic acid, which was collected in a fifth graduated cylinder.

A volume of 0.5 ml from each of the five graduated cylinders was placed in 15 ml of either a dioxane scintillator (100 g of naphthalene, 7 g of FPO\textsuperscript{1} and 300 mg of dimethyl-POPOP\textsuperscript{1} in 1 liter of dioxane) or a toluene scintillator (5.5 g of POP, 100 mg of dimethyl-POPOP, 800 ml of toluene, and 200 ml of Triton-X-100). The radioactivity was counted in a Tricarb Scintillation Spectrometer Model 3320 (Packard Instrument Co., Downers Grove, Ill., 60515) with automatic external standardization, automatic background subtract, and windows set for simultaneous counting of tritium and carbon 14. Each vial was counted for 100 min so as to approach a counting error of less than 5% in all vials, including those obtained from the second and fifth cylinders, which routinely contained negligible amounts of radioactivity.

The remaining 9.5 ml of the third and fifth graduated cylinders were adjusted to pH 6.0 by adding 1.9 ml of 0.3M sodium acetate buffer (pH 6.0) and titrating by the dropwise addition of 1N sodium hydroxide. The volumes were recorded at this point as “final volume.” Each unknown was composed of 3 ml of the material from the third cylinder, and each blank consisted of 3 ml of the material from the respective fifth cylinder. The catecholamine standards consisted of 1 ml of an aqueous solution containing 0.1 μg of the respective catecholamine, plus 2 ml of the 0.3M acetate buffer (pH 6.0). All solutions were at 25°C.

The fluorophores were developed and measured in a room with constant lighting and constant temperature, used only for fluorometry. No smoking was permitted. To develop the fluorophores, 0.1 ml of fresh potassium ferricyanide solution (2.5 g/liter) was added to each tube. After exactly 3 min, 1 ml of fresh alkaline-ascorbate was added to each tube. [The alkaline-ascorbate was made by adding 2.5 ml of ascorbic acid (20 g/liter) to 22.5 ml of 5N sodium hydroxide.] Exactly 3 min later, the fluorescence was determined in a Model 110 filter fluorometer equipped with a High Sensitivity Conversion Kit, with filter 405 as the excitation (primary) filter and 65A as the emission (secondary) filter (G. K. Turner Associates, Palo Alto, Calif. 94303). At exactly 6 min the fluorescence was again read, with the 2A and 47B filters in combination for the excitation wavelength and the 2A-15 filter for the emission wavelength. Intermittently, spectra were checked with a Mark I spectrophotofluorometer (Farrand Optical Co., Mt. Vernon, N. Y. 10550). Thus, each plasma sample provided data symbolized as follows:

**Fluorescent Readings**

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<th>Filter set</th>
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<td>n\textsubscript{2}</td>
</tr>
<tr>
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<td>e\textsubscript{2}</td>
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<td>o\textsubscript{1}</td>
<td>o\textsubscript{2}</td>
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<tr>
<td>Sample</td>
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<td>u\textsubscript{2}</td>
</tr>
<tr>
<td>Sample blank</td>
<td>b\textsubscript{1}</td>
<td>b\textsubscript{2}</td>
</tr>
</tbody>
</table>

**Isotopic Calculations**

\[
\text{Norepinephrine-}\text{H} = \frac{(e\textsubscript{1} - o\textsubscript{2})}{(n\textsubscript{1} - o\textsubscript{1})} - \frac{(e\textsubscript{2} - o\textsubscript{2})}{(n\textsubscript{2} - o\textsubscript{1})}
\]

and

\[
\text{Epinephrine} = \frac{(u\textsubscript{2} - b\textsubscript{1}) - [\text{Norepinephrine}]}{(e\textsubscript{1} - o\textsubscript{1})}
\]

From the fluorescent readings, the weights of norepinephrine and epinephrine in the sample cuvet were calculated from the following formulas:

Plasma norepinephrine, μg/liter =

\[
\frac{1000(30[E5] - [E5])}{[FV]} = \frac{1000([N5] - [N3])}{30[NSA]}
\]

and

Plasma epinephrine, μg/liter =

\[
\frac{1000([E5] - [E5])}{[FV]} = \frac{1000([N5] - [N3])}{30[NSA]}
\]

**Results**

The total percentage of isotope recovered in all five cylinders of samples from four different experiments are shown, for each catecholamine, in Table 1. The percentage of each catecholamine recovered in the third cylinder is also shown in Table 1 for the same experiments. The percentage recovery of

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\textsuperscript{1} Abbreviations used: FPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene.
Table 1. Percentage of Radioactive Catecholamine Recovered

<table>
<thead>
<tr>
<th>Time period</th>
<th>In all five cylinders, (±SD)</th>
<th>In third cylinder, (± SD)</th>
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</tr>
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<tr>
<td></td>
<td>Nor</td>
<td>Epi</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>97 ± 4</td>
<td>96 ± 11</td>
<td>6</td>
</tr>
<tr>
<td>II</td>
<td>97 ± 5</td>
<td>90 ± 4</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>88 ± 5</td>
<td>88 ± 5</td>
<td>12</td>
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<tr>
<td>IV</td>
<td>97 ± 4</td>
<td>102 ± 8</td>
<td>6</td>
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</table>

isotope in the third cylinder of duplicate analyses of the same plasma differed by less than 2% for norepinephrine, and less than 3% for epinephrine.

Blood was obtained from the antecubital veins of 20 persons who had been quietly lying down for at least 30 min in the morning. Eleven of these were hospitalized hypertensive patients and nine were normotensive persons in good apparent health. The results for catecholamines are shown in Figure 3.

Discussion

Because the highest concentration of catecholamines is present in the third cylinder, use of the third cylinder increases the accuracy of the fluorometric determination as compared to the results obtained when the eluates are developed from a pool of the third and fourth cylinders. While the latter method gives a more complete elution, it also yields a more dilute sample to measure. By knowing the percentage of added radioactive catecholamine eluted in the third cylinder, one can calculate the amount of endogenous catecholamine originally present in the plasma sample. Similarly, the isotopic data permit the use of less than 10 ml of plasma. However, use of such small samples will decrease the accuracy and sensitivity of the method, unless one is working with a catecholamine-rich plasma such as that from the adrenal (or even renal) veins. Accordingly, when drawing blood from the antecubital vein of a relaxed recumbent subject one should attempt to obtain at least 20 ml of plasma, since a greater quantity of catecholamine per sample will contribute to greater fluorometric accuracy and sensitivity.

Figure 1 indicates why 5 to 20% of the total radioactivity may appear in the fourth cylinder as a “tail” from the third cylinder “peak.” Similarly, another 5 to 35% of the added radioactivity of either catecholamine may appear in the first cylinder. A sudden increase in the percentage of an isotope appearing in the first cylinder might suggest isotope deterioration, but such deterioration can be detected by routine paper chromatographic purity checks performed every one to three months.

Note that the radioactive catecholamines are added only after the plasma has been separated from the remainder of the sample, since radioactive catecholamines may penetrate platelets and erythrocytes (1–3). In fact, there is some evidence suggesting that norepinephrine and epinephrine enter the erythrocytes of the same person at difference rates (4). There is also data demonstrating that radioactive L-norepinephrine will penetrate the erythrocytes of different persons at different rates (5). Finally, available radioactive catecholamines are racemic, and the isomers perhaps penetrate red cells at different rates, an additional confounding factor.

In this method, the plasma blanks are made in a manner that eliminates the difficulties attendant upon adding reagents out of sequence (i.e., “unoxidized” blanks), as well as the difficulties attendant upon forming a sample blank to which some reagents may be added only after a significant delay (i.e., “faded”) or sometimes not at all. Our previous experience with the latter two methods for making plasma catecholamine blanks resulted in high and low blank values, respectively, as noted by other workers (3, 6, 7). The variability in blank values from method to method encompasses several different methods of preparing plasma blanks, several different types of fluorescence stabilizers, and, in at least one case, the preparation of two different blanks by two different methods, whose arithmetic average fluorescence is

![Graph](image-url)

Fig. 3. Plasma catecholamine concentrations for hypertensive and normotensive groups do not differ significantly.

The bars indicate group means and the brackets indicate ±1 SD.
used as the blank value in calculations (8). These factors no doubt contribute to the wide range of normal values previously reported (Table 2). The normal values obtained by the present method tend to be higher than those obtained by other methods for trihydroxyindoles. This is probably partly due to the low plasma blanks obtained with the present method but is also due in part to the use of a correction factor for loss of catecholamine during chromatography.

Gerst et al. (29) have shown that the fluorescent intensity of the trihydroxyindole fluorophore is temperature dependent. Fortunately, the fluorescence intensity-temperature relationship is linear. The slope is identical for aqueous standards and aqueous blanks. Plasma eluates and eluate blanks also have an identical slope, although it is different from the slope of the aqueous solutions. In the present experiment, samples and the standards were at the same temperature as their respective blanks (25°C). Thus, the temperature effect can be an additional reason for using a sample blank as described in this method.

In addition to the data presented in the results section, a number of clinical experiences with this method had suggested to us that the substances being measured are in fact plasma epinephrine and plasma norepinephrine:

- For instance, in studies of plasma catecholamines obtained by catheterization of the left adrenal vein, with simultaneous central aortic sampling, it has been possible on several occasions to determine that the sampling catheter had recoiled out of the adrenal vein and into the renal vein. These determinations were based upon gross differences in the plasma catecholamine concentrations, and were all subsequently confirmed by examining the films taken during the angiographic procedure.

- Analysis of these samples from the adrenal vein (to be reported elsewhere) reveals the adrenal gland to be secreting predominantly epinephrine, as would be expected.

- Only 15% of the aortic samples in the above experiment contained measurable amounts of norepinephrine, and 70% contained measurable amounts of epinephrine. In contrast, samples of venous blood similarly obtained in the recumbent resting state have detectable amounts of norepinephrine in approximately 50% of the samples and epinephrine in 79%. This would seem to indicate that the arteriolar vascular bed is releasing norepinephrine, as would be expected.

- We have studied a patient with systemic lupus erythematosus and Raynaud's phenomenon. Indwelling catheters were placed in both a vein and an artery of the right arm. A control sample of venous blood showed no measurable norepinephrine but an epinephrine concentration of 2.39 µg/liter. Arteriography was accompanied by

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### Table 2. Reported Mean Values for Human Plasma Catecholamines

<table>
<thead>
<tr>
<th>Year</th>
<th>Reference</th>
<th>Method*</th>
<th>Norepinephrine</th>
<th>Epinephrine</th>
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<td>E.D.</td>
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<td>E.D.</td>
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* E.D., ethylenediamine condensation; THI, trihydroxyindole method; Iso, isotopic; Enz, isotopic enzymic conversion.

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pain and the venous plasma epinephrine concentration rose to 5.06 µg/liter, still without demonstrable norepinephrine. Approximately 1.0 mg of reserpine was then injected intra-arterially, and after 1 min the venous sample showed 42.65 µg of norepinephrine per liter, but no detectable epinephrine. A simultaneous sample of arterial blood contained 28.33 µg of norepinephrine per liter, but no detectable epinephrine, thus demonstrating a release of norepinephrine from the hand as would be expected from the known pharmacologic effects of reserpine. Twenty minutes after reserpine, the norepinephrine of the venous blood sample from that extremity had decreased to 17.68 µg/liter.

- We have had the opportunity to study a totally adrenalectomized patient, whose plasma contained norepinephrine but no detectable epinephrine.

In conclusion, the present plasma catecholamine method can be used by any laboratory now doing fluorometric analyses of urinary catecholamines and having access to a scintillation counter capable of simultaneously determining carbon and tritium labels.

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References


