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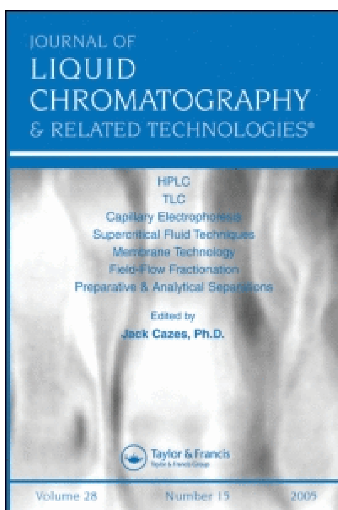

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EVALUATION OF FOUR METHODS FOR CATECHOLAMINE ANALYSIS IN PLASMA OBTAINED FROM SUBJECTS DURING INCREMENTAL CYCLE ERGOMETRY

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ABSTRACT

Plasma was collected from a subject during incremental cycle ergometry in order to determine the anaerobic threshold. Norepinephrine and epinephrine were extracted using alumina, strong-cation exchange/alumina, or weak-cation exchange, and then separated using reversed-phase ion-pair chromatography. Amperometric detection was used following chromatography for all three types of extractions (Methods A, B and C), while coulometric detection was only used following chromatography and alumina extraction (Method D). The upper limit of linearity was the lowest for Method C. The extraction for Method B took about twice the amount of time as the other extractions. The standard curve for Methods A, B and D was linear to 5000 ng/L. The lowest limit of quantification and relative standard deviation was obtained using Method D. In summary, Method D was the best method for this purpose.

INTRODUCTION

The inflection in blood lactate concentration that occurs during increased work may be caused by muscle anaerobisis, fiber recruitment or hormonal control

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via catecholamines (1). Therefore, changes in plasma catecholamine concentrations are quantitated as part of the process of determining their influence on the lactate threshold during graded exercise. Initially, plasma catecholamine concentrations are low because the subject is at rest. By the time the subject reaches volitional exhaustion, plasma catecholamine concentrations are significantly above normal concentrations.

Plasma catecholamine concentration can be determined by several methods as described in three excellent reviews (2 3 -4). "However, it is now very clear from the ever-growing volume of publications on the subject, that high performance liquid chromatography with amperometric or fluorometric detection is emerging as a pre-eminent tool for metabolic fingerprinting of these important biomolecules (2)." Besides amperometric detection, coulometric detection can be used (3). Sample pretreatment is required to remove endogenous interferences (2,3). Catecholamines have been extracted using alumina (5 ,6), weak-cation exchange (7), strong-cation exchange followed by alumina (8), boric acid gel (9) and organic solvents (10 ,11).

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Our review of the literature revealed that none of the HPLC methods with electrochemical detection (ECD) meet all of our criteria for quantitating norepinephrine and epinephrine concentrations in plasma of subjects during incremental cycle ergometry (2-11). For this purpose, the limit of quantitation must be low enough to allow for the determination of basil norepinephrine and epinephrine levels. This is especially difficult for epinephrine because basil levels are frequently less than 100 ng/L (10,11). The method must be accurate and have a relative standard deviation of less than 5% for norepinephrine and epinephrine. Accurate and precise concentrations are required to determine the onset of the anaerobic threshold. The range of linearity must be large, because the concentration of plasma norepinephrine and epinephrine in these subjects increases above basil values by a factor of about 10 to 20. This is especially

important for norepinephrine because the level of norepinephrine can be as high as 5000 ng/L. Finally, the method must be practical because there are typically about 15 samples per subject. The most common shortcomings of previously reported methods was the limit of detection that was too high for epinephrine and upper limit of linearity for norepinephrine was not high enough. To the authors knowledge, this is the first direct comparison of these methods and should provide researchers or clinicians new to this field with a valuable comparison of electrochemical methods for catecholamine analysis.

Therefore, the purpose of this research was to ascertain which of the four methods (Figure 1) described here, if any, meet all of the criteria, as described in the preceding paragraph, for determining the concentration of plasma norepinephrine and epinephrine in subjects who have exercised to volitional exhausting during incremental cycle ergometry. In all cases, separations were done using a reversed-phase ion-pair HPLC. Three types of extractions were used with amperometric detection: **1.** aluminum oxide (Method A), **2.** strong-cation in series with aluminum oxide (Method B) and **3**. weak-cation (Method C). The fourth method was an aluminum oxide extraction followed by coulometric detection (Method D). We did not evaluate boric acid gel because this gel is relatively expensive when compared to aluminum oxide. We did not evaluate liquid-liquid extraction, because this extraction is limited due to relatively poor selectivity of this extraction compared to alumina, although the recovery is higher (10).

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EXPERIMENTAL

Materials

Reduced glutathione (96%), sodium 1-octanesulfonate (98%), 3,4-dihydroxybenzylamine hydrobromide (98%), and 70% (w/v) perchloric acid

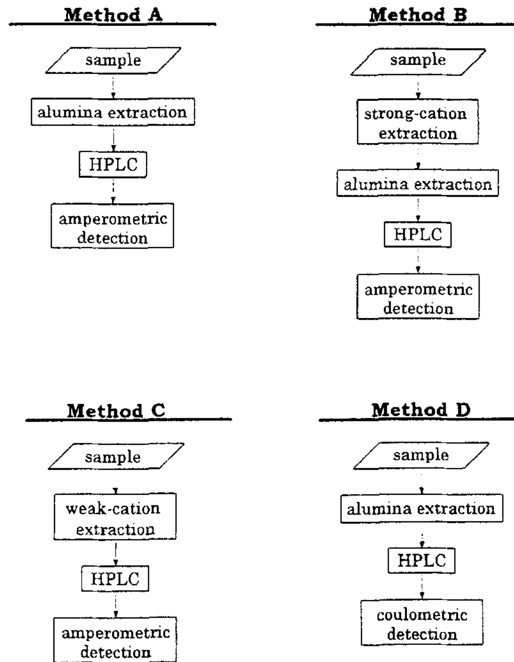

FIGURE 1. Methods Evaluated for the Quantification of Plasma Catecholamine Concentrations During Incremental Cycle Ergometry.

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(99.999%) were purchased from Aldrich Chemical Company (Milwaukee, WI). HPLC grade monobasic potassium phosphate, water, methanol, and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA) as well as Gelman 0.20-μm x 45-mm Nylaflo membranes and 13- x 75-mm Vacutainer blood collection tube containing 0.048 mL of a 15% tripotasssium ethylenediaminetetreacetic acid. Ultrapure grade tris-(hydroxymethyl)aminomethane (TRIS) was supplied by Research Organics (Cleveland, OH). Ethylene glycol-bis(β-aminoethylether) N,N,N'-tetraacetic acid (97%, EGTA) was obtained from Sigma Chemical Company (St. Louis, MO). Microfilters, RC58 membranes, a octadecyldimethylsilyl, 3-μm, 3.2-x100-mm cartridge column and holder, acid

washed aluminum oxide, norepinephrine bitartrate, epinephrine bitartrate, and an electrode polishing kit were obtained from Bioanalytical Systems (W. Lafayette, IN). An octadecyldimethylsilyl, 3-µm, 4-x100-mm glass-lined column was obtained from Scientific Glass Engineering (Austin, TX). Strong-cation and weak-cation exchange solid-phase extraction columns, each containing 100 mg of sorbent, and a solid-phase extraction manifold were acquired from Supelco, Inc. (Bellefonte, PA). All other chemicals were reagent grade.

Methods

Sample collection. An indwelling catheter was placed in a vein of the subject's antecubital fossa the morning after an overnight fast. A mixture containing 2.5 g of glutathione, 3.0 g of EGTA, and 33.5 mL of distilled water was prepared in a 50-mL Erlenmeyer flask with a ground-glass stopper. The mixture was continuously stirred during use. During exercise to volitional exhaustion on a cycle ergometer, 3 mL of whole blood was collected during the last 30 s of each 2-min work stage and immediately after maximal exercise for analysis. The blood was transferred to a 13-x100-mm tube containing 100 USP units of lithium heparin. A 70-µL aliquot of glutathione and EGTA mixture was added to the whole blood and gently rocked. The blood was centrifuged at 1000g for 5 min. The plasma was stored in 1.5-mL polyethylene Eppendorf micro test tubes at -70°C.

Pooled Plasma Samples. Pooled plasma A was prepared from one subject at rest. Blood was collected in six 13- x 75-mm Vacutainer tubes via venipuncture of a vein in the subject's antecubital fossa. The Vacutainer tubes were centrifuged at 1000 g for 10 min and the resulting plasma was pooled. Aliquots (1.2 mL) of pooled plasma were stored in Eppendorf tubes at -70°C. Pooled plasma B was prepared by pooling all the plasma obtained while exercising a subject to volitional exhaustion. Pooled plasma C was prepared similarly but a different subject was used.

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Aluminum Oxide Extraction. This extraction was performed as previously described (6) with the following exceptions: **1.** A pH 7.4 phosphate buffer was prepared by dissolving 0.472 g of potassium monobasic phosphate, 1.72 g of sodium dibasic phosphate, 0.42 g of sodium bicarbonate, 1.46 g of sodium chloride, 0.95 g of sodium metabisulfite, 0.31 g of reduced glutathione, and 0.38 g of EGTA in 150 mL of HPLC grade water. The pH was adjusted to a pH of 7.4 with 1 M HCl or 1 M NaOH as required. The solution was transferred to a 200-mL volumetric flask. The flask was filled to the mark with HPLC grade water. **2.** A combined stock solution containing 1 mg/L of norepinephrine and 1 mg/L epinephrine in 0.1 M perchloric acid was prepared from the 100 mg/L solution of norepinephrine and 100 mg/L solution of epinephrine (6) using 1.0-mL disposable plastic pipets and a 100-mL volumetric flask. This solution was stored at $4^{o}C$. **3.** Polypropylene centrifugation tubes (15-mL) were used for the dilutions of the norepinephrine and epinephrine solution containing 1 mg/L of each catecholamine, and for the extractions. **4.** The combined stock solution was diluted using disposable plastic pipets and 0.1 M perchloric acid. Standards were prepared from the resulting dilutions of combined stock solution by adding 10-μL aliquots (using a micropipet with disposable plastic tips) of each dilution to 1.0 mL of the pH 7.4 phosphate buffer. Standards were prepared before each use. **5.** All plasma samples were centrifuged for 4 min in a Beckman Microfuge E. **6.** The extraction was performed by rocking for eight minutes. **7.** The aluminum oxide was washed three times with 5-mL of vacuum degassed HPLC grade water.

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Strong-Cation/Aluminum Oxide Extraction. This extraction was done as described elsewhere (8) with minor modifications: **1.** Standards were prepared in accordance with the procedure described under *Aluminum Oxide Extraction.* **2.** The samples or standards were extracted under a vacuum of 400 Pa (3 mm of Hg) at a rate of ~100 μL every 5 seconds. **3.** The catecholamines were eluted into 15-mL polypropylene centrifugation tubes under a vacuum of 400 Pa at a rate of ~100 μL every 5 seconds. **4.** An aluminum oxide extraction was then performed

as described under *Aluminum Oxide Extraction* except 2 mL of TRIS buffer was added to the eluate followed by 50 mg of aluminum oxide.

Weak-Cation Extraction. Slight alterations were made to Supelco's extraction procedure (7): **1.** Standards were prepared in accordance with the procedure described under *Aluminum Oxide Extraction.* **2.** An aliquot of sample (0.75 mL) was diluted with an equal volume of HPLC grade water in a 1.5-mL polyethylene Eppendorf micro test tube. The internal standard was then added. **3.** The samples or standards were extracted under a vacuum of 400 Pa (3 mm of Hg) at a rate of ~100 μL every 15-20 seconds. **4.** The catecholamines were eluted into a 1.5-mL polyethylene Eppendorf micro test tube.

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Amperometric Detection. Chromatographic conditions were established as recommended by Bioanalytical Systems (6) using the 3.2-x100-mm column. The Bioanalytical Systems LC4A detector was set to 1 nA/V x 1 (B) and +0.65V. The mobile phase flow was set to 1.2 mL/min. The mobile phase was conditioned by recirculation as required. The glassy carbon electrode was polished in accordance with the manufacturer's procedure. The mobile phase was continuously sparged with helium. The electrodes were connected in the dual series mode.

Coulometric Detection. The mobile phase was a 50 mM, pH 2.6 phosphate buffer containing 2.0 mM sodium 1-octanesulfonate, 60 mL/L of methanol, 40 mL/L of acetonitrile and 0.25 mM Na_2EDTA. The mobile phase was pumped through the 4-x100-mm column at a flow of 0.8 mL/min. The potentials of the ESA 5100A coulometric detector with a 5011 analytical cell and a 5021 conditioning cell were set as follows: conditioning cell = +0.35V, detector 1 = +0.05V, and detector 2 = -0.30V.

Method Evaluation. The method evaluation protocol of reference 12 was followed. Recovery was determined by adding 500 pg of norepinephrine and 500 pg of epinephrine to 1.0 mL of a pooled plasma sample. The slope (m), intercept

(b), and correlation coefficient (r) were determined using a linear regression. The limit of detection was determined as described elsewhere (12). The limit of quantification was determined by calculating the mean of phosphate buffer blanks and adding 10 times the standard deviation of the mean (13). The pooled relative standard deviation (13) for norepinephrine concentration determined using Method A was calculated from the relative standard deviation for pooled plasma-A-I, pooled plasma-A-II and the standards(Table 1) (14). The calculation was similar for determining the pooled relative standard deviation for epinephrine except the pooled plasma-A-I and A-II values were not available, because the values were below the limit of quantification. For Method B, the pooled relative standard deviation was calculated using pooled plasma-B and pooled plasma-C (Table 1). For Method C, only the 1000 ng/L standard was used so a pooled relative standard deviation was not calculated. For Method D, the pooled relative standard deviation was determined using the pooled plasma-A and the standards (Table 1).

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RESULTS AND DISCUSSION

Method A.

The standard curve was linear to 5000 ng/L for both norepinephrine (r = 0.996) and epinephrine (r = 0.999). The average concentration of the 100, 500, or 1000 ng/L norepinephrine and epinephrine STD-I (Table 1) was not statistically different from the expected value of 100, 500, and 1000 ng/L, respectively, at the 0.01 significance level (15). The same was true for all three STD-II's (Table 1). The average concentration of norepinephrine in pooled plasma-A (A-I of Table 1) using one standard curve was not statistically different from the concentration of norepinephrine found in a second extraction of pooled plasma-A (A-II of Table 1) using a different standard curve at the 0.01 significance level (15). The limit of

TABLE 1.

Results for Methods A, B, C AND D[1].

	Sample	Nor[2] ± RSD[4] (n)	Epi[3] ± RSD[4] (n)
Method A[1]	100 ng/L STD-I[5]	100 ± 5.0 (5)	108 ± 7.4 (5)
	100 ng/L STD-II[5]	103 ± 7.9 (2)	90 ± 9.0 (2)
	Average[6]	102 ± 1.9 (2)	99 ± 12.9 (2)
	500 ng/L STD-I[5]	539 ± 5.4 (5)	498 ± 13.0 (5)
	500 ng/L STD-II[5]	530 ± 1.5 (2)	462 ± 1.5 (2)
	Average[6]	534 ± 1.3 (2)	480 ± 5.3 (2)
	1000 ng/L STD-I[5]	1047 ± 6.7 (6)	1040 ± 5.6 (6)
	1000 ng/L STD-II[5]	1033 ± 3.1 (2)	954 ± 5.3 (2)
	Average[6]	1040 ± 1.0 (2)	997 ± 6.1 (2)
	pooled plasma-A-I[7]	292 ± 2.8 (4)	< LOQ[8] (4)
	pooled plasma-A-II[7]	286 ± 3.2 (4)	< LOQ[8] (4)
Method B[1]	pooled plasma-B[7]	647 ± 5.4 (4)	255 ± 8.1 (4)
	pooled plasma-C[7]	397 ± 4.6 (4)	134 ± 5.7 (4)
Method C[1]	1000 ng/L STD[5]	987 ± 9.6 (3)	887 ± 8.5 (3)
Method D[1]	25 ng/L STD[5]	25 ± 6.2 (6)	25 ± 5.0 (5)
	100 ng/L STD[5]	106 ± 3.0 (4)	96 ± 3.2 (6)
	500 ng/L STD[5]	498 ± 1.6 (6)	494 ± 2.1 (6)
	1000 ng/L STD[5]	943 ± 3.1 (5)	988 ± 4.6 (6)
	pooled plasma-A[7]	253 ± 8.8 (4)	36 ± 7.9 (4)

[1]c.f. Figure 1, [2]norepinephrine concentration (ng/L), [3]epinephrine concentration (ng/L), [4]relative standard deviation, [5]standard prepared as described in the section on methods, [6]Average of STD-I and STD-II, [7]pooled plasma prepared as described in the section on methods, and [8]limit of quantification.

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TABLE 2.

Comparison of the Methods for Norepinephrine Quantification.

	LOD[2]	LOQ[3]	Linearity[4]	RSD[5] (n)
Method A[1]	14	29	5000	5.1 (24)
Method B[1]	6	11	5000	5.0 (8)
Method C[1]	ND[6]	ND[6]	1000	9.6 (3)
Method D[1]	6	8	5000	3.8 (21)

[1]c.f. Figure 1, [2]limit of detection, [3]limit of quantification, [4]upper limit of linearity, [5]relative standard deviation, and [6]not determined for this method.

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detection was 14 ng/L (n=4) and the limit of quantification was 29 ng/L (n=4) for norepinephrine (Table 2). The limit of detection was 30 ng/L (n=4) and the limit of quantification was 43 ng/L (n=4) for epinephrine (Table 3). The pooled relative standard deviation was 5.1% (n=24) for norepinephrine concentrations (Table 2) and 9.0% (n=16) for epinephrine concentrations (Table 3). The recovery relative to dopamine is 98 ± 6% for norepinephrine (n=3) and 96 ± 4% (n=4) for epinephrine. We found that chromatographic conditions had to be optimized in order to prevent an endogenous compound from coeluting with norepinephrine and a different endogenous compoumd from coeluting with epinephrine (Figure 2).

Method B.

This standard curve was linear to 5000 ng/L for both norepinephrine (r = 0.995) and epinephrine (r = 0.999). The limit of detection was 6 ng/L (n=2) and the limit of quantification is 11 ng/L (n=2) for norepinephrine (Table 2). The limit of detection was 10 ng/L (n=2) and the limit of quantification is 21 ng/L (n=2) for epinephrine (Table 3) (13). Further evaluation was not done because we felt the method was too time consuming. A relatively large broad peak eluted before norepinephrine (Figure 3).

TABLE 3.

Comparison of the Methods for Epinephrine Quantification.

	LOD[2]	LOQ[3]	Linearity[4]	RSD[5] (n)
Method A[1]	30	43	5000	9.0 (16)
Method B[1]	10	21	5000	7.0 (8)
Method C[1]	ND[6]	ND[6]	1000	8.5 (3)
Method D[1]	5	7	5000	3.9 (21)

[1]c.f. Figure 1, [2]limit of detection, [3]limit of quantification, [4]upper limit of linearity, [5]relative standard deviation, and [6]not determined for this method.

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Method C.

This standard curve is linear to about 1000 ng/L for norepinephrine (r = 0.999) and 1000 ng/L for epinephrine (r = 0.999). Typical chromatograms are shown in Figure 3. Further evaluation was not done because the method did not meet our criteria for the upper limit of linearity for norepinephrine. Compared to Method A and B, Method C did not provide a clean extraction as demonstrated by the noisy baseline (Figure 4).

Method D.

The standard curve was linear to 5000 ng/L for norepinephrine (r = 0.999) and epinephrine (r = 0.999). The average concentration of the 100, 500, or 1000 ng/L norepinephrine and epinephrine standard (Table 1) was not statistically different from the expected value at the 0.01 significance level. The limit of detection was 6 ng/L (n=5) and the limit of quantification is 8 ng/L (n=5) for norepinephrine (Table 2). The limit of detection was 5 ng/L (n=5) and the limit of quantification was 7 ng/L (n=5) for epinephrine (Table 3). The pooled relative standard deviation was 3.8% (n=21) for norepinephrine concentrations (Table 2)

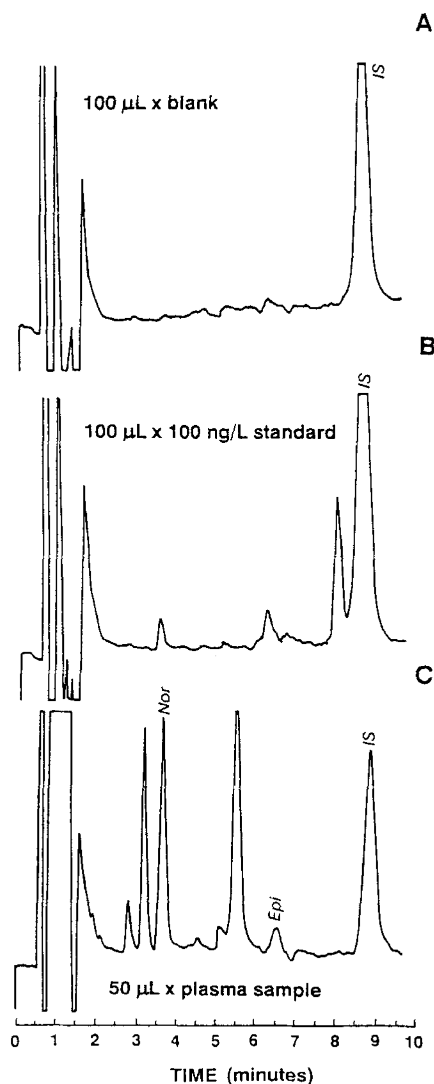

FIGURE 2. Method A Chromatograms: Samples were extracted using alumina and following HPLC separation the compounds were detected amperometrically as described in the methods section. Phosphate buffer blank with internal standard (IS) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Extracted plasma sample taken from a subject after 20 minutes of exercise on the ergometer (C).

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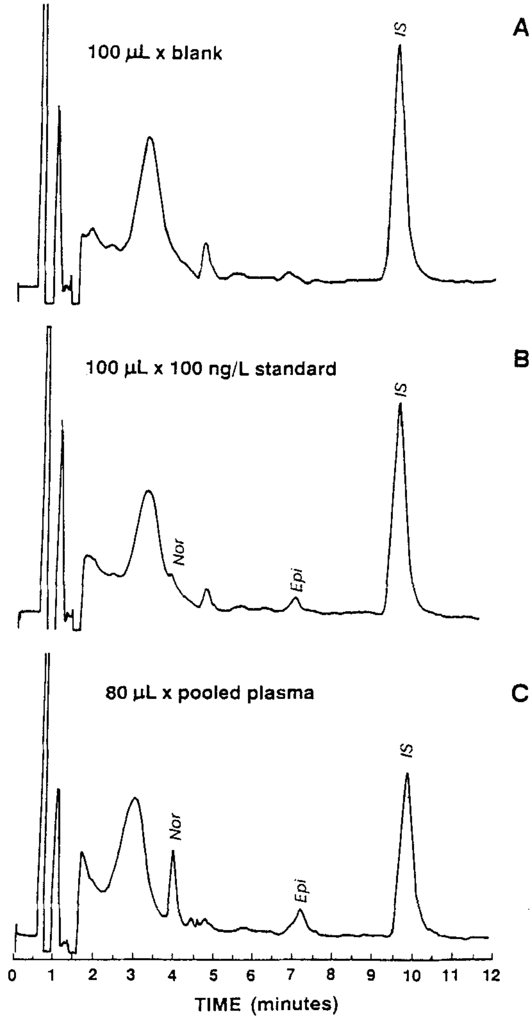

FIGURE 3. Method B Chromatograms: Samples were extracted using strong-cation exchange and then alumina as described in the methods section. Following HPLC separation the compounds were detected amperometrically. Phosphate buffer blank with internal standard (IS) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Extracted pooled plasma-B (Table 1) (C).

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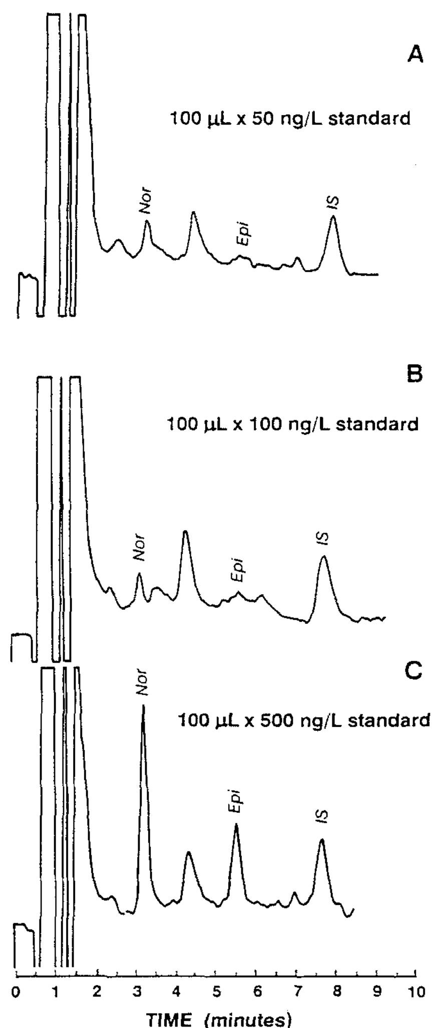

FIGURE 4. Method C Chromatograms: Samples were extracted using weak-cation exchange and following HPLC separation the compounds were detected amperometrically as described in the methods section. Standard containing 50 ng/L of norepinephrine (Nor) and epinephrine (Epi) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Standard containing 500 ng/L of norepinephrine (Nor) and epinephrine (Epi) (C).

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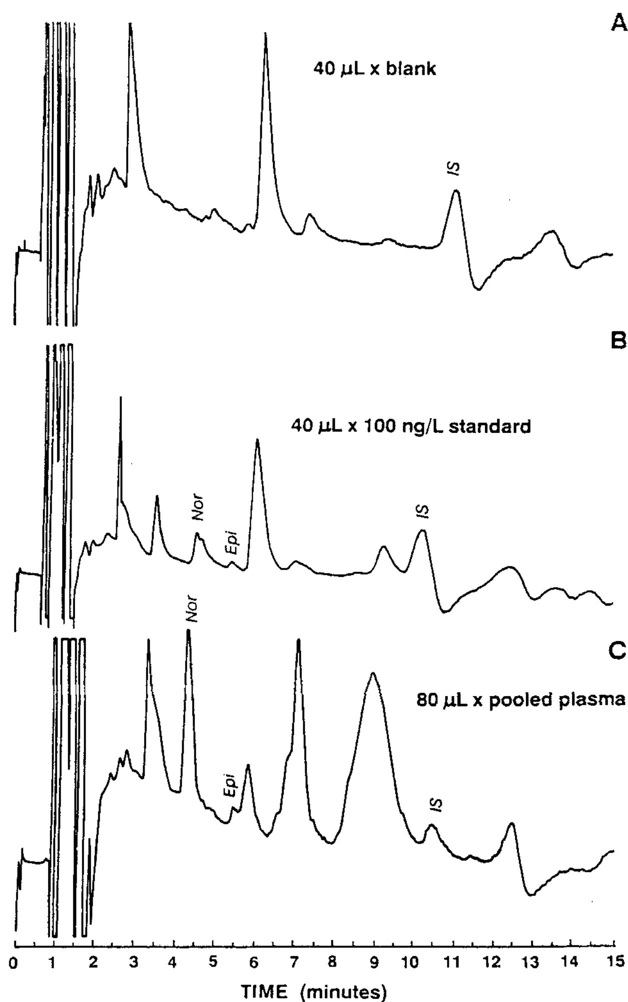

FIGURE 5. Method D Chromatograms: Samples were extracted using alumina and following HPLC separation the compounds were detected coulometrically as described in the methods section. Phosphate buffer blank at twice the gain with internal standard (IS) (A). Standard containing 100 ng/L of norepinephrine (Nor) and epinephrine (Epi) (B). Extracted pooled plasma-A (Table 1) (C).

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and 3.9% (n=23) for epinephrine concentrations (Table 3). The recovery for the aluminum oxide extraction was the same as for Method A. The chromatographic conditions were carefully optimized to prevent compounds from coeluting with norepinephrine and epinephrine (Figure 5).

Summary.

The aluminum oxide extraction required about 1 hour for six samples; however, the combined strong-cation exchange and aluminum oxide extraction required 2 hours to complete for the same number of samples. Given amperometric detection, the advantage to doing the strong-cation/aluminum oxide extraction was that the limit of quantification is lower for norepinephrine and epinephrine and the peak that elutes just before epinephrine was greatly minimized. However, Method B was not further evaluated, because it was too time consuming. Method C was not completely evaluated, because the standard curve was not linear over the required range.

Method D was the best of the four methods described here (Table 2 and 3) for determining plasma norepinephrine and epinephrine concentrations in exercise research samples as well as clinical samples. The aluminum oxide extraction was completed in the shortest amount of time. The limit of quantification for norepinephrine and epinephrine was the lowest. The pooled relative standard deviation was the lowest for norepinephrine and epinephrine. The standard curve was linear over the required range.

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