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Note

Proposal for the standardization of the calibration method for the assay of plasma catecholamines

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Since 1980, over 2000 publications have dealt with assays of three plasma catecholamines (CAs), norepinephrine (NE), epinephrine (E) and dopamine (DA). In their review of the various assay techniques, Holly and Makin [1] classified and discussed the various methods. The main techniques include high-performance liquid chromatography (HPLC) with fluorimetric detection or electrochemical detection (ED), gas chromatography, and radiochemical–radioenzymic techniques using phenylethanolamine-N-methyltransferase or catechol-O-methyltransferase. HPLC techniques for plasma CA analyses are similar to assays of urinary samples [2,3]. However, direct comparison of results is difficult owing to differences in analytical methodologies used. The HPLC protocols employed for quantitative analysis of plasma CA levels in-

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volve several steps, one of which, the establishment of calibration curves, has not been examined critically. Certain authors calibrate their systems using extraction of aqueous standards, generally in acidic solution, others use spiked pooled plasma. In our opinion these two approaches are not equivalent. We have, therefore, undertaken a study to compare these two procedures by assaying the two types of standard at the same concentrations by HPLC with electrochemical detection.

EXPERIMENTAL

Reagents

Norepinephrine bitartrate, dopamine hydrochloride and alumina acid type WA-1 were obtained from Sigma (St. Louis, MO, U.S.A.), and epinephrine base (E) from Merck (Darmstadt, F.R.G.). The internal standard (I.S.), 3,4-dihydroxybenzylamine hydrobromide (DHBA), was purchased from Aldrich (Milwaukee, WI, U.S.A.) and sodium octane sulphonate from Fluka (Buchs, Switzerland). HPLC-grade methanol was purchased from Carlo Erba (Milan, Italy). All other chemical reagents were obtained from Merck.

Apparatus

The liquid chromatographic system consisted of a Model 114 M Beckman pump (Beckman, Gagny, France), a Model U6K injector (Waters/Millipore, Saint Quentin, France) and a 250×4.6 mm I.D. column of 5 μm average particle size Ultrasphere ODS (Beckman).

A Model 5100 A Coulochem ESA coulometric detector (Cunow, Clichy, France) was equipped with a guard cell (Model 5020) and an analytical cell (Model 5011) with a positive dual potential: the first detector at +0.00 V and the second at +0.27 V. The potential of the guard cell was set at +0.27 V. Calculations were made on the basis of peak heights measured by an electronic integrator (Spectra-Physics, La Verpillère, France).

The mobile phase consisted of a 92:8 (v/v) mixture of 0.1 *M* sodium acetate, 0.05 *M* citric acid containing 50 mg of $EDTANa_2$ and 225 mg of octane sulphonic acid sodium salt per litre (aqueous phase pH 4.8) and methanol. It was filtered through a 0.22-μm membrane filter and degassed prior to use.

Samples

Human plasma was obtained from healthy adults. Blood was drawn into heparinized tubes and centrifuged at 1000 *g* for 10 min. Plasma aliquots were placed in polypropylene tubes and stored at −80°C until analysed.

The 10 m*M* stock solutions of the standards containing NE, E and DA and the internal standard DHBA were prepared in 0.1 *M* perchloric acid; aliquots

were frozen at −20°C for three months; 10 μM solutions were prepared daily in 0.1 M perchloric acid.

For the aqueous standards, 2 ml of triple-distilled water with and without spiking were extracted; for the plasma standards, 2.0 ml of human plasma with and without spiking were extracted (the latter in duplicate). Spiking was performed using a 10 μM solution of the three catecholamines so as to obtain 2, 5, 10, 20, 30 and 50 nM solutions. All assays were performed using a 12 nM solution of internal standard.

Extraction

The extraction procedure was a modification of the techniques of Davis et al. [4] and Mefford et al. [5]: 25 mg of alumina were added to each tube of the series, with 25 pmol of internal standard DHBA. The pH was adjusted to 8.60 ± 0.02 with 800 μl of 1 M Tris–HCl buffer (pH 8.6) containing 2% (w/v) EDTA. The tubes were agitated for 10 min. After rapid sedimentation, the supernatant was discarded and the alumina was washed twice with water. The

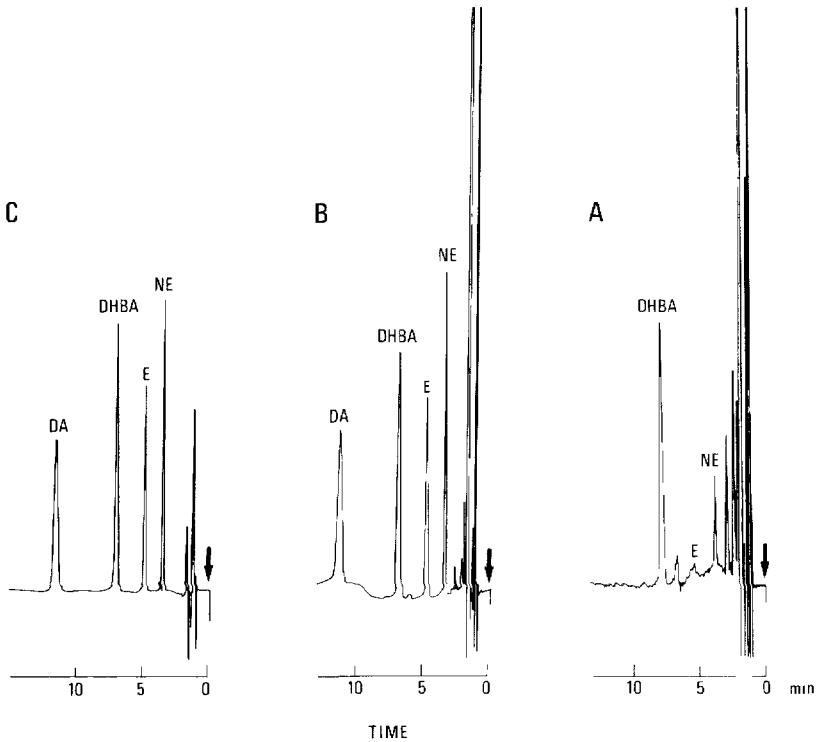

Fig. 1 Chromatograms of human plasma (A), the same plasma spiked with 10 nM standard (B), and the extract of 10 nM aqueous solution of standard (C). Peaks: NE = norepinephrine; E = epinephrine; DHBA = internal standard; DA = dopamine.

catecholamines were desorbed from the alumina using 100 μl of 0.1 M perchloric acid. After 2 min of agitation and centrifugation for 10 min at 3000 g, 50 μl of the clear supernatant were injected.

TABLE I

ABSOLUTE RECOVERIES OF CATECHOLAMINES

Values obtained from an aqueous solution (10 nM) with respect to the non-extracted standards (100 nM in order to take into account the volume ratios).

Compound	Recovery (%)
Norepinephrine	67.6
Epinephrine	68.3
3,4-Dihydroxybenzylamine	68.5
Dopamine	68.6

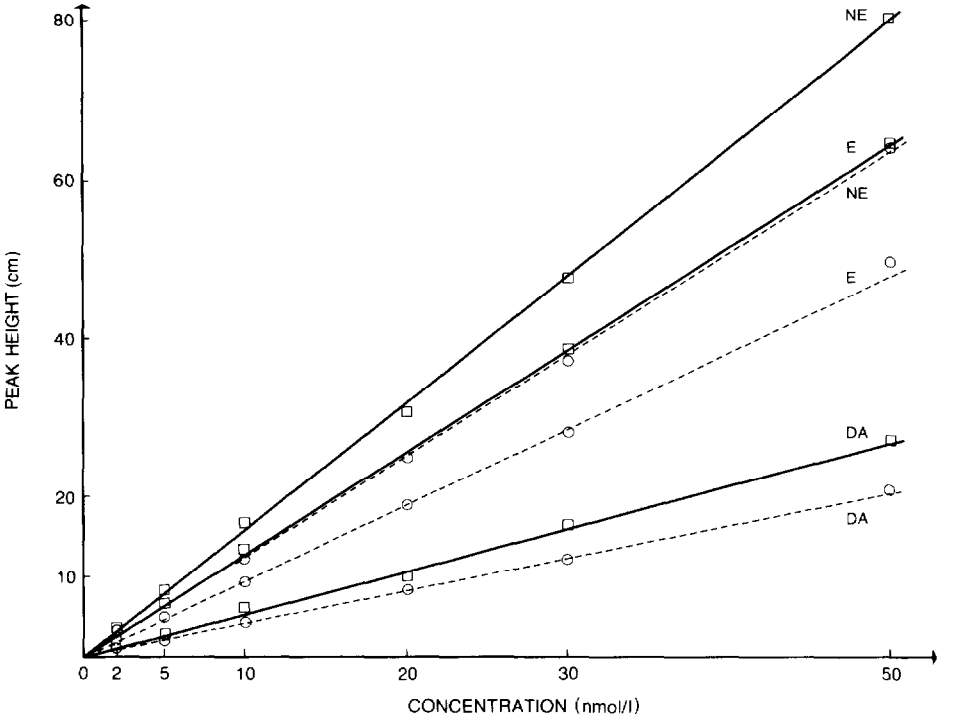

Fig. 2. Calibration curves for the three catecholamines (absolute recoveries): continuous lines are for aqueous standards extracted, and dashed lines for spiked human plasma extracted (values corrected for the endogenous levels). The results are averaged from triplicate analyses.

RESULTS

Fig. 1 shows typical chromatograms of a plasma sample, the same plasma spiked with 10 n*M* standards, and the extract of a 30 n*M* aqueous solution of standards.

The absolute extraction recovery for the aqueous solution of the three catecholamines and the internal standard was 65–70% (Table I) depending on the concentration studied. However, for the same concentration, the results for a given catecholamine did not differ by more than 2%. These values were obtained by comparing the heights of the peaks in the extracted sample with those of the non-extracted mixture of the corresponding concentration. Fig. 2 shows the calibration curves for the aqueous standards and spiked plasma samples in terms of absolute recoveries. The curves corresponding to the relative recoveries (with respect to DHBA) are given in Fig. 3 and the linear regression equations in Table II. Quantification was based on the use of peak heights.

The calibration curves for the extracts of aqueous standards and the spiked plasma obviously differ in terms of both absolute and relative recoveries. The extraction efficiencies for aqueous standards were always higher than those for the spiked plasma samples. The slopes of the calibration extracts using spiked plasma were lower than those for the aqueous standards: 92%, 84% and 84%, respectively, for NE, E and DA. The use of the latter values always resulted in erroneously high levels. For example, the following concentrations were found for an unknown plasma sample: 109.0% for NE, 117.0% for E and 116.3% for DA. The difference was most marked for E.

DISCUSSION

Radioenzymic techniques are both sensitive and specific for CA assays. However, lower cost and increasing simplicity of operation have prompted interest in HPLC analyses. The use of the internal standard DHBA generally corrects for losses during extraction. Bouloux et al. [7] described a systematic investigation of some of the common problems encountered in adsorption and desorption of CAs from plasma on aluminas, the most common extraction method used for the HPLC analysis of CAs. This extraction step may be preceded by deproteinization; both strategies have been envisaged by Davis et al. [4]. It may constitute the sole purification step prior to injection into a chromatographic apparatus [8], or extraction may be preceded by purification on ion-exchange resin [9] or boric acid gel [10].

The multiplicity of techniques and modifications of methods reported for plasma CA assays reflects the persistence of various problems. HPLC–ED has given results similar to those obtained by gas chromatography–mass spectrometry (for the determination of brain CAs) [11], and the comparison of the HPLC–ED results with those obtained by radioenzymic analysis (REA) re-

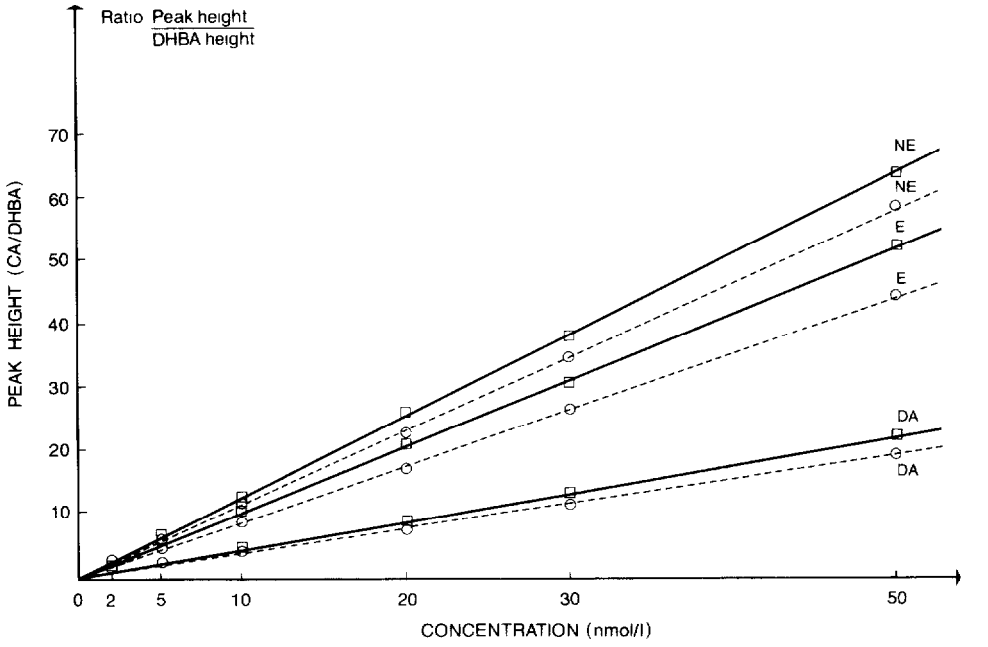

Fig. 3. Calibration curves for the three catecholamines (relative recoveries): continuous lines are for aqueous standards extracted, and dashed lines for spiked plasma extracted (values corrected for the endogenous levels). The results are averaged from triplicate analyses.

TABLE II

LINEAR REGRESSION EQUATIONS

	Equation	Correlation coefficient, r
NE		
Aqueous standard	$y=0.127x+0.0185$	0.9999
Plasma	$y=0.117x-0.00157$	0.9997
E		
Aqueous standard	$y=0.099x+0.145$	0.9984
Plasma	$y=0.0836x+0.0411$	0.9994
DA		
Aqueous standard	$y=0.0454x-0.00839$	0.9998
Plasma	$y=0.0383x+0\ 00361$	0 9995

vealed excellent correlation [12,13]. Nevertheless, the results obtained with the latter techniques are at variance with those reported by Causon et al. [14], who found that te REA values were generally higher than those obtained by HPLC–ED.

However, along with these correlations, Hjemdahl [15] described an inter-laboratory comparison of various plasma CA assays and protocols that revealed considerable problems [15,16]. This is probably due to the calibration since these authors used the recovery results for E and NE added to the basal plasma pool. In the different techniques used by the various laboratories cited, calibration was carried out with a mixture of standards in aqueous solution [8, 9, 16–21], with 0.1 *M* phosphate buffer (pH 7.0) [4], or with spiked plasma pools [22–25].

In addition to the three main CAs, Mefford et al. [5] also assayed dihydroxyphenylethylene glycol (DOPAC), which is unstable in the extracts, and calibrated their method with respect to the extraction yield of the internal standard. Plasma spiking resulted in recoveries of only 75.4±7.7% for NE, 72.2±3.8% for E, and 68.2±5.4% for DA. This could be due to CA binding to plasma proteins, especially albumin and alpha-1 and alpha-2 globulins [26–29]. These bound CAs would then escape extraction. Thus the volume of the basal plasma pool used for the calibration curve is important. In order to verify this, we conducted a series of experiments in which various volumes of plasma were spiked with the same amount of standards. The desorption was carried out as indicated in Experimental. The results (Table III) show that recoveries (calculated with respect to DHBA) decrease significantly with increasing plasma volume. Since 50 mg of alumina were used for the extraction of 4 ml of plasma, the other reagents being varied to maintain the protocol ratios, the results obtained cannot be explained by the saturation of the active sites on alumina.

The results discussed in this paper demonstrate that the two calibration methods are not identical: the slope of the calibration curve obtained with spiked plasma was lower and thus gave higher concentrations than those obtained with spiked water or a buffer. The average height of the DHBA peak was found to be 12.6 cm in the extract of the aqueous solution and 10.9 cm in the extract of spiked plasma, i.e. 86% recovery. The respective absolute recov-

TABLE III

PERCENTAGE RECOVERIES OF CATECHOLAMINES FROM DIFFERENT VOLUMES OF PLASMA SPIKED WITH THE SAME AMOUNT OF STANDARDS

Compound	Recovery (%)		
	1.0 ml	2.0 ml	4.0 ml
Norepinephrine	99.7	93.3	92.2
Epinephrine	99.6	92.6	86.8
Dopamine	108 0	102.0	101.0

eries of NE, E and DA were 74.5%, 81.3% and 77.4%. These values, and the comparison of Figs. 2 and 3, demonstrate that:

(1) The extent of adsorption of CAs and the internal standard on plasma proteins is not identical.

(2) The use of DHBA as the internal standard corrects only partially for the adsorption of CAs on plasma proteins since it is adsorbed to a lesser extent. Thus DHBA is not an ideal internal standard when spiked plasma extracts are used. However, its use is justified when extracts of aqueous standards are used for calibration.

The differences observed cannot, in our opinion, be entirely explained by the "volume displacement" error due to the volume occupied by the protein itself, which may result in slightly increased concentrations of CAs in protein-free filtrates [30].

The findings discussed in this paper may help to explain interlaboratory variations in plasma CA levels. In conclusion, the results of this study are in favour of the use of calibration with aqueous standards rather than spiked plasma samples for the analysis of plasma catecholamines.

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