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IMPROVED QUANTIFICATION OF PLASMA CATECHOLAMINES BY THE
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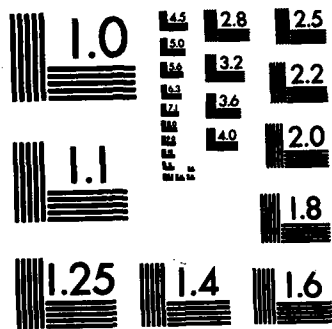
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IMPROVED QUANTIFICATION OF PLASMA CATECHOLAMINES BY THE RADIOENZYMIC KIT METHOD

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James P. Ellis, Jr., Ph.D.
John W. Burns, Ph.D.

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USAF SCHOOL OF AEROSPACE MEDICINE
Aerospace Medical Division (AFSC)
Brooks Air Force Base, Texas 78235



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The Office of Public Affairs has reviewed this report, and it is releasable to the National Technical Information Service, where it will be available to the general public, including foreign nationals.

This report has been reviewed and is approved for publication.

James P. Ellis, Jr.
JAMES P. ELLIS, JR., Ph.D.
Project Scientist

Raymond G. Troller
RAYMOND G. TROLLER, Colonel, USAF, MC
Supervisor

Roy L. Dehart
ROY L. DEHART
Colonel, USAF, MC
Commander

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ing; (a) quantification of certain plasma samples which could not be satisfactorily quantified by conventional options, (b) an apparent gain in analytical precision in virtually all other analyses, and (c) a gain in economy of approximately 25%.

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IMPROVED QUANTIFICATION OF PLASMA CATECHOLAMINES
BY THE RADIOENZYMIC KIT METHOD

INTRODUCTION

A recent literature search of procedures currently used to quantify catecholamines in physiological fluids revealed widespread use of the radioenzymic assay described by Peuler and Johnson (1). Popularity of the Peuler-Johnson procedure may be due, in part, to its use in conjunction with the commercial kit marketed by Upjohn Diagnostics under the tradename CAT-A-KIT. In the Upjohn procedure (2) each "unknown" and control specimen is analyzed with and without addition of an internal standard; this requirement virtually doubles laboratory costs of analysis. The latter feature is emphasized here for two reasons. First, although Peuler and Johnson specified inclusion of internal standards for each and every specimen, the necessity for that usage was not actually documented (1). Second, we encountered a problem using the Peuler-Johnson procedure for measuring catecholamine levels in plasma from severely stressed swine and found that a satisfactory solution was to depart from the prescribed use of internal standards. In addition to facilitating the analysis of problematic swine plasma, this revised procedure has other important benefits applicable to catecholamine analyses in clinical chemistry.

In view of the foregoing, the purpose of this report includes the following: (a) to recount difficulties we initially encountered in the prescribed use of CAT-A-KITs for swine plasma analyses, (b) to document our unsuccessful attempts to overcome those difficulties by options suggested in procedural notes (2) furnished with each CAT-A-KIT, (c) to describe our departure from the prescribed procedure, (d) to validate that departure through intermethod/interlaboratory comparisons, (e) to demonstrate how the revised procedure might also impact favorably on human catecholamine analyses, and (f) to suggest benefits that derive from use of the revised procedure.

MATERIALS AND METHODS

Radioenzymic Assays

Except for the revised handling of internal standards detailed in this report, our use of CAT-A-KITs for plasma epinephrine (E) and norepinephrine (NE) analyses was in accord with instructions furnished by Upjohn Diagnostics (Kalamazoo, Mich.). Briefly, those instructions (2) specify the following procedural steps: (a) the appropriate addition of plasma and solutions to assay tubes so as to give duplicate "blank" tubes and duplicate "sample" and "sample + standard" tubes for each control and unknown plasma, (b) addition to each tube of a buffered reaction mixture containing tritiated methylating agent [(^3H -methyl) S-adenosyl-L-methionine, or ^3H -SAM] and methylating enzyme (catechol-O-methyltransferase, or COMT), (c) incubation of all tubes at 37°C for 60 min, (d) removal of interfering substances by solvent extraction, (e) separation of the tritiated methoxy derivatives (metanephrines) by thin-

layer chromatography (TLC), (f) transfer of the TLC zones to scintillation vials, (g) elution of the metanephrines from the silica gel, followed by their oxidation to ³H-vanillin, (h) extraction of the ³H-vanillin into scintillation counting fluid, (i) measurement of the radioactivity of each vial, and (j) calculation of catecholamine concentrations of each plasma through use of the following equation:

$$\left[\frac{\text{cpm "sample" - cpm "blank"}}{\text{cpm "sample + standard" - cpm "sample"}} \right] \times \frac{\text{quantity "standard"}}{\text{volume "sample"}} \quad (1)$$

In equation (1), the quantity of each catecholamine is usually 100 pg and the volume of plasma is 0.050 ml; concentrations are pg/ml. For present terminology, the numerator and denominator of the bracketed portion of equation (1) are denoted "net plasma" (or NP) and "net standard" (or NS), respectively. Also, radioactivity measurements are herein expressed as dpm.

Our departure from the Upjohn procedure (2) was not in the procedure per se, but in the handling of the individual NSs within a given assay. Namely, instead of dividing the NP of a sample by its respective NS, a single, composite NS was derived from the individual NSs in each assay for each catecholamine. The composite NS was denoted "mean net standard" (or MNS) and was computed in the following manner: (a) the mean and standard deviation (S.D.) of the individual NSs in a given assay (except controls) were determined, (b) those NSs which fell more than 1 S.D. away from the mean were rejected, and (c) the remaining ones were averaged to give the MNS. To complete the calculation of final catecholamine concentrations, each NP was divided by the MNS and the quotient multiplied by the unbracketed factors of equation (1).

To validate use of the MNS concept, simultaneous intermethod/interlaboratory analyses were performed on a set of 48 plasma samples previously obtained from acceleration-stressed swine. Comparisons were limited to plasma NE determinations since the radioenzymic procedure (3) used in the second laboratory was based on the highly specific methylation of NE by the enzyme phenylethanolamine-N-methyltransferase (PNMT).

Plasma Samples

Catecholamine data reported here were either extracted from two recently concluded experimental studies (4, 5) or obtained by further analysis of plasma collected from those studies. In those studies, human subjects were tested at relatively mild exercise levels (4) and miniature swine were exposed to rather high acceleration (G) levels (5).

For special analyses reported here, three pools (denoted S-I, S-II, and S-III) of swine plasma were prepared so as to give strikingly different catecholamine concentrations. Importantly, those pools were not prepared by spiking plasma with catecholamine mixtures, but by combining plasma samples obtained from stressed and unstressed swine.

RESULTS

Initial Analytical Difficulties

In our initial attempt to use CAT-A-KITs to measure NE and E levels in plasma of G-stressed swine, we encountered marked variation of NSs in certain specimens, most frequently and most markedly when "apparent" catecholamine concentrations exceeded 10 ng/ml. Variations were of two types, with some NSs being very low (even negative) and others very high. As might be expected, rather bizarre plasma concentrations of catecholamines resulted from calculations.

Unsuccessful Attempts to Correct Problem by Conventional Means

Procedural notes (2) accompanying each CAT-A-KIT specified two options for improving the analysis of specimens having high catecholamine concentrations: to dilute the specimen and to increase the quantity of standards. To ascertain whether either of those options might improve quantification of the problematic plasma, we prepared and used three pools of swine plasma with widely differing catecholamine concentrations. Those assessments included the following analyses: (a) undiluted aliquots of all three pools, (b) 1:3 dilution of the two pools having high catecholamine concentrations (pools S-II and S-III), and (c) all diluted and undiluted aliquots at two levels of standards (i.e., 500 pg and the prescribed 100 pg). In addition to those analyses, undiluted aliquots of pools S-II and S-III were analyzed a second time 2 days later to ascertain interassay variation of NP and NS measurements.

In Table 1, data under the NP and NS columns show the effects of specimen dilution and enriched standards, respectively; data within parentheses reflect interassay variation. With respect to sample dilution, expression of NPs as dpm/ μ l plasma shows that dilution did not result in a proportionate decrease in radioactivity. The disparity was especially marked for the E assay of pool S-II (which was 42% higher than expected), but was substantial for the other three examples (higher by 15% to 18%). The finding of disproportionately higher NPs in diluted samples has since been repeatedly confirmed in our laboratory.

An examination of the NSs in Table 1 reveals that use of a greater quantity of standard did not improve the quantification of E and NE in pools S-II and S-III. For example, NSs of both catecholamines were not only erratic at both quantity levels in diluted as well as undiluted aliquots of pools S-II and S-III, but the NSs found upon reanalysis of undiluted samples were strikingly different from those found upon first analysis. Although the NSs in those repeat analyses were not greatly different from the presumably acceptable NSs found for pool S-I (i.e., having NE and E concentrations that were within the normal range for swine), they were adjudged too variable to be quantitatively sound.

In Table 1 it is also noted that interassay duplication of the exceptionally high NPs of pools S-II and S-III was not only much better than that of the respective NSs, but was well within the limits specified by Upjohn Diagnostics (2) for its control plasma (which typically give NPs much lower than those of

TABLE 1. RADIOACTIVITIES OF INTERNAL CATECHOLAMINE STANDARDS
I. EFFECTS OF SAMPLE DILUTION AND ENRICHED STANDARDS

Swine Pool	Sample dilution	NP (dpm)		NS (dpm/pg)	
		(per vial)	(per μ l P) ^b	(100 pg)	(500 pg)
<u>Norepinephrine</u>					
S-I	Undiluted	788	16	36.6	37.5
S-II	Undiluted	16,187 (14,499) ^a	324 (290)	16.0 (30.4)	20.2 (29.3)
	1:3 Dil.	6,371	382	46.5	44.0
S-III	Undiluted	57,486 (55,984)	1,150 (1,120)	16.9 (36.4)	6.1 (33.7)
	1:3 Dil.	22,066	1,324	22.2	45.4
<u>Epinephrine</u>					
S-I	Undiluted	1,005	20	54.7	53.2
S-II	Undiluted	2,506 (2,132)	50 (43)	46.5 (38.4)	39.5 (39.9)
	1:3 Dil.	1,182	71	60.0	62.3
S-III	Undiluted	32,521 (31,659)	650 (633)	42.9 (58.2)	33.2 (42.9)
	1:3 Dil.	12,416	745	44.5	64.1

^a Values in parentheses obtained from a second assay.

^b P denotes plasma.

pools S-II and S-III). In this connection, it is noteworthy to mention that our frequent finding of good interassay duplication of exceptionally high NPs suggested the soundness of those values and pointed to the possibility that, in instances of a plasma having an obviously aberrant NS, it might be feasible to substitute a NS derived from other samples analyzed at the same time.

Validation of MNS Usage

Results obtained from the intermethod analysis of plasma NE are summarized in Figure 1. As shown in the upper graph of the figure, NE concentrations determined by the COMT procedure (1, 2) in our laboratory and computed from MNSs

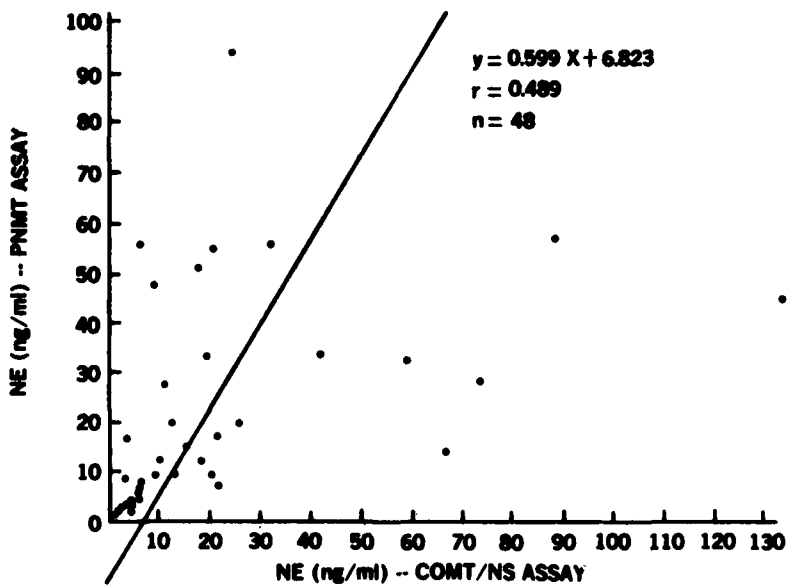
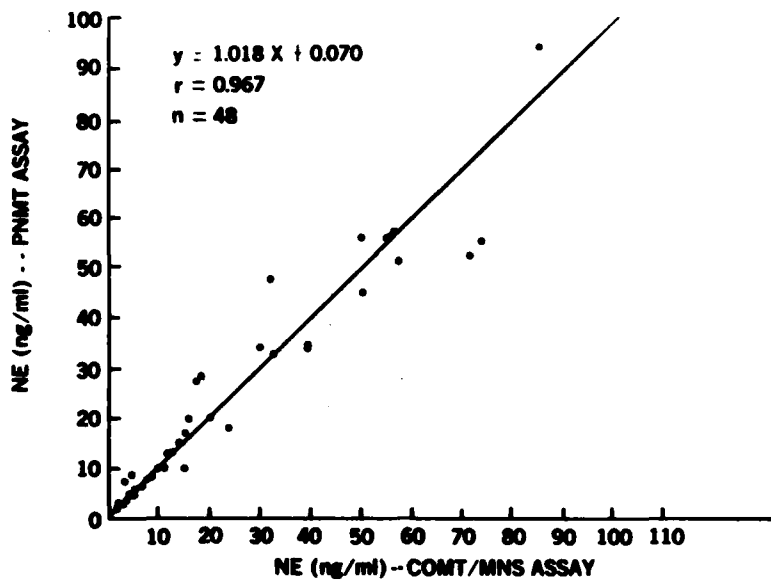


Figure 1. Interlaboratory comparisons of plasma NE determinations. Plasma NE concentrations determined by PNMT- versus COMT-catalyzed enzymic reactions. COMT/NS and COMT/MNS denote computations based on prescribed (1) and modified procedures, respectively. The diagonal line is regression.

(i.e., COMT/MNS) correlated quite closely with those determined by the PNMT procedure ($r=0.967$). In sharp contrast, NE concentrations computed in the conventional manner (2) correlated rather poorly with PNMT-based values ($r=0.489$). Interestingly, the opposing influences of very low and very high NSs on computed catecholamine levels are clearly reflected in the lower graph of Figure 1 by the wide scattering of values on both sides of the regression line.

Data from the intermethod comparisons validated use of a MNS not only for those specimens within an assay having an obviously aberrant NS, but also for samples within an assay having a presumably acceptable NS. Although not discernible from comparisons in Figure 1, several plasma samples having a NS that fell within 1 S.D. of the MNS gave NS-computed concentrations that differed substantially from MNS-computed values. Moreover, in those instances, the COMT/MNS values agreed closely with PNMT values. These findings suggest that the marked interplasma variation of NSs may not principally be due to inherent differences between plasma samples per se, but to analytical variation.

Typical Analysis of Human and Swine Plasma

Catecholamine data summarized in Table 2 illustrate computations of MNSs for bonafide sets of human and swine plasma and demonstrate the effectiveness of their use in comparison with the conventional use of individual NSs (1,2). As a matter of general interest, the set of swine samples was collected over a period of about 30 min, during which time the healthy swine was briefly exposed to +7 G_z (test A) and, a few minutes later, to +5 G_z (test B); samples 1-4 were obtained immediately before G, during G, immediately after G, and 2 min later. The human samples were drawn over a period of approximately 90 min from a healthy man immediately before (#1) and at the peak (#2) of four relatively mild levels of treadmill exercise.

With respect to the swine data in Table 2, it was not surprising to find the need to reject three or four NSs from the final MNS computation. However, the finding that one of the divergent E standards occurred in a baseline sample clearly indicated that aberrancy among NSs was not due entirely to high catecholamine levels. Moreover, the same finding points out just as clearly the need for an intervention such as the MNS.

Another interesting observation of the swine data in Table 2 is that, although the individual NSs differed substantially from one sample to the next, the differences did not follow a consistent sample-to-sample pattern. On the contrary, variations among NSs were quite erratic. This observation points further to the likelihood that the variations were largely analytical, not biological, in nature.

Similar conclusions concerning variation of NSs may be drawn from the example of human data given in Table 2. However, because the exercise-induced increases in catecholamine levels of human plasma were so slight in comparison with the G-induced increases in swine plasma, the human data illustrate more clearly the need for modifying the prescribed usage (1, 2) of NSs. For example, from NE analyses of the two test A samples, the NPs suggest an increase of more than 20%; however, the NS-computed concentrations of NE suggest an increase of less than 10%. The reason for the disparity is quite obvious; the NSs of the

TABLE 2. TYPICAL ANALYSES OF HUMAN AND SWINE PLASMA

Sample identification	NE				E				
	radioactivity		conc. (pg/ml)		radioactivity		conc. (pg/ml)		
	NP (dpm/tube)	NS (dpm/pg)	using NS	using MNS	NP (dpm/tube)	NS (dpm/pg)	using NS	using MNS	
Swine #52 baseline	1,075	38.39	560	563	1,188	48.40 ^a	491	574	
test A	1	1,298	37.36	695	679	1,462	41.26	709	706
	2	33,592	41.77	16,084	17,583	12,923	40.40	6,398	6,241
	3	75,302	25.10 ^a	60,002	39,415	32,121	13 ^a	12,815	15,514
	4	10,406	39.99	5,204	5,447	4,858	.14	2,362	2,346
test B	1	2,842	37.49	1,516	1,488	1,962	82	895	948
	2	12,349	46.58 ^a	5,302	6,464	5,698	63 ^a	2,343	2,752
	3	13,377	34.24	7,814	7,002	3,906		1,932	1,886
	4	3,893	29.94 ^a	2,600	2,038	2,450	9 ^a	1,361	1,183
Initial MNS (+S.D.)		36.76 (+6.37)				43.36 (+4.74)			
Final MNS (+S.D.)		38.21 (+2.56)				41.41 (+1.40)			
Human DR baseline	959	51.72 ^a	371	410	186	43.82	85	84	
test A	1	1,107	42.66 ^a	519	474	228	40.92	111	102
	2	1,365	49.02	557	584	271	45.68	119	122
test B	1	1,086	43.90	495	465	190	39.54 ^a	96	85
	2	1,844	48.20	765	789	514	51.38 ^a	200	231
test C	1	1,549	47.00	659	663	233	42.92	108	105
	2	1,949	42.99 ^a	907	834	392	39.12 ^a	200	176
test D	1	1,207	47.68	506	517	307	47.93	128	138
	2	3,383	45.50	1,487	1,448	522	45.97	227	234
Initial MNS (+S.D.)		46.52 (+3.02)				44.14 (+4.04)			
Final MNS (+S.D.)		46.72 (+1.79)				44.54 (+2.49)			

^aExcluded from computation of Final MNS.

two samples were markedly different (almost representing the extremes for the nine determinations).

Intrasubject versus Intersubject Variation in NSs

Although not mentioned earlier, each MNS used for calculating COMT/MNS catecholamine concentrations in the intermethod appraisal (Fig. 1) was derived from NSs of plasma from the same animal since only samples from one animal were analyzed at a time. Consequently, that appraisal did not validate use of a single MNS for computing catecholamine levels in plasma from different animals. On the issue of intra- and intersubject variations in internal catecholamine standards, Peuler and Johnson (1) pointed out that variations in the inhibitory capability of plasma were less marked between samples from the same individual than between samples from different individuals. Although those investigators did not document those differences in their report (1), their assertions nevertheless raised a serious question on the validity of deriving a MNS from NSs of plasma from different individuals. For this reason, we deemed it necessary to ascertain whether intersubject variation in NSs was substantially greater than the relatively small intrasubject variation we had consistently found theretofore.

Standardization data obtained in connection with our recently concluded exercise study (4) seemed ideally suited for comparing intra- and intersubject differences in NSs, principally because that study featured the collection of 9 blood samples from each of 9 experimental subjects. Those data are summarized in Table 3. The notation mNS (not MNS) signifies usage of initial (not final) MNSs, as required for the desired comparisons.

A particularly noteworthy observation from data in Table 3 is that coefficients of variation (CV) for intersubject variations in mNSs were not strikingly different from CVs for intrasubject variations in NSs. As a matter of fact, for NE, intersubject variation was substantially less than the average intrasubject variation.

The NSs of control specimens are included in Table 3 for two reasons. First, the finding that interassay CVs for those radioactivities were comparable to the intersubject (interassay) CVs for the mNSs suggests the latter variations were largely analytical in nature--perhaps more so than for intrasubject (intra-assay) variation. Second, as data from each subject are tabulated in the order of sample analysis, the control data demonstrate the possible encounter of a technical problem starting with the assay of samples from subject SR. For example, for the last four subjects (Table 3), the mNSs of unknowns and NSs of controls not only tended to be lower for both catecholamines, but the ones for E were distinctly and decisively lower than the corresponding ones for NE; for equal quantities, radioactivities are typically higher for E than for NE (1, 2).

DISCUSSION

In the present study, we did not exhaustively evaluate the two options suggested by Upjohn Diagnostics (2) for overcoming the difficulties we had

TABLE 3. RADIOACTIVITIES OF INTERNAL CATECHOLAMINE STANDARDS
 II. INTRA- AND INTERSUBJECT VARIATIONS IN HUMAN PLASMA

Subject I.D.	Plasma (n)	NE			E			NS ^a of controls			
		mNS ^a	S.D. ^a	CV(%)	mNS ^a	S.D. ^a	CV(%)	NE	E		
JS	9	48.78	3.30	6.76	48.01	3.80	7.91	57.14	59.76		
JM	9	52.90	1.80	3.40	58.82	2.01	3.41	58.58	64.02		
RG	9	52.49	3.51	6.69	55.32	6.69	12.10	61.52	59.61		
FR	9	52.59	5.02	9.54	49.03	5.87	11.98	58.22	59.23		
DB	9	51.09	3.40	6.66	49.54	1.81	3.66	58.18	59.36		
SR	9	49.34	4.66	9.45	46.61	5.05	10.84	55.60	51.14		
JW	9	51.86	1.98	3.82	47.85	3.56	7.43	56.76	49.74		
DR	9	46.52	3.02	6.49	44.14	4.04	9.16	50.66	45.64		
LP ^b	5	51.43	3.36	6.54	44.10	5.13	11.63	59.74	54.62		
Intrasubject average				6.59				8.68			
Intersubject mean		50.78				49.27				57.38	55.90
S.D.		2.14				4.89				3.05	5.97
CV(%)		4.21				9.93				5.31	10.68

^aValues are dpm/pg.

^bSubject did not complete experiment.

encountered in quantifying catecholamines in the plasma of severely stressed swine. Neither did we validate the preferential use of MNSs as thoroughly as we would have preferred. Yet, we believe sufficient information was obtained on both of those important issues.

Even had sample dilution proved a suitable solution to our problem, we might still have looked upon it with disfavor, largely because of its requirement for repeat analyses. The latter factor can be a decisive one for assays as costly and as time-consuming to perform as the COMT-based assay (1, 2).

We believe data summarized in Figure 1 unequivocally demonstrate the need for screening NSs and for implementing the use of a mean internal standard of some sort. We do not believe it essential, however, to reject NSs solely on

the basis of exceeding the initially computed mean by more than 1 S.D.; that choice, in our opinion, should rest with the individual investigator. Actually, the 1 S.D. limit suggested here was the third such rejection principle which we examined. We first examined the feasibility of using fixed acceptance limits which did not vary from one assay to the next and which were arbitrarily set from accumulated data. We then adopted the more statistically acceptable principle of rejecting NSs which fell more than 2 S.D. from the initially computed mean. We found that principle well suited for analyses of human plasma; however, the infrequent finding in swine plasma analyses that too wide a range of "acceptable" NSs was used for computing final MNSs prompted abandonment of the 2 S.D. principle and adoption of the one specified here. To illustrate the difference in rejecting NSs on the two latter bases from the typical analyses given in Table 2, it may be readily deduced from the four sets of NSs that not one NS fell more than 2 S.D. from its respective mean. That finding alone is quite acceptable; however, we believe it less acceptable to use a standard derived from individual values which vary within so wide a range as 25.10 - 46.58 dpm/pg (e.g., swine plasma NE).

We recognize the strong possibility that usage of a MNS may be limited. For example, our experience has been restricted to analyses of plasma from healthy human and animal subjects. Plasma from unhealthy subjects might have widely differing inhibitory capabilities, which would likely preclude use of a MNS. Too, although it appears that computation of a MNS from NSs of different individuals is valid, the same may not be true for combining NSs of different species.

Undoubtedly the greatest benefit we have realized from use of MNSs is the analysis of plasma which would otherwise be unquantifiable by the Upjohn procedure (2). In this connection, although the lower graph of Figure 1 shows widely divergent COMT/NS values of NE, it does not (indeed, cannot) show the extreme cases which we encountered--cases involving, for example, a negative NS.

But even for those analyses in which a NS was not obviously aberrant, we believe that use of the MNS principle was beneficial as it probably enhanced analytical precision to a substantial degree. Two factors support that belief. First, from a purely theoretical point of view, it is clear from equation (1) that NSs figure just as decisively as NPs in calculation of catecholamine concentrations. Second, although not documented here, we have consistently found substantially less intra-assay variation in NPs than in NSs. Interestingly, the latter observation is supported by the example of "typical data" which has appeared in every brochure (2) from Upjohn Diagnostics that we have received over the past four years. In that example (2), if one were to take the duplicate radioactivities and re-express them as NPs and NSs, intra-assay CVs for the NPs of E and NE would be 5% and 8%, respectively, whereas CVs for the respective NSs would be 8% and 18%. In their evaluation of CAT-A-KITs, Tasseron et al. (6) also found intra-assay (within-run) CVs of radioactivities for both catecholamines to be at least twice as high for "sample + standard" as for "sample" alone.

Greater economy of operation has been a more recent benefit which we have realized since implementing the MNS principle. The savings derive partly by singly rather than doubly analyzing each internal standard and partly by

utilizing the assay tubes thereby liberated for the analysis of additional unknowns. Consequently, we estimate our usage of three instead of four assay tubes per unknown has resulted in an approximate savings of 25%--ostensibly with perhaps a gain in precision.

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