Optimal Collection and Storage Conditions for Catecholamine Measurements in Human Plasma and Urine

Frans Boomsma,1 Gootzen Alberts, Loes van Eijk, Arie J. Man in 't Veld, and Maarten A. D. H. Schalekamp

Improvements in methodologies for measuring concentrations of catecholamines (CA) have led to an increasing use of these compounds as markers in the screening of patients and in long-term clinical trials. Because of the associated logistical problems, we have investigated the unresolved question of optimal conditions for sample preparation and for storage of plasma and urine samples. Results show that blood should be centrifuged within 1 h after collection; the use of a refrigerated centrifuge is not necessary. Once plasma is prepared, CA are stable for 1 day at 20 °C, 2 days at 4 °C, 1 month at −20 °C (or 6 months with added glutathione), and up to 1 year at −70 °C. CA are stable at 4 °C for 1 month in unpreserved urine and for 4 months in urine preserved with EDTA and sodium metabisulfite. In acidified urine, CA were nearly unchanged after 1 year at 4 and −20 °C.

Indexing Terms: norepinephrine • epinephrine • dopamine • sample handling • variation, source of

The improvements made in recent years in methodologies for measuring catecholamines (CA) have made it possible to use CA concentrations as biochemical markers, both in routine screening of patients and during long-term and large-scale clinical trials.2 At the same time, however, the associated logistical problems, especially with such trials, again raise still-unresolved questions about optimal conditions for preparation of plasma and urine specimens during storage at different temperatures for prolonged periods of time. Blood is generally collected through an indwelling catheter into tubes containing an antioxidant (heparin, EDTA, or EGTA) and an antioxidant (glutathione, sodium metabisulfite, or ascorbic acid). A delay in centrifugation of blood apparently led to an immediate de-

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2 Nonstandard abbreviations: CA, catecholamines; NE, norepinephrine; E, epinephrine; DA, dopamine; HPG, heparinized plasma containing extra glutathione; HP, heparinized plasma; UA, acidified urine; UO, unpreserved urine; and UB, urine preserved with EDTA and sodium metabisulfite.
cay in measured plasma CA (1), whereas others reported unchanged CA after a delay of 1 h (2, 3), 3 h (4), or even 7–24 h (5). Often a refrigerated centrifuge is used, but whether or not this is really necessary is not clear. According to one report (1), plasma should be frozen immediately; others claim that plasma CA are stable at room temperature for 22 h, even in the absence of added antioxidant (2). Reports on the stability of plasma CA at −20 °C vary from 11 days to 240 days (1–4, 6–11). Regarding lower storage temperatures, Sever (3) saw no decrease after 6 months at <−50 °C; others report a decrease after 4 months at −70 °C (12). Most of the references cited gave results for only norepinephrine (NE) and epinephrine (E); dopamine (DA) was only rarely considered. Reports on the stability of CA in urine are scarce. At room temperature, CA are stable with EDTA and glutathione as preservatives; at −30 °C, they are stable in unpreserved urine, as well as in urine preserved by acidification or with EDTA and glutathione, for 57 days (13). At −80 °C, NE and DA are reportedly stable for 13 months in urine preserved with EDTA and sodium metabisulfite (14).

We have addressed these questions by measuring CA (NE, E, and DA): (a) after various delays in centrifugation of blood kept at 20 and 4 °C; (b) after various delays in freezing of plasma kept at 20 and 4 °C; (c) after centrifugation of blood at 20 and 4 °C; (d) after storage at various temperatures for up to 1 year in heparinized plasma with and without added glutathione; and (e) after storage at various temperatures for as long as 1 year in unpreserved urine, acidified urine, or urine preserved with EDTA and sodium metabisulfite. To measure CA, we used sensitive and reproducible HPLC methods, in which CA are concentrated from plasma or urine by liquid–liquid extraction and derivatized with the selective fluorogenic agent 1,2-diphenylethlenediamine (15, 16).

Materials and Methods

Materials

NE, E, DA, and α-methylnorepinephrine were obtained from Sigma Chemical Co. (St. Louis, MO), EDTA and sodium metabisulfite from Merck (Darmstadt, Germany), and reduced L-glutathione was from Fluka (Buchs, Switzerland). 1,2-Diphenylethlenediamine was prepared as described before (15).

Blood was obtained from human volunteers and from patients with essential hypertension. Procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983. Unless otherwise stated, blood was collected in chilled heparinized polystyrene 10-mL tubes containing 12 mg of glutathione and centrifuged within 15 min at 4 °C (10 min, 3000 × g). Plasma was stored at −70 °C unless otherwise stated.

Portions of urine of healthy volunteers were collected and pooled in polyethylene containers; preservatives were added as described in the Results section.

Apparatus

The instrumentation for chromatography consisted of a Kratos SF-400 pump (Applied Biosystems, Ramsey, NJ), a Kontron (Milan, Italy) 460 autosampler equipped with a 200-μL injection loop, a Shimadzu (Kyoto, Japan) RFR 535 spectrofluorometer (excitation at 350 nm, emission at 480 nm), and a Merck-Hitachi D-2500 integrator (Hitachi, Tokyo, Japan). Separations were performed on 3-μm (particle size) Cp™ MicroSpher C18 [100 mm × 4.6 mm (i.d.)] columns from Chrompack (Bergen op Zoom, The Netherlands).

Catecholamine Determinations

CA concentrations in 1 mL of plasma were determined by HPLC with fluorometric detection after liquid–liquid extraction and derivatization with the fluorogenic agent 1,2-diphenylethlenediamine as described previously (15). A similar method was used to determine CA concentrations in urine (16). Interassay coefficients of variation of both methods were 3–7 %.

Statistics

Analysis of variance, Student’s paired t-test, and Wilcoxon’s signed rank test were used for comparisons between groups. Stability during storage was investigated by trend-analysis, by using Spearman rank correlation coefficients and by regression analysis. Values of P <0.05 were considered significant.

Results

Effects of time-lapse (a) between collection and centrifugation of blood and (b) between preparation and freezing of plasma. Blood was obtained from six healthy volunteers into heparinized tubes containing glutathione. One part of each blood sample was centrifuged without delay. Portions of the remainder were centrifuged after 4, 24, or 48 h of standing at 4 °C or at 20 °C; the plasmas then obtained were immediately frozen at −70 °C (designated B). One part of the plasmas obtained after immediate centrifugation was immediately frozen at −70 °C (designated A); portions of the remaining plasmas were frozen after 4, 24, or 48 h of standing at 4 or 20 °C (designated P). CA concentrations in all samples A, B, and P from each volunteer were measured in the same assay. Results (Table 1) clearly show that plasma, once prepared, can be left at 4 °C for at least 48 h and at 20 °C for 24 h without any changes in measured CA concentrations. An increase in time between blood collection and centrifugation, however, is critical: Keeping blood at 4 °C leads to an increase in measured NE and E concentrations; keeping blood at 20 °C results in decreases in measured NE, E, and DA concentrations.

To further evaluate how fast these last-named phenomena occur, we centrifuged aliquots of blood from eight more healthy volunteers and froze the supernates immediately, and we left part of the aliquots at 4 or 20 °C for 30 min, 1, 2, 3, and 4 h before centrifuging and freezing. Results for plasma CA measurements in these samples are shown in Table 2; because of the nonnormality of the distributions, median levels are also reported. The data show that a time lag of 30 min or 1 h between blood collection and centrifugation does not
Table 1. Effect of Time between Collection and Centrifugation of Blood, and between Preparation and Freezing of Plasma on Measured Concentrations of Catecholamines

<table>
<thead>
<tr>
<th>Time, h*</th>
<th>NE (mean ± SD), nmol/L</th>
<th>E (mean ± SD), nmol/L</th>
<th>DA (mean ± SD), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>P, 4 °C</td>
<td>1.73 ± 0.45</td>
<td>0.17 ± 0.05</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>1.76 ± 0.45</td>
<td>0.16 ± 0.06</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>24</td>
<td>1.74 ± 0.42</td>
<td>0.16 ± 0.06</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>48</td>
<td>1.72 ± 0.44</td>
<td>0.17 ± 0.06</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>P, 20 °C</td>
<td>1.70 ± 0.45</td>
<td>0.17 ± 0.07</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>1.71 ± 0.42</td>
<td>0.19 ± 0.07</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>24</td>
<td>1.56 ± 0.37b</td>
<td>0.15 ± 0.04</td>
<td>0.12 ± 0.05b</td>
</tr>
<tr>
<td>B, 4 °C</td>
<td>2.19 ± 0.58b</td>
<td>0.19 ± 0.07b</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>4</td>
<td>2.45 ± 0.48b</td>
<td>0.21 ± 0.08b</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>24</td>
<td>2.44 ± 0.51b</td>
<td>0.19 ± 0.07b</td>
<td>0.14 ± 0.07</td>
</tr>
<tr>
<td>48</td>
<td>0.98 ± 0.33b</td>
<td>0.09 ± 0.04b</td>
<td>0.05 ± 0.01b</td>
</tr>
<tr>
<td>B, 20 °C</td>
<td>0.66 ± 0.18b</td>
<td>0.06 ± 0.07b</td>
<td>0.03 ± 0.02b</td>
</tr>
</tbody>
</table>

*Sample types were: A, blood centrifuged immediately after collection and the plasma frozen immediately at −70 °C; P, blood centrifuged immediately after collection and the plasma frozen at −70 °C after standing under the indicated conditions; B, blood centrifuged immediately after standing under the indicated conditions, with the plasma then separated and frozen immediately at −70 °C.

b Significantly different from A (P < 0.05).

n = 8.

Table 2. Effect of Time between Collection and Centrifugation of Blood on Measured Concentrations of Catecholamines

<table>
<thead>
<tr>
<th>Time, h</th>
<th>NE (mean ± SD (and median))</th>
<th>E (mean ± SD), nmol/L</th>
<th>DA (mean ± SD), nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.85 ± 1.24 (1.59)</td>
<td>0.24 ± 0.10 (0.20)</td>
<td>0.11 ± 0.03 (0.11)</td>
</tr>
<tr>
<td>4 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.82 ± 1.15 (1.64)</td>
<td>0.22 ± 0.10 (0.18)</td>
<td>0.11 ± 0.02 (0.11)</td>
</tr>
<tr>
<td>1</td>
<td>1.88 ± 1.17 (1.61)</td>
<td>0.25 ± 0.14 (0.19)</td>
<td>0.11 ± 0.03 (0.12)</td>
</tr>
<tr>
<td>2</td>
<td>2.09 ± 1.25 (1.79)</td>
<td>0.24 ± 0.10 (0.20)</td>
<td>0.13 ± 0.03 (0.14)</td>
</tr>
<tr>
<td>3</td>
<td>2.09 ± 1.18 (1.88)</td>
<td>0.25 ± 0.10 (0.22)</td>
<td>0.14 ± 0.02 (0.13)</td>
</tr>
<tr>
<td>4</td>
<td>2.31 ± 1.21 (2.04)</td>
<td>0.25 ± 0.11 (0.20)</td>
<td>0.14 ± 0.02 (0.14)</td>
</tr>
<tr>
<td>20 °C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>1.78 ± 1.13 (1.60)</td>
<td>0.25 ± 0.11 (0.22)</td>
<td>0.12 ± 0.03 (0.12)</td>
</tr>
<tr>
<td>1</td>
<td>1.74 ± 0.98 (1.55)</td>
<td>0.22 ± 0.10 (0.21)</td>
<td>0.12 ± 0.04 (0.11)</td>
</tr>
<tr>
<td>2</td>
<td>1.59 ± 0.82 (1.47)</td>
<td>0.19 ± 0.06 (0.18)</td>
<td>0.10 ± 0.03 (0.10)</td>
</tr>
<tr>
<td>3</td>
<td>1.39 ± 0.58 (1.26)</td>
<td>0.17 ± 0.07 (0.15)</td>
<td>0.11 ± 0.02 (0.12)</td>
</tr>
<tr>
<td>4</td>
<td>1.22 ± 0.37 (1.11)</td>
<td>0.15 ± 0.07 (0.15)</td>
<td>0.09 ± 0.02 (0.09)*</td>
</tr>
</tbody>
</table>

*Significantly different from t = 0 (P < 0.05).

n = 8.

result in changes in measured concentrations of CA, but 2 h is already too long.

Preparation of plasma in a refrigerated centrifuge or at room temperature. Blood was obtained from 19 volunteers and patients and divided into two portions. One portion was centrifuged in a refrigerated centrifuge at 4 °C; the other was centrifuged at the same time in a similar centrifuge at room temperature. The concentra-

ations of NE, E, and DA measured in the plasmas prepared by cold centrifugation (means ± SD: 1.22 ± 0.49, 0.19 ± 0.14, and 0.10 ± 0.06 nmol/L, respectively) were not different from the concentrations measured in the plasmas prepared at room temperature (1.25 ± 0.50, 0.19 ± 0.14, and 0.10 ± 0.04 nmol/L, respectively).

Stability of CA in specimens stored at various temperatures. For determining the stability of CA in plasma, we pooled plasma prepared from blood collected into heparinized tubes containing glutathione (HPG pool). Another pool was made of plasma prepared from heparinized blood without glutathione (HP pool). To be able to clearly discern decreases in plasma concentrations of E and DA, which are rather low under normal circumstances, we enriched both pools in E and DA. The pools were aliquotted into Eppendorf vials and stored at various temperatures (37, 20, 4, −20, and −70 °C). The concentrations of NE, E, and DA measured without delay, were 2.51, 1.23, and 1.47 nmol/L, respectively, in the HP pool; 2.63, 1.27, and 1.44 nmol/L, respectively, in the HPG pool. At various times thereafter, ranging from 1 day to 1 year, concentrations were measured again. Results, expressed as percentages of the originally determined concentrations, are presented in Figure 1. In both pools CA concentrations were already decreased after 1 day at 37 °C, 2–3 days at 20 °C, and 1 week at 4 °C. The protective effect of extra glutathione became most obvious during storage at −20 °C. In the HGP pool, CA remained stable for up to 200 days; in the HP pool a decrease was already evident after 1 month. At −70 °C, no obvious loss of CA was seen for 1 year, neither in HP nor in HPG plasma. Trend and regression analysis indicated a slight downward trend for NE in HP (trend and regression) and in HGP (only with regression), and no significant changes in E and DA. Variations in HGP were smaller than in HP.

For investigating the stability of CA in urine, a pool made of urine portions of healthy volunteers was enriched in E to better allow follow-up of E concentrations (which are usually rather low). The pool was then divided into three portions: One portion was acidified with 5 mL of 6 mol/L HCl per liter (pH 3.2; henceforth designated UA); to another portion was added 250 mg of EDTA and 250 mg of sodium metabisulphite per liter (designated UB); and the third portion received no further treatment (designated UO). All three portions were aliquotted into Eppendorf vials and stored at 37, 20, 4, −20, and −70 °C. CA concentrations were measured after various times of storage (up to 1 year), and expressed as a percentage of the values determined immediately, which were 0.34, 0.25, and 1.86 mmol/L for NE, E, and DA, respectively, in all three portions. As shown in Figure 2, in both UO and UB, CA are stable for 1–2 days at 37 °C and for 4–8 days at 20 °C, and NE and DA are stable for longer than E. In UA, CA are stable for 3 weeks at 37 °C and for >2 months at 20 °C, with NE and E being more stable than DA. The stability at 4, −20, and −70 °C was investigated with trend and regression analysis. In UO, E started to decrease after 35 days at 4 °C, whereas NE and DA were more stable. At −20 and
-70 °C, NE and E showed a slight downward trend, whereas DA remained stable. In UB, CA were stable up to 4 months at 4 °C and thereafter slowly decreased. At -20 and -70 °C, the pattern was the same as in UO: a slight downward trend for NE and E, and DA remained stable. CA were most stable in UA. E showed a small but clear downward trend at all three storage temperatures (the least at -20 °C); DA was stable at all three temperatures and NE at 4 and -20 °C. Remarkably, NE showed a slight downward trend at -70 °C.

Discussion

In our own university hospital setting, we routinely measure CA in plasma; samples are collected in specially prepared heparinized glutathione-containing tubes, brought to the nearby laboratory on ice, and centrifuged within 15 min in a refrigerated centrifuge. The plasma is immediately stored at -70 °C in an ultrafreezer, and measurements are performed within 2 weeks. Urine is collected in containers with EDTA and sodium metabisulfite, kept in a refrigerator between collections, and, when collection is completed, aliquots are stored at -20 °C until assayed within 2 weeks. Regularly we receive from other centers samples for CA measurements that have not been handled similarly for various reasons, e.g., no specially prepared tubes or no refrigerated centrifuge available, no centrifuge nearby at all, no ultrafreezer available, samples left or sent by mail at room temperature, or samples that for logistical reasons during clinical trials have been stored for prolonged periods in freezers or ultrafreezers. We undertook the present study to answer questions regarding the validity of CA measurements in such samples.

The results show that in plasma, once prepared, CA are stable for some time (24 h at 20 °C; 48 h at 4 °C), so that immediate freezing is not imperative. In accord
with this finding, the use of a refrigerated centrifuge is not necessary. However, the time between blood collection and the preparation of plasma is quite critical; if the time exceeds 1 h, CA values increase (when blood is kept at 4 °C) or decrease (when kept at 20 °C). The most likely explanation is that, at 20 °C, CA are taken up by the erythrocytes and (or) platelets (17, 18), leading to a decrease in the plasma concentration. Because the uptake of CA in erythrocytes is reported to be temperature dependent (17), this process would be much slower at 4 °C than at room temperature. However, at 4 °C, blood constituents such as erythrocytes may break down, releasing their CA content, and thereby leading to an increase in plasma CA concentration. The sometimes visually discernible more reddish color of plasma, prepared after leaving blood on ice for longer periods, than of plasma prepared from the same blood immediately, supports this explanation. Alternatively, cryoprecipitation of plasma proteins might also be a cause of increases in CA concentrations.

CA are stable for a year in plasma stored at −70 °C, although there is a small but significant downward trend for NE. The results are slightly better in HPG than in HP, suggesting that the addition of extra glutathione is preferable. In urine, even if unpreserved, CA are more stable than in plasma. This is probably due mostly to the much higher concentrations, which are inherently more stable than low concentrations (19). The fact that CA are stable in urine at 20 °C for at least 4 days, even without preservatives, means that collection of 24-h portions can be done without cooling between collection times, and preservatives can be added later, if deemed necessary. For long-time storage, preservation by acidification gives best results, and storage at −20 °C is sufficient.

When the antioxidative capacity (whether inherent or as a result of added preservatives) is near its end, a chaotic process may ensue and results may vary greatly. In one experiment we measured CA in unpreserved urine in five replicates after 3 weeks at 20 °C and found values for NE, E, and DA that varied from 5% to 90% of the original concentrations. No such variation was found in similar experiments with urine preserved by acidification or by EDTA and sodium metabisulfite.

Two things should be kept in mind when interpreting the results of this study. First, the various conditions have been studied singly. In combination, e.g., leaving plasma for 4 days in a refrigerator and then storing at −20 °C, breakdown of CA may be much more accelerated than when stored without delay at −20 °C. Second, the samples were all measured once after the first thawing. Repeated thawing and freezing will greatly increase the breakdown of CA.

The results of this study agree with some previous reports but are at variance with others. The main reason for the discrepancies is probably that we used highly sensitive and reproducible methods for the determination of CA concentrations, whereas especially in some of
the older studies the methods used were too insensitive to detect small decreases in CA concentrations.

Recommendations

In summary, our results lead us to the following recommendations:

**Plasma preparation and transport**
- Blood should be centrifuged within 1 h after collection.
- A refrigerated centrifuge is not necessary for the preparation of plasma.
- For measurements within 4 weeks, heparinized plasma is good enough; thus, blood can be collected into standard tubes.
- Extra glutathione is necessary only when prolonged storage is foreseen at −20 °C and is advantageous for storage at −70 °C; when it is used, it could be added after centrifugation.
- During transport, plasma samples should not be left more than 1 day at 20 °C or 2 days at 4 °C. Transport by mail should be in cooled containers or on solid CO₂.

**Plasma storage**
- Plasma should be stored no longer than 1 month (heparinized plasma) or 6 months (with extra glutathione) at −20 °C.
- Plasma CA are stable up to 1 year at −70 °C, preferably in heparinized plasma with extra glutathione.

**Urine collection and storage**
- Because CA are stable even in unpreserved urine for 4 days, refrigeration between collection periods is not necessary (at least not for this reason).
- Acidification is the best method for prolonged preservation (almost no change for up to 1 year even at 4 °C).
- With EDTA and sodium metabisulfite as preservatives, CA show only a very slight downward trend in the course of 1 year.
- Preservatives can be added after collection is completed.

The results of this study show that it is not necessary to have access to specially prepared, nonstandard blood-collection tubes, refrigerated centrifuges, and ultrafreezers to prepare and temporarily store plasma and urine samples for reliable CA measurements. However, in view of the chaotic degradation process that may occur when the antioxidative capacity is near its end, it seems advisable to minimize as much as possible any destabilizing conditions.

References