Dilution of Plasma with Tris Buffer Increases Measured Catecholamines in Plasma

Jean-Louis Cuche, Françoise Seiz, Gérard Ruget, Monique Gentil, and Christophe Gaudin

We investigated the effects of dilution of plasma samples on the measured concentrations of catecholamines. Diluting samples of human plasma 10-, 50-, and 100-fold with Tris buffer (100 mmol/L, pH 8.6) improved analytical recovery of internal standards, suggesting that it decreases the commonly observed inhibition of methylation in radioenzymatic assays of catecholamines in plasma. However, the dilution is not associated with a proportional decrease in counted radioactivity. This extra amount of radioactivity, which is unlikely to be nonspecific in origin, accounts for a significant increase in the calculated catecholamine concentration. Tentatively, we suggest that Tris buffer releases both catecholamines and conjugated catecholamines bound to some unidentified low-molecular-mass component of plasma.

The radioenzymatic method for measuring CAs in plasma has gained broad acceptance as the method of reference (1). CAs are methylated by COMT (EC 2.1.1.6), from rat liver (2), which transfers a tritiated methyl group from S-adenosylmethionine to catechola. The O-methylated compounds are extracted into organic solvents (usually toluene/isoamyl alcohol), then separated by thin-layer chromatography on the basis of the polarity of their side chains. Finally, those compounds having a beta-hydroxyl group are oxidized with periodate. These four steps contribute to the specificity of this assay. The procedure described by Peuler and Johnson is now used by several investigators (1, 4).

However, Hortnagl et al. (5) reported that the methylation was less efficient in plasma than in water, and they postulated the existence of an endogenous inhibitor of CAs methylation. Because this hypothetical inhibitor apparently varies from one plasma to another, internal standards are usually included in each sample. The concentration of CAs in each plasma is calculated from the radioactivity (counts/min, or cpm) as follows (6):

\[
\text{CAs concentration} = \frac{\text{cpm "sample"} - \text{cpm "blank"}}{\text{cpm "sample + standard"} - \text{cpm "blank"}} \times \text{quantity (ng) of added standard}} \text{volume (\mu L) of sample}
\]

The value measured for an internal standard (cpm "sample + standard" – cpm "sample") provides a simple index of the methylation for each plasma, when compared with that for an external standard, i.e., when plasma is replaced by the same volume of water. Expressing the internal standard value as a percentage of the external standard value indicated an apparent inhibition of methylation by about 25% in a population of healthy subjects (7), by 60% in uremic patients (7), and by more than 100% in dogs during intravenous injection of norepinephrine (8).

Johnson et al. (9) proposed diluting the plasma sample to decrease this inhibition. When the internal standard value of a given plasma is very low, diluting the sample brings the value for the internal standard back toward its usual limits, or within the same range of variation for a given series of measurements. Thus, by bringing the efficiency of methylation to within the same order of magnitude for sets of samples, one can more appropriately intercompare the measured amine concentrations (8, 10). This reasoning relies on an assumption of strict parallelism between the dilution factor, the amount of CAs present in the diluted sample, and thus the final radioactivity. Unfortunately, this assumption does not seem to be valid. According to Ellis and Burns (6), "the dilution did not proportionately decrease the radioactivity counted"; in contrast, we have reported far higher apparent concentrations of conjugated CAs in diluted plasmas than in nondiluted plasmas (7, 8, 10).

We undertook the present study to gain further insights into this problem. We report our data because what appears to be a purely technical problem may conceal a phenomenon with potential physiological significance.

Materials and Methods

We measured CAs by a radioenzymatic assay based on that described by Peuler and Johnson (4), as slightly modified (10) and routinely used in our laboratory. A feature of this assay, unique to the assay of plasma, is the inclusion of plasma in the incubation mixture without an initial extraction of the CAs or deproteinization. The COMT was prepared from liver extract according to the procedure proposed by Boren et al. (3); this enzyme preparation was devoid of dopa-decarboxylase activity, and it introduced a negligible amount of additional protein in the incubation mixture. Thus our assay is based on the same original report (4) that forms the basis of the Upjohn "CAT-A-KIT" system, but all reagents, including COMT, are prepared in our laboratory.

Conjugated CAs were measured as deconjugated compounds after enzymatically catalyzed hydrolysis by treatment with either glucuronidase (EC 3.2.1.31) or sulfatase (EC 3.1.6.1)—both from Sigma Chemical Co., St. Louis, MO—according to procedures already described (7, 11).

To minimize the effect of interassay variation, we assayed...
all aliquots from a given plasma (diluted or nondiluted, hydrolyzed or nonhydrolyzed) in the one assay.

Plasma was sampled from nine resting healthy subjects.

In a first series of measurements, CAs and conjugated CAs were measured both in undiluted plasma and in plasma diluted 10-, 50-, and 100-fold with Tris buffer (100 mmol/L, pH 8.6). In a preliminary study, we had observed that plasmas from five subjects gave results that were less variable and higher when diluted with Tris buffer than when diluted with water.

In a second series of measurements, we assayed deproteinized samples. We mixed nine volumes of plasma with one volume of 2 mol/L perchloric acid for 1 min, and centrifuged (15 min, 5000 rpm). CAs and conjugated CAs were measured in undiluted supernatants and after dilution 10-, 50-, and 100-fold with Tris buffer.

Statistical significance was tested with either one-way analysis of variance or Student's t-test.

Results

Effects of dilution of plasma: Our data for CAs and conjugated CAs in undiluted plasma samples are in agreement with previous reports (11-16). However, dilution of plasma samples with Tris buffer was associated with an increase of the calculated concentrations of CAs, glucuro-conjugated CAs, and sulfo-conjugated CAs (Table 1).

Table 1. Effects on CAs Values Measured after Diluting Plasma of Control Subjects with Tris Buffer

<table>
<thead>
<tr>
<th>Catecholamines, n = 9</th>
<th>Undiluted</th>
<th>10-fold</th>
<th>50-fold</th>
<th>100-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA</td>
<td>32 (10)</td>
<td>273 (136)</td>
<td>1867 (535)</td>
<td>5850 (1201)</td>
</tr>
<tr>
<td>NE</td>
<td>329 (32)</td>
<td>597 (103)</td>
<td>1126 (371)</td>
<td>3242 (1518)</td>
</tr>
<tr>
<td>EPI</td>
<td>49 (10)</td>
<td>296 (149)</td>
<td>798 (360)</td>
<td>1910 (785)</td>
</tr>
<tr>
<td>Glucuro-conjugated catecholamines, n = 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>37 (14)</td>
<td>259 (92)</td>
<td>6235 (1590)</td>
<td>10 454 (1978)</td>
</tr>
<tr>
<td>NE</td>
<td>392 (23)</td>
<td>4096 (199)</td>
<td>21 904 (2037)</td>
<td>40 020 (3327)</td>
</tr>
<tr>
<td>EPI</td>
<td>25 (5)</td>
<td>236 (81)</td>
<td>4911 (1461)</td>
<td>5633 (1750)</td>
</tr>
<tr>
<td>Sulfo-conjugated catecholamines, n = 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td>4681 (591)</td>
<td>10 236 (1346)</td>
<td>22 956 (5009)</td>
<td>43 119 (4638)</td>
</tr>
<tr>
<td>NE</td>
<td>688 (100)</td>
<td>1 439 (206)</td>
<td>5996 (1390)</td>
<td>9675 (963)</td>
</tr>
<tr>
<td>EPI</td>
<td>421 (25)</td>
<td>1903 (147)</td>
<td>11 232 (1410)</td>
<td>16 860 (1288)</td>
</tr>
</tbody>
</table>

Significantly different from results for nondiluted samples at *p <0.05, ^<0.01, and ^<0.001.

Fig. 1. Radioactivity (counts/min) for catecholamines (O, n = 9), sulfo-conjugates (A, n = 6), and glucuro-conjugates (M, n = 6) in blank (B), nondiluted plasma (ND), and in plasma diluted 10-, 50-, or 100-fold. Shaded areas show detection limit for each amine, arbitrarily defined as twice the blank value.

![Graph](https://via.placeholder.com/150)

Fig. 2. Change in norepinephrine concentrations in nine plasma samples after dilution.

<table>
<thead>
<tr>
<th>Results below the limit of sensitivity of the assay, according to the blank value</th>
</tr>
</thead>
<tbody>
<tr>
<td>o</td>
</tr>
<tr>
<td>&gt;2 (cpm &quot;blank&quot;)</td>
</tr>
<tr>
<td>&lt;2 (cpm &quot;blank&quot;)</td>
</tr>
</tbody>
</table>

Fig. 2. Change in norepinephrine concentrations in nine plasma samples after dilution.

Deproteinization of plasma: Deproteinization did not change the concentration of CAs or glucuro-conjugated CAs.
Effects of the dilution of supernates: Dilution of deproteinized supernates with Tris buffer (10-, 50-, and 100-fold) induced a comparable increase in the computed concentrations of both CAs and conjugated CAs (data not reported). However, the concentrations of DA and EPI were significantly higher in diluted supernates than in undiluted plasma, whereas the concentrations of glucuro-conjugated DA and EPI were significantly lower, perhaps because of some hydrolysis of these two glucuro-conjugates by the perchloric acid.

Discussion

Clearly, dilution of plasma with Tris buffer is associated with an increase in the calculated concentrations of amines (Table 1), secondary to a nonproportional decrease in radioactivity for most compounds measured (Figure 1), confirming the suggestion of Ellis and Burns (6). This could have resulted from either retention of nonspecific radioactivity or release of "masked" CAs and conjugated CAs by dilution with Tris buffer.

Unfortunately we have no direct evidence to determine which of these is the case. However, the retention of nonspecific radioactivity seems a remote possibility, for the following reasons:

1. The radioenzymatic assay is very specific. The excess unincorporated [3H]-S-adenosylmethionine remained in the organic phase, whereas the radioactivity of O-methylated products was in the acid phase. To check for a possible contamination, we washed the aqueous layer with toluene/isooamyl alcohol (3/2 by vol). Any unknown tritiated compound remaining should not migrate from the origin in thin-layer chromatography, the O-methylated products being separated on the basis of the polarity of their side chains. Furthermore [3H]normetanephrine and [3H]metanephrine were oxidized into [3H]vanillin under the effect of sodium metaperiodate. We have shown that the amount of [3H]vanillin yielded became constant after a 10-min reaction time (unpublished personal data).

2. If, despite all this, a nonspecific radioactivity might be included in these steps and affect the specificity of the radioenzymatic assay, this hypothetical contamination should present the same pattern of response to the dilution for all compounds measured. But this is not so (Figure 1).

3. If the [3H]-S-adenosylmethionine is of less than its usual purity, nonspecific radioactivity is increased, and this is revealed by an increase in the blanks' values. As shown in Figure 1, the radioactivity of the blanks was within normal values.

Because diluting the plasma samples with Tris buffer did not induce a proportional decrease of the radioactivity counted, and because this effect was unlikely to be due to the retention of nonspecific radioactivity, a Tris-induced release of CAs and conjugated CAs from plasma has to be considered.

Two phenomena must be discussed. The first is related to inhibition of methylation. As expected, and shown in Figure 3, the 10-fold dilution was associated with a statistically significant increase in the analytical recovery of internal standard, and the inhibition of CAs and glucuro-conjugated CAs vanished. As far as sulfo-conjugates are concerned, a 30% inhibition remained after a 10-fold dilution, and was not decreased by further dilution. This dilution-lifted inhibition had a functional role, because the concentrations of seven of nine compounds measured in 10-fold diluted samples were significantly higher than those measured in undiluted plasma.

The second phenomenon is independent of the first and is related to the presence of Tris buffer only, because the calculated concentrations of conjugated CAs, at least, were higher in 50- and 100-fold than in 10-fold diluted samples. To explain this phenomenon, we suspect that the dilution with Tris buffer might weaken CA binding; it is well known that CAs are bound to plasma proteins (17). When this weakening became rupture, more of the amines became measurable. It is interesting to note that a similar hypothesis has been proposed to explain the Tris-induced release of insulin bound to globulin in vitro at pH 8.6 (18). To investigate this hypothesis, we made a series of measurements on deproteinized, then-diluted plasmas. We found basically the same type of response, thus ruling out a major role for large proteins that had been precipitated by perchloric acid. If the hypothesis of a releasing effect induced by Tris is still valid, one should suspect a role for plasma components of low molecular mass that are present in the supernatant fluid after treatment with perchloric acid.

In conclusion, our data for human plasma diluted with Tris buffer (100 mmol/L, pH 8.6) confirm that the dilution diminishes the inhibition of methylation that commonly is observed when radioenzymatic assay is used to measure catecholamines. They also confirm that dilution does not induce a proportional decrease of the radioactivity counted. This extra radioactivity is unlikely to be nonspecific, but instead related to amines unmasked. We propose a releasing effect of both CAs and conjugated CAs, bound to a low-molecular-mass component of plasma, to explain this phenomenon.

This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, Conseil Scientifique de la Faculté de Médecine Paris-Ouest (Université René-Descartes) and Association HYPERART.

References
Percentile Estimates of Reference Values for Total Protein and Albumin in Sera of Premature Infants (<37 Weeks of Gestation)

Stanley H. Zlotkin and Charles W. Casselman

We measured the concentrations of total protein and albumin in sera of 281 well-fed premature infants, gestational ages 22–36 weeks, and calculated reference values from the 10th to 90th percentiles. The mean serum albumin concentration (27.6 ± 4.4 g/L, X ± SD) and total protein concentration (49.2 ± 6.7 g/L) at a postnatal age of 14.5 days were lower than reference values for full-term infants. We detected a significant positive correlation between albumin concentration and gestational age (r = 0.34, p < 0.01) and total protein concentration and gestational age (r = 0.43, p < 0.01). Even though albumin values were low, generalized edema was not present. We conclude that values for total protein and albumin in the preterm infant are lower than in the full-term infant but are an expected physiological response to premature birth.

Reference values of many analytes—including IgG, prealbumin, thyroxin, and hemoglobin—differ between premature and full-term infants (1–4). Clinical reference values for total protein and albumin have been determined in several studies for adults (5, 6) and for several ranges of ages within childhood (7, 8); however, reference values for the infant born prematurely are largely unavailable. In the one earlier study that addressed this issue, few preterm infants were studied, and preterm infants were defined as those born after less than 39 weeks of gestation (9). Because it is now widely accepted that preterm infants are by definition born in less than 37 weeks of gestation, values from this study are not necessarily applicable for current use.

We and others (10, 11) have observed that when the reference values for full-term newborns are applied to the premature infant, values for serum albumin and total protein consistently fall below those for the full-term infant. Yet, other than mild subcutaneous edema of the hands and feet in the first few postnatal days, premature infants who maintain consistently "low" values for serum total protein and albumin are notably free of generalized edema (12).

Our objective here was to evaluate the effect of gestational age and nutrient intake on plasma total protein and albumin concentrations in preterm infants.

Subjects and Methods

Selection of Subjects

A retrospective review was completed on 286 infants, all of whom received total parenteral nutrition (TPN) in our neonatal intensive-care unit during an 18-month period from January 1984 to June 1985. We selected infants receiving TPN because they received a standard nutrient intake and blood tests were performed on these infants on a regular basis. Infants were included in this retrospective analysis if their admitting diagnoses gave no indication of liver disease. During analysis of the data, infants were excluded from the study if there was any biochemical or clinical evidence of liver dysfunction. Infants were excluded if their serum bilirubin concentrations exceeded 260 µmol/L.

CLINICAL CHEMISTRY, Vol. 33, No. 3, 1987 411