Modified Handling of Internal Catecholamine Standards in Radioenzymic Assays

James P. Ellis, Jr. and John W. Burns

A departure from a radioenzymic procedure widely used for plasma catecholamine assay is reported, in which we use "mean net standards" instead of individual internal standards. Interlaboratory/intermethod analyses validated use of the modified principle and demonstrated its preferential use over the conventional principle. Also demonstrated is use of the modified procedure for calculating catecholamine concentrations of several plasma samples collected from an exercising man and an acceleration-stressed swine. Benefits already derived from use of the procedure include: (a) quantification of certain plasma samples that could not be satisfactorily quantified by conventional options, (b) an apparent gain in analytical precision in virtually all other analyses, and (c) costs of analysis reduced by about 25%.

Additional Keyphrases: swine • "mean net standards" • stress • "kit" methods • economics of laboratory operation • exercise, effects of

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). The popularity of this procedure may be due, in part, to its use in conjunction with the commercial kit marketed by Upjohn Diagnostics under the trademark CAT-A-KI'T. In the Upjohn procedure (2) each "unknown" and control specimen is analyzed with and without addition of an internal standard—a requirement that virtually doubles laboratory costs of analysis. We emphasized the latter feature for two reasons. First, although Peuler and Johnson specified that internal standards be included for each and every specimen, it was not actually documented that this is essential (2). Second, we encountered a problem in using the Peuler-Johnson procedure for measuring catecholamine concentrations 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 assay of swine plasma, this revised procedure has other important benefits applicable to catecholamine analyses in clinical chemistry.

In view of the foregoing, we here recount the difficulties we initially encountered in the prescribed use of the CAT-A-KIT for assay of swine plasma, document our unsuccessful attempts to overcome those difficulties by options suggested in procedural notes (2) furnished with each CAT-A-KIT, describe our departure from the prescribed procedure, validate that departure through intermethod/interlaboratory comparisons, demonstrate how the revised procedure might also favorably affect human catecholamine analyses, and suggest benefits that derive from use of the revised procedure.

Materials and Methods

Radioenzymic assays: Except for the revised handling of internal standards detailed below, our use of CAT-A-KITs for plasma epinephrine and norepinephrine analyses was in accord with instructions furnished by Upjohn Diagnostics, Kalamazoo, MI 49001. Briefly, those instructions (2) specify the following procedural steps: (a) 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-SAM)3 and methylating enzyme (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 monoxygenase derivatives (methanephrines) by thin-layer chromatography; (f) transfer of the chromatographic zones to scintillation vials; (g) elution of the metanephrines from the silica gel, followed by their oxidation to (3H)vannilin; (h) extraction of the (3H)vannilin into scintillation counting fluid; (i) measurement of the radioactivity of each vial; and (j) calculation of catecholamine concentrations of each plasma as follows:

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

In equation 1, the quantity of each catecholamine is usually 100 pg and the volume of plasma is 50 μL; concentrations are expressed as picograms per milliliter (ng/L). For present terminology, the numerator and denominator of the bracketed portion of equation 1 are denoted "net plasma" (NP) and "net standard" (NS), respectively. Also, radioactivity measurements are herein expressed as dpm.

Data handling: Our departure from the Upjohn procedure (2) was not in the procedure per se, but in the handling of the individual NS values within a given assay; namely, instead of dividing the NP of a sample by its respective NS, we derived a single, composite NS from the individual NS values in each assay for each catecholamine. The composite NS, denoted "mean net standard" (MNS), was computed as follows: (a) the mean and SD of the individual NS's in a given assay (except controls) were determined, (b) those NS values more than 1 SD from the mean were rejected, and (c) the remaining values were averaged to give the MNS. To complete the calculation of final catecholamine concentrations, each NP was divided by the MNS and the quotient was multiplied by the unbracketed factors of equation 1.

To validate the use of the MNS concept, we performed simultaneous intermethod/interlaboratory analyses on a set of 48 plasma samples previously obtained from acceleration-stressed swine. Comparisons were limited to determinations of plasma norepinephrine since the radioenzymic procedure


Received July 6, 1982; accepted Aug. 24, 1982.

3 Nonstandard abbreviations: 3H-SAM, (3H-methyl)S-adenosyl-L-methionine; COMT, catechol-O-methyltransferase, EC 2.1.6; NP, "net plasma" value; NS, "net standard" value; and MNS, "mean net standard" value.
(3) used in the second laboratory was based on the highly specific methylation of norepinephrine by the enzyme phenylethanolamine-N-methyltransferase (EC 2.1.1.28).

Plasma samples: Catecholine data reported here were either taken from two recently conducted experimental studies (ms. submitted for publication) or obtained by further analysis of plasma collected from those studies. In those studies, human subjects were tested at relatively mild exercise levels, and miniature swine were exposed to high accelerations (g).

For special analyses reported here, three pools (S-I, S-II, and S-III) of swine plasma were prepared so as to give strikingly different catecholamine concentrations. Importantly, these pools were not prepared by adding catecholamine mixtures to the plasma, 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 concentrations of norepinephrine and epinephrine in plasma of g-stressed swine, we encountered marked variation of NS's in certain specimens, most frequently and most markedly when "apparent" catecholamine concentrations exceeded 10 ng/mL. Variations were of two types, with some NS values being very low (even negative) and others very high. As might be expected, rather bizarre data on plasma concentrations of catecholamines resulted from the calculations. Procedural notes (2) accompanying each CAT-A-KIT specified two options for improving the assay of specimens having high catecholamine concentrations: to dilute the specimen, and to increase the quantity of the standards used. To ascertain whether either of those options might improve quantification of the problematic plasma, we prepared three pools of swine plasma with widely differing catecholamine concentrations and used them as follows: (a) undiluted aliquots of all three pools, (b) threelfold dilutions of the two pools having high catecholamine concentrations (pools S-II and S-III), and (c) all diluted and undiluted aliquots at two quantities of standards (i.e., 500 pg and the prescribed 100 pg). In addition, we also assayed undiluted aliquots of pools S-II and S-III a second time two days later to ascertain the between-assay variation in NP and NS measurements.

Table 1 shows the effects of specimen dilution and enriched standards. Expression of NP's as disintegrations per minute (dpm) per microliter of plasma shows that dilution did not proportionately decrease the radioactivity counted. The disparity was especially marked for the epinephrine assay of pool S-II (42% higher than expected), but was also substantial for the other three examples (higher by 15 to 18%). The finding of disproportionately higher NPs's in diluted samples has since been repeatedly confirmed in our laboratory.

Also, use of a greater quantity of standard did not improve the quantification of epinephrine and norepinephrine in pools S-II and S-III. For example, not only were the NS's of both catecholamines erratic at both quantities in diluted as well as undiluted aliquots of pools S-II and S-III, but also the NS values found when we re-assayed undiluted samples were strikingly different from those of the first assay. Although the NS's in those repeat analyses did not differ greatly from the presumably acceptable NS's found for pool S-I (i.e., having catecholamine concentrations within the normal range for swine), we judged them to be too variable to be quantitatively sound.

The between-assay duplication of the exceptionally high NPs's of pools S-II and S-III not only was much better than that of the respective NS's, but also was well within the limits specified by Upjohn Diagnostics (2) for its control plasma (which typically gave NP values much lower than those for pools S-II and S-III). In this connection, our frequent finding of good between-assay duplication of exceptionally high NP values 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 an NS derived from other samples assayed at the same time.

Validation of MNS Usage

Results obtained from the intermethod analysis of plasma for norepinephrine are summarized in Figure 1. As shown in the upper graph of the figure, norepinephrine concentrations determined by the COMT procedure (1, 2) in our laboratory and computed from MNSs (i.e., COMT/MNS) correlated quite closely with those determined by the phenylethanolamine-N-methyltransferase procedure (r = 0.967). In sharp contrast, norepinephrine concentrations computed in the conventional manner (2) correlated rather poorly with values based on that procedure (r = 0.489). The opposing influences of very low and very high NS values on computed catecholamine concentrations are clearly reflected in the lower graph of Figure 1 by the wide scatter in the values.

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

---

Table 1. Effects of Sample Dilution and Enriched Standards

<table>
<thead>
<tr>
<th>Plasma pool</th>
<th>Sample</th>
<th>Per vial NP, dpm</th>
<th>Per µL of plasma</th>
<th>NS, dpm/pg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Norepinephrine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-I</td>
<td>Undil.</td>
<td>788</td>
<td>16</td>
<td>36.6</td>
</tr>
<tr>
<td>S-II</td>
<td>Undil.</td>
<td>16 187 (14 499)*</td>
<td>324 (290)</td>
<td>16.0 (30.4)</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>6 371</td>
<td>382</td>
<td>46.5</td>
</tr>
<tr>
<td>S-III</td>
<td>Undil.</td>
<td>57 486 (55 984)</td>
<td>1150 (1120)</td>
<td>16.9 (36.4)</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>22 066</td>
<td>1324</td>
<td>22.2</td>
</tr>
<tr>
<td><strong>Epinephrine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-I</td>
<td>Undil.</td>
<td>1 005</td>
<td>20</td>
<td>54.7</td>
</tr>
<tr>
<td>S-II</td>
<td>Undil.</td>
<td>2 506 (2 132)</td>
<td>50 (43)</td>
<td>46.5 (38.4)</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>1 182</td>
<td>71</td>
<td>66.0</td>
</tr>
<tr>
<td>S-III</td>
<td>Undil.</td>
<td>32 521 (31 659)</td>
<td>650 (633)</td>
<td>42.9 (58.2)</td>
</tr>
<tr>
<td></td>
<td>1:3</td>
<td>12 416</td>
<td>745</td>
<td>44.5</td>
</tr>
</tbody>
</table>

* Values in parentheses were obtained from a second assay and reflect between-assay variation.
from comparisons in Figure 1, several plasma samples having an NS value within one standard deviation of the MNS gave NS-computed concentrations that differed substantially from MNS-computed values. However, in those instances, the COMT/MNS values agreed closely with phenylethanolamine-N-methyltransferase values. These findings suggest that the marked interplasma variation in NS values may not be due principally to inherent differences between plasma samples per se, but to analytical variation.

Intrasubject vs Intersubject Variation

Each MNS used for calculating COMT/MNS catecholamine concentrations in the intermethod appraisal (Figure 1) was derived from NS's of plasma from the same animal, because we only assayed samples from one animal at a time. Consequently, that appraisal did not validate use of a single MNS value for computing catecholamine concentrations in plasma from different animals. 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 they did not document those differences in their report, their assertions nevertheless raised a serious question about the validity of deriving an MNS from NS values for plasma from different individuals. We therefore deemed it necessary to ascertain whether intersubject variation in NS values was substantially greater than the relatively small intrasubject variation we had consistently found before.

Standardization data obtained in connection with our recently concluded exercise study (manuscript submitted for publication; see above) seemed ideally suited for comparing intra- and intersubject differences in NS's; those data are summarized in Table 2. Note that the coefficients of variation for intersubject variations in mNS's (initial MNS calculation) were not strikingly different from those for intrasubject variations in NS's. In fact, for norepinephrine, intersubject variation was substantially less than the average intrasubject variation.

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

### Table 2. Intra- and Intersubject Variations in Norepinephrine (NE) and Epinephrine (E) in Human Plasma (dpm/pg)

<table>
<thead>
<tr>
<th>Subject</th>
<th>NE</th>
<th>E</th>
<th>NS of controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mNS</td>
<td>SD</td>
<td>CV, %</td>
</tr>
<tr>
<td>JS</td>
<td>48.78</td>
<td>3.30</td>
<td>6.76</td>
</tr>
<tr>
<td>JM</td>
<td>52.90</td>
<td>1.80</td>
<td>3.40</td>
</tr>
<tr>
<td>RG</td>
<td>52.49</td>
<td>3.51</td>
<td>6.69</td>
</tr>
<tr>
<td>FR</td>
<td>52.59</td>
<td>5.02</td>
<td>9.54</td>
</tr>
<tr>
<td>DB</td>
<td>51.09</td>
<td>3.40</td>
<td>6.66</td>
</tr>
<tr>
<td>SR</td>
<td>49.34</td>
<td>4.66</td>
<td>9.45</td>
</tr>
<tr>
<td>JW</td>
<td>51.86</td>
<td>1.98</td>
<td>3.82</td>
</tr>
<tr>
<td>DR</td>
<td>46.52</td>
<td>3.02</td>
<td>6.49</td>
</tr>
<tr>
<td>LP</td>
<td>51.43</td>
<td>3.36</td>
<td>6.54</td>
</tr>
</tbody>
</table>

Intrasubject average | 6.59  | 8.68

Intersubject mean | 50.78 | 2.14 | 4.21

---

* Initial mean NS, not final MNS.  
  a n = 5 for this subject, who did not complete the experiment; n = 9 for all other subjects.  
  c SD = 3.05 dpm/pg, CV = 5.31%.  
  d SD = 5.97 dpm/pg, CV = 10.68%.
Typical Assays of Human and Swine Plasma

In this communication we cannot adequately illustrate use of the MNS concept for the assay of bona fide samples of human plasma. However, an expanded version of this report is available (5) that includes a table of pertinent data obtained from assays of several samples drawn successively from an exercising man and from an acceleration-stressed swine. In addition to illustrating the two-step procedure of computing a MNS and the difference between NS- and MNS-computed concentrations of catecholamines, the tabulated data suggest more clearly the likelihood that the interplasma variations in NS's are more analytical than biological.

Discussion

In the present study, we did not exhaustively evaluate the two options suggested by Upjohn Diagnostics (2) for overcoming the difficulties we had encountered in quantifying catecholamines in the plasma of severely stressed swine. Neither did we validate the preferential use of MNS values as thoroughly as we would have preferred. Still, we believe we obtained sufficient information 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 the data summarized in Figure 1 unequivocally demonstrate the need for screening NS's and for implementing the use of a mean internal standard of some sort. We do not believe it essential, however, to reject NS's solely on the basis of exceeding the initially computed mean by more than one standard deviation; that choice, in our opinion, should rest with the individual investigator. Actually, the 1-SD limit suggested here was the third such rejection principle we examined. We first examined the feasibility of using fixed acceptance limits that did not vary from one assay to the next and that were arbitrarily set from accumulated data. We then adopted the more statistically acceptable principle of rejecting NS's more than 2 SD from the initially computed mean. This principle was well suited for analyses of human plasma; however, our infrequent finding in swine plasma analyses that too wide a range of "acceptable" NS's was used for computing final MNS's prompted adoption of the 1-SD criterion.

We recognize that use of an MNS may well 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 probably would preclude use of an MNS. Too, although computation of an MNS from NS values of different individuals appears to be valid, the same may not be true for combining NS values of different species.

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

But even for those analyses in which a NS was not obviously aberrant, we believe that use of the MNS principle probably enhanced analytical precision substantially. Two considerations support that belief. First, from a purely theoretical point of view, it is clear from equation 1 that NS values figure just as decisively as NP values in calculation of catecholamine concentrations. Second, although not documented here, we have consistently found substantially less within-assay variation in NP values than in NS values, an observation supported by the example of "typical data" that has appeared in every brochure (2) from Upjohn Diagnostics that we have received during the past four years. In that example (2), if one were to take the duplicate radioactivities and re-express them as NP and NS values, intra-assay CV's for the NP's of epinephrine and norepinephrine would be 5 and 8%, respectively, whereas CV's for the respective NS's would be 8 and 18%. In their evaluation of CAT-A-KIT's, Tasseron et al. (4) also found intra-assay (within-run) CV's of radioactivities for both catecholamines to be at least twice as high for "sample + standard" as for "sample" alone.

Since implementing the MNS principle, we have also realized greater economy of operation. The savings derive partly from assaying each internal standard singly rather than doubly and partly by using the assay tubes thereby liberated for the analysis of additional unknowns. Consequently, we estimate that our use of three instead of four assay tubes per unknown has resulted in an approximate savings of 25%, and perhaps a gain in precision.

We gratefully acknowledge the contributions of Dr. Michael G. Ziegler for furnishing phenylethanolamine-N-methyltransferase-based analyses, of S/Sgt Gary W. Moore for performing many COMT-based analyses, and of Ms. Laura Wilcox for expert secretarial assistance.

References

2. Brochure, CAT-A-KIT™ (Catecholamines Radioenzymatic Assay Kit 3H), Upjohn Diagnostics, Division of the Upjohn Co., Kalamazoo, MI (undated).

CLINICAL CHEMISTRY, Vol. 29, No. 1, 1983 147