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Evaluation of a simple plasma catecholamine extraction procedure prior to high-performance liquid chromatography and electrochemical detection

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ABSTRACT

The modified extraction method for catecholamines described in this study is reproducible, simple, rapid, economical and relatively hazard-free. This method is based on the principle that plasma catecholamines are selectively adsorbed on acid-washed alumina at pH 8.6 and then eluted at a pH between 1.0 and 2.0. No statistically significant differences were obtained by using either 0.5 or 1.0 ml of plasma with 0.5 or 1.0 ml of Tris buffer. A 15-min mixing time during the adsorption and desorption steps was found to be practical, but any standardized time up to 1 h can be used. If the washing step was omitted, the catecholamines could not be eluted from the acid-washed alumina. To prevent dilution, the alumina had to be centrifuged and not aspirated to dryness after the washing step. An amount of 50 mg of WA-4 alumina was found to be the most practical in this study. Extracted or unextracted plasma as well as catecholamine standards were stable for four months at −20°C.

INTRODUCTION

Sample clean-up prior to high-performance liquid chromatography with electrochemical detection (HPLC–ED) is necessary for plasma catecholamine determination, because many other components exist in plasma samples that may interfere with the analysis. The most commonly used extraction procedure is based on the principle that the plasma catecholamines are selectively adsorbed on acid-washed alumina at pH 8.6 and eluted at a low pH between 1.0 and 2.0 [1,2]. This principle has been widely applied using different plasma volumes, alumina brands, alumina amounts, buffer mixing times and washing times. Other commonly altered variables include either aspiration or centrifugation of the alumina or the use of different acids (volume, pH and molarities) [1–23]. In 1984, Hjemdahl [13] observed that variability existed both within and between different methods employed within the same laboratory as well as between laboratories.

In our laboratory, we routinely assay for catecholamines in plasma obtained from both humans and animals subjected to various stresses. We therefore had

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the additional problem of not knowing if the constraints of the extraction procedure impose an upper limit on the detection of plasma catecholamines as we expected levels between 10 and 100 times higher than normal, resting, human values.

The aims of this study were three-fold.

(1) To attempt to clarify whether the differences in catecholamine concentrations described in the literature could be due to the amount of alumina, to the quality of the alumina, to the volume of plasma used or to laboratory procedures.

(2) To adopt a consistent procedure for plasma catecholamine extraction.

(3) To determine whether the addition of an antioxidant, such as reduced glutathione, significantly reduces oxidation of the catecholamines between obtaining and centrifugation of the blood sample. Some authors claim the necessity for an antioxidant [8,12,16] whereas others do not [9–11,13,18,21,22].

EXPERIMENTAL

Catecholamine standards were prepared by using a cold 0.1 *M* perchloric acid (pH 1.2) solution as the solvent. The standard concentrations were 0–50 ng/ml for norepinephrine (NE) and epinephrine (E) and 0–100 ng/ml for dopamine (DA) (Sigma, Poole, U.K.). The acid-washed alumina (WA-1 and WA-4) was obtained from Sigma, and the BAS from Bioanalytical Systems (West Lafayette, IN, U.S.A.).

Tris–EDTA (tris[hydroxymethyl]aminomethane–ethylenediaminetetraacetic acid disodium salt) buffer (1.5 *M*; pH 8.6) was prepared by dissolving 45 g of Tris and 5 g of EDTA in 200 ml of glass-distilled water. The pH was adjusted with concentrated hydrochloric acid (Merck, Darmstadt, Germany).

The monochloroacetic acid (MCAA) mobile phase consisted of 1.4% MCAA, 0.47% sodium hydroxide and 0.075% EDTA. The pH was adjusted to 3.0 with either MCAA or sodium hydroxide as required. Prior to use, sodium octylsulphate (6 mg%) was added to the mobile phase. Chemicals were purchased from Merck. The mobile phase was filtered through a 0.4-μm membrane filter and degassed by stirring for 15 min under vacuum.

The same pool of plasma was used as a control throughout this study. The catecholamine content was determined by using a standard catecholamine curve.

Sample collection

Blood was drawn from rabbits and humans into lithium heparin vacutainers and then immediately transferred to a 5-ml tube containing 100 μl of reduced glutathione (4 m*M*) and centrifuged (except where the effect of no added glutathione and delay in centrifugation was tested). Plasma from the rabbits was pooled but the human plasma was not pooled. All plasma was frozen at -20°C within 10 min of blood sampling.

Chromatography

The liquid chromatograph was a Varian Vista 5000 HPLC system (Varian, Walnut Creek, CA, U.S.A.). The MCAA mobile phase was run at a flow-rate of 1.0 ml/min in a closed system. The stationary phase was a 5-μm reversed-phase ODS Spherisorb column (250 mm × 4.6 mm I.D.) with a 5-μm reversed-phase guard column (5.0 mm × 4.6 mm I.D.). The column temperature was maintained at 35°C and the room temperature at 25°C.

An LC-4B amperometric detector set at 1 nA and a TL-5 transducer with a glassy-carbon working electrode were used (Bioanalytical Systems). The applied potential was +650 mV *vs.* Ag/AgCl. A linear 1200 recorder set at 100 mV recorded changes in the electrode potential (Linear Instruments, Reno, NV, U.S.A.). A 100-μl sample containing the extracted catecholamines was injected using a Hamilton syringe into a 100-μl loop fitted onto a Rheodyne injector (Rheodyne, Cotati, CA, U.S.A.).

Modified extraction procedure

The samples were extracted on a minicolumn (65 mm × 15 mm I.D.) with a built-in filter as well as top and bottom caps (Isolab, Akron, OH, U.S.A.) as follows:

(1) Weigh 50 mg of alumina (WA-4).

(2) Add 0.5 ml of plasma, catecholamine standards or controls.

(3) Add 1.0 ml of Tris buffer (pH 8.6, 1.5 *M*).

(4) Rotate for 15 min.

(5) Wash the alumina three times with distilled water, aspirating the alumina to dryness between washes through the bottom of the minicolumns.

(6) Centrifuge the alumina to dryness.

(7) Add 200 μl of perchloric acid (pH 1.2, 0.1 *M*).

(8) Mix and stand for 15 min.

(9) Centrifuge the perchloric acid containing the extracted catecholamines into an Eppendorff tube at 1000 *g* for 3 min (by fitting the end of the minicolumn tightly onto the Eppendorff tube).

(10) Freeze the extract in the Eppendorff tube at −20°C.

Other procedures

This modified extraction procedure was compared with the extraction procedure described by BAS [3]. The major difference between the two methods was that in the former method the entire extraction took place in one minicolumn, whereas in the latter the alumina, plasma and buffer are first rotated in a test-tube. The alumina is then washed in the test-tube by aspirating the supernatant through the top, and the alumina slurry is transferred into an adaptor containing a micro-filter. These extraction methods were compared using the same chromatographic procedure and the same pool of plasma. The modified extraction using the chromatographic procedure was also compared with the radioenzymic assay kit (Cat-a-Kit Code TRK 895, Amersham, U.K.), by assaying the same twenty

human samples, as well as the reference standards included with the radioenzymic kit.

Statistical analysis

The means ($\bar{x}$) and standard deviations (S.D.) were calculated. Statistical analysis was done using a one-way analysis of variance, and significant differences were assessed by using the Student t-test: $p < 0.05$ was regarded as statistically significantly different.

The significance of the correlation coefficient (r) was determined using the t distribution with $n-2$ degrees of freedom.

RESULTS

Comparison between the catecholamine extraction procedure as described by BAS [3] and the modified method developed in this study

No statistically significant differences in the catecholamine concentration were found between the two extraction procedures for the same pool of rabbit plasma (repeated ten times) (Table I). The mean recovery for the modified method was 60.8 ± 5% for norepinephrine, 68.7 ± 3% for epinephrine and 94 ± 5% for dopamine.

Comparison between different volumes of Tris buffer and plasma using 50 mg of alumina

A comparison using different volumes of identical plasma and Tris buffer showed that the epinephrine concentration measured when using 1.0 ml of plasma with 1.0 ml of Tris was significantly increased ($p < 0.001$) when compared with that using 0.25 ml of plasma with 0.25 ml of Tris and decreased ($p < 0.001$) when compared with 0.25 ml of plasma with 0.5 ml of Tris. No other statistically significant differences in the catecholamine concentrations were found (Table II).

TABLE I

COMPARISON BETWEEN THE CATECHOLAMINE EXTRACTION PROCEDURE [3] AND THE MODIFIED METHOD

Compound	Concentration (mean ± S.D.) (ng/ml)	n
Extraction according to ref. 5		
Norepinephrine	7.07 ± 0.82	10
Epinephrine	24.28 ± 1.81	10
Modified extraction		
Norepinephrine	6.99 ± 1.74	10
Epinephrine	23.13 ± 2.23	10

TABLE II

COMPARISON BETWEEN DIFFERENT VOLUMES OF TRIS BUFFER AND PLASMA USING 50 mg OF ALUMINA

Procedure	Concentration (mean ± S.D.) (ng/ml)		*n*
	Norepinephrine	Epinephrine	
(a) 1.0 ml of plasma + 1.0 ml of Tris	6.99 ± 1.74	23.13 ± 2.23[a,b]	10
(b) 0.5 ml of plasma + 0.5 ml of Tris	7.67 ± 0.24	24.82 ± 1.77	9
(c) 0.25 ml of plasma + 0.25 ml of Tris	6.69 ± 0.62	14.24 ± 1.36[a]	10
(d) 0.5 ml of plasma + 1.0 ml of Tris	6.91 ± 0.69	23.94 ± 2.10	10
(e) 0.25 ml of plasma + 0.5 ml of Tris	6.63 ± 0.32	31.24 ± 0.93[b]	9

[a] Epinephrine concentration was significantly decreased ($p < 0.001$).

[b] Epinephrine concentration was significantly increased ($p < 0.001$) between procedures a and e.

Assessment of the adsorption, washing and eluting steps

The catecholamine concentration in 0.5 ml of plasma using 0.5 ml of Tris and 50 mg of alumina was used to determine the effects of different time-periods of adsorption, washing and elution as well as the re-use of the columns.

No statistically significant differences were found between the following procedures for ten replicate analyses.

(1) A 0.5-ml volume of plasma, 0.5 ml of Tris and 50 mg of alumina were allowed to mix during the adsorption step for 15, 30 or 60 min.

(2) Alumina was allowed to stand in water during the washing step for 15, 30 or 60 min.

(3) Elution was carried out with perchloric acid for 15, 30 or 60 min by rotating or standing.

(4) New or re-washed minicolumns were used with new, old or no 0.2-μm filters (an extra micro-filter was attached to the bottom end of the minicolumn).

It is important to note that no peaks were observed on the chromatograms when the alumina was not washed with water after the adsorption step.

A comparison between centrifuging and aspirating the alumina to dryness showed that centrifuging was the better method, because 10–50 μl of fluid could be trapped in the alumina after aspiration.

Comparison between different storage periods at −4°C of 50 mg of alumina with 0.5 ml of Tris before extraction

Storage significantly decreased ($p < 0.001$) the concentration of the epinephrine determined when the plasma was extracted using alumina and Tris that had been stored together for four months at −4°C. Therefore it is desirable to mix the alumina and Tris buffer just prior to the extraction procedure (Table III).

TABLE III

COMPARISON BETWEEN DIFFERENT STORAGE PERIODS OF 50 mg OF ALUMINA WITH 0.5 ml OF TRIS AT −4°C BEFORE ANALYSIS

Storage period	Concentration (mean ± S.D.) (ng/ml)		*n*
	Norepinephrine	Epinephrine	
No storage	7.67 ± 0.24	24.82 ± 1.77[a]	9
24 h	6.17 ± 1.25	19.75 ± 4.26	6
Three weeks	6.73 ± 0.53	23.75 ± 2.36	6
Four months	7.53 ± 2.30	13.98 ± 1.87[a]	5

[a] Epinephrine concentration was significantly decreased ($p < 0.001$) between no storage and a four-month storage.

Comparison between different brands and amounts of alumina using the same pool of standards

The same pool of norepinephrine, epinephrine and dopamine standards was extracted using different amounts and brands of alumina. Catecholamine peak heights (mm) were significantly increased ($p < 0.05$) when 50 mg of BAS alumina was compared with 50 mg of WA-1 and WA-4 for epinephrine and dopamine (Table IV). Standard peak heights (mm) for 100 mg of alumina (WA-1, WA-4 or BAS) were significantly decreased ($p < 0.01$) when compared with 25 mg and 50 mg of alumina (Table IV).

TABLE IV

COMPARISON BETWEEN DIFFERENT AMOUNTS OF ALUMINA

The same pool of catecholamine standards was used and the peak heights were compared. $n = 10$ in all cases.

Brand of alumina	Amount of alumina (mg)	Peak height (mean ± S.D.) (mm)		
		Norepinephrine	Epinephrine	Dopamine
WA-1	25	132.00 ± 11.95	65.10 ± 14.29	93.70 ± 24.90
WA-4	25	133.30 ± 8.17	68.40 ± 7.40	98.00 ± 11.18
BAS	25	145.90 ± 3.28	89.70 ± 6.68	124.10 ± 9.09
WA-1	50	129.90 ± 3.14	60.90 ± 6.05	91.80 ± 9.30
WA-4	50	126.90 ± 7.03	57.80 ± 4.85	87.10 ± 7.20
BAS	50	136.20 ± 8.25	77.00 ± 3.91	114.20 ± 4.09
WA-1	100	27.30 ± 9.96	13.30 ± 4.90	16.00 ± 8.40
WA-4	100	27.63 ± 4.17	12.50 ± 1.93	13.40 ± 2.60
BAS	100	64.70 ± 30.49	24.10 ± 8.04	26.90 ± 14.76

TABLE V

CONSTANTS AND INTERCEPTS OF THE EQUATION OF THE STANDARD CURVES USING DIFFERENT AMOUNTS OF WA-4 ALUMINA

Equation given as $y = ax + b$; $n = 10$ in all cases.

Compound	Amount of alumina (mg)	y-Intercept	Constant (a)
Norepinephrine	10	3.10	13.27
	25	1.48	16.46
	50	−1.48	16.19
	100	−0.74	8.17
Epinephrine	10	1.64	6.47
	25	0.82	7.64
	50	2.89	6.71
	100	0.17	3.79
Dopamine	10	0.43	3.61
	25	1.97	4.26
	50	1.61	3.95
	100	−0.75	2.35

Standard curves using different amounts of WA-4 alumina

Table V indicates that the extraction procedure was less sensitive when 100 mg of WA-4 alumina was used. However, individual standard curves using 10, 25, 50 or 100 mg of alumina showed a significant correlation between peak height (mm) and concentration (ng) with $r = 0.999$ ($p < 0.005$) for norepinephrine, epinephrine and dopamine. The data were best represented by the linear equation $y = ax + b$ (Table V).

TABLE VI

COMPARISON OF THE PLASMA CATECHOLAMINE CONCENTRATIONS BETWEEN DIFFERENT AMOUNTS OF WA-4 ALUMINA

Amount of alumina (mg)	Concentration (mean ± S.D.) (ng/ml)		n
	Norepinephrine	Epinephrine	
10	8.62 ± 0.94	24.42 ± 2.77[a]	6
25	8.93 ± 0.56	22.87 ± 1.88	6
50	8.23 ± 0.79	19.87 ± 1.63	6
100	7.40 ± 0.75	13.17 ± 1.34[a]	6

[a] Epinephrine concentration was significantly decreased ($p < 0.02$) between 100 mg and 10 mg of WA-4 alumina.

Comparison of the plasma catecholamine concentration using different amounts of WA-4 alumina

The plasma catecholamine concentration using the same pool of plasma was determined by using 10, 25, 50 or 100 mg of WA-4 alumina. A decreasing trend in catecholamine concentration was observed between 25 mg of alumina and 100 mg of alumina for norepinephrine. Epinephrine was significantly decreased ($p < 0.02$) between 100 mg of alumina and 10 mg of alumina (Table VI).

Validation of the modified extraction procedure and the chromatographic method used in this study

There were no statistically significant differences between catecholamine concentrations measured using the modified extraction procedure with the HPLC–ED method described in this study and the radioenzymic assay using catecholamine reference standards available with the radioenzymic assay kit and individual plasma samples obtained from twenty humans (Table VII). The values obtained were significantly higher than those quoted in the literature, but this was expected as the blood was drawn from stressed volunteers.

Blood collection and handling for the catecholamine determination

No statistically significant differences in the concentration of catecholamines measured were evident when ten identical plasma samples without glutathione were compared with ten plasma samples containing reduced glutathione over a four-month period. There was also no statistically significant difference in the catecholamine concentration measured when blood samples were centrifuged immediately after sampling or centrifuged up to 30 min later.

Plasma and standard catecholamine storage with and without catecholamine extraction

The catecholamine concentrations of extracted and unextracted plasma (blood taken in reduced glutathione) and standard samples were found to be stable at $-20°C$ over a four-month period. A significant correlation between peak height (mm) and catecholamine concentrations (ng) was obtained with $r = 0.829$–0.989

TABLE VII

COMPARISION BETWEEN THE HPLC–ED METHOD AND THE RADIOENZYMIC ASSAY

Method	Concentration (mean ± S.D.) (ng/ml)			*n*
	Norepinephrine	Epinephrine	Dopamine	
HPLC–ED	1.69 ± 0.56	1.03 ± 0.27	0.52 ± 0.11	20
Radioenzymic	1.37 ± 0.52	1.44 ± 0.58	0.70 ± 0.37	20

TABLE VIII

EFFECT OF TIME ON STORAGE OF PLASMA AND STANDARD CATECHOLAMINE SAMPLES AT −20°C

Storage period	Concentration (mean ± S.D.) (ng/ml)		*n*
	Norepinephrine	Epinephrine	
No storage	7.67 ± 0.24	24.82 ± 1.77[a]	10
Three weeks	6.29 ± 0.08	22.37 ± 0.31	10
Four months	8.39 ± 0.63	20.07 ± 1.79[a]	10

[a] Epinephrine concentration was significantly decreased ($p < 0.001$) between no storage and four months of storage.

($p < 0.05$) between the various determinations done over the time-period (Table VIII).

DISCUSSION

The modified extraction procedure used in this study demonstrated no statistically significant differences when compared to the one described in ref. 3 (Table I). The modified procedure is simple, reproducible and rapid compared with the latter method because the minicolumns consist of one component, whereas the adaptors used in the other method [3] consist of five components that have to be put together. In this method [3] the alumina, plasma and buffer are first rotated in a test-tube. The supernatant is aspirated from the top and the alumina slurry is subsequently transferred to an adaptor. This is a possible source of error, which is eliminated in the modified method.

Anton and Sayre [1,2] established that non-acid-washed alumina resulted in poor recoveries, whereas acid-washed Woelm N acitivity Grade 1 alumina produced good recoveries with norepinephine (66 ± 15%) and epinephrine (67 ± 13%). Mean recoveries obtained with the modified method used in this study were 60.8 ± 5% for norepinephrine, 68.7 ± 3% for epinephrine and 94 ± 5% for dopamine. Anton and Sayre [1], Bouloux *et al.* [5], Causson and Carruthers [6], Carruthers *et al.* [7], Hjemdahl *et al.* [12] and others [3,4] obtained similar recoveries. Bouloux *et al.* [5] demonstrated that acid-washed alumina prepared in the same fashion as that of Anton and Sayre [1,2] compared well with Sigma's WA-1 and WA-4, with the latter producing the best results. Bouloux *et al.* [5] obtained an intra-assay precision of 7.7% for norepinephrine, 8.0% for epinephrine and 9.8% for dopamine. In this study, for amounts of 25 and 50 mg of WA-4 alumina (Table VI), intra-assay precisions of 6–10% for norepinephrine and *ca.* 8% for epinephrine were found.

In this study is was found that increasing the amount of alumina to 100 mg resulted in significant decreases ($p < 0.05$) in the sensitivity of the measurement of standard catecholamine content (Table IV). Anton and Sayre [1,2] used constant amounts of alumina, but Bouloux *et al.* [5] found that the plasma catecholamine recovery was independent of the amount of alumina added. Eriksson and Persson [8] established that the adsorption of the catecholamines on the alumina was linear from 0.1 to 400 pmol. The present study showed that the same pool of plasma with an individual standard curve (0–50 ng/ml) for each amount of alumina showed no stastistically significant difference in the catecholamine concentration when the amount of alumina was between 10 and 50 mg (Table VI). However, a decreasing trend in the norepinephrine concentration was found between 25 and 100 mg of alumina, and the epinephrine concentration was significantly decreased ($p < 0.02$) between 10 and 100 mg of alumina (Table VI). This indicated that sensitivity loss occurred with larger amounts of alumina, even though the standard curves were linear ($r = 0.997$) (Table V). The amounts, as well as the brands, of alumina used by various authors varied. In this study it was found that all extractions should be standardized on a brand as well as an amount of alumina.

Anton and Sayre [1,2], Bouloux *et al.* [5] and BAS [3,4] agreed that the adsorption of the catecholamines on the alumina was optimal in the pH range 8.2–8.9. In this study, pH 8.6 was used for the adsorption of the catecholamines on the alumina.

No standardization of time when mixing the alumina, plasma and Tris buffer was found in the literature. In 1988 it was found [4] that immediate mixing was necessary. Bouloux *et al.* [5] showed that optimal adsorption occurred within 5 min. In this study, no significant difference in catecholamine concentrations was found when the mixing period was 15, 30 or 60 min during the adsorption step.

Different workers have used different volumes and ratios of plasma and Tris buffer [1–23]. In this study, no statistically significant differences were found when using 0.5 or 1.0 ml of plasma with 0.5 or 1.0 ml of Tris buffer. However, the epinephrine concentration measured when 1.0 ml of plasma was used with of 1.0 ml of Tris was significantly increased ($p < 0.001$) when compared with 0.25 ml of plasma and 0.25 ml of Tris, and decreased ($p < 0.001$) when compared with 0.25 ml of plasma and 0.5 ml of Tris (Table II).

No peaks were observed on the chromatograms when the alumina was not washed with water after the desorption step because, in the presence of Tris buffer, the addition of perchloric acid did not lower the pH sufficiently for elution to take place. This agreed with the observation that desorption of catecholamines from the alumina occurred at pH 1.0 [2]. Allowing the alumina to stand for 15, 30 or 60 min with water, as well as washing it three times, did not result in any statistically significant differences in the catecholamine concentrations. The alumina should be washed well with water to remove all traces of the Tris buffer.

Studies on different acids and molarities for desorption of the catecholamines

from the alumina favoured 0.05—0.5 *M* perchloric acid [1–3]. Bouloux *et al.* [5] obtained maximal desorption within 10 s using phosphoric acid (0.25 *M*). In this study, no statistically significant differences were found whether the alumina plus perchloric acid was allowed to stand or was rotated for 15, 30 or 60 min.

The effects of storing alumina plus Tris buffer at −4°C are not clear in the literature. In 1980, Wenk and Greenland [23] indicated that reduced recoveries were obtained by storing the eluent and alumina overnight at −4°C, whereas overnight storage of the eluent at −20°C had no adverse effect on the concentration of the catecholamines measured. Eriksson and Persson [8] showed that the plasma or eluent could be stored at −4°C with or without the acid, provided that no alumina was present. In this study, a significant decrease ($p<0.02$) was obtained for epinephrine concentration when the Tris that was stored with the alumina at −4°C for four months prior to being used was compared with the Tris and alumina added immediately prior to the catecholamine extraction (Table III). No other significant differences were observed when the alumina and Tris were stored at −4°C for shorter periods of time.

In some studies [3,4] the water and perchloric acid were removed from the alumina by centrifugation, whereas other workers aspirated the supernatant. In this study, 10–50 μl of fluid were found to be trapped in the alumina when the supernatant was aspirated. This caused dilution of the catecholamine concentrations and therefore centrifugation was preferred.

The results obtained by different workers with the HPLC–ED and the radioenzymic methods compared favourably [8,9,12,14,15,19,20]. No significant differences in the mean catecholamine concentration of individuals (undergoing the same exercise) and reference standards were obtained between the radioenzymic method and the modified extraction method with the HPLC–ED method in this study (Table VII). However, the radioenzymic method used here was tedious, expensive and hazardous owing to the use of radioactive material.

It is not clear from the literature whether degradation of plasma catecholamines occurs *in vitro*, even though MAO and COMPT are not present in plasma. Bouloux *et al.* [5] and Carruthers *et al.* [7] found that blood samples should be centrifuged immediately in the cold. In this study, no significant differences in the catecholamine concentrations were obtained when the blood was centrifuged immediately or up to 30 min after blood sampling. No significant differences were observed using heparinized blood or heparinized blood containing reduced glutathione (4 m*M*).

Carruthers *et al.* [7] and Hallman *et al.* [11] observed a decrease in the catecholamine concentration when the plasma was stored for several months at −20°C, but Goldstein and co-workers [9,10] did not observe any decreases when samples were stored for nine months in liquid nitrogen. Bouloux *et al.* [5] demonstrated no loss in norepinephrine content, with a 40% decrease in epinephrine concentration over six weeks at −20°C. In the present study, no significant difference in catecholamine concentration was found when the extracted as well as the

non-extracted catecholamine standards and plasma were stored at −20°C for four months (Table VIII).

This study has thus shown that catecholamine extractions can be done simply and quickly with only three critical factors: (i) the brand of alumina; (ii) the amount of alumina; and (iii) the pH of the alumina just prior to the desorption step.

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