Stability of Plasma Free Metanephrines during Collection and Storage as Assessed by an Optimized HPLC Method with Electrochemical Detection, *Jacques J. Willemsen,[1] C.G.J. (Fred) Sweep,[1] Jacques W.M. Lenders,[2] and H. Alec Ross[1,3*]* (Departments of [1] Chemical Endocrinology, [2] General Internal Medicine, and [3] Endocrinology, University Medical Center Nijmegen, 6500 HB Nijmegen, The Netherlands; * address correspondence to this author at: Department of Chemical Endocrinology 530, University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands; fax 31-24-3541484, e-mail a.ross@ace.umcn.nl)

There is substantial evidence that plasma concentrations of the free (unconjugated) metanephrines metanephrine (MN) and normetanephrine (NMN) are better than other indices of catecholamine excess for detecting pheochromocytomas *(1–3)*. However, it currently is unknown how stable these compounds are after blood collection and after separation of plasma as well as during storage. To investigate this, we modified the original method by Lenders et al. *(4)*, which consists of HPLC with electrochemical detection, preceded by a prepurification step on cation-exchange columns, to increase the procedural recovery and thereby sensitivity. The principal adjustments were as follows:

Before plasma was passed through the cation-exchange column, 1 mL of Aqua Dest and 135 μL of a solution of 0.2 mol/L ammonium acetate (pH 6.0) were added to 1 mL of plasma. The 135 μL of ammonium acetate solution included 100 μL of internal standard solution [108.7 nmol/L 3-ethoxy-4-hydroxyphenylethanolamine oxalate (EHPEA)] and 35 μL of ammonium acetate containing, only for addition experiments, MN and NMN. The calibrator mixture consisted of 19.5 nmol/L MN, 18.1 nmol/L NMN, and 108.7 nmol/L EHPEA in the aforementioned ammonium acetate solution, of which 140 μL was injected directly. After column elution, dried residues were dissolved in 150 μL of the ammonium acetate solution, of which 140 μL was injected. In all plasma samples assayed, MN and NMN peaks were completely separated from surrounding peaks by virtue of a slight increase in polarity of the mobile phase.

Within-assay SDs, estimated from duplicate measurements of various samples (n = 11) containing 98–351 pmol/L MN and 129–350 pmol/L NMN, were 12.3 pmol/L for MN and 11.6 pmol/L for NMN (mean CVs, 7.0% and 4.5%, respectively). Single measurements of a control sample in 15 separate runs gave mean (SD) values of 207 (22.9) pmol/L (CV, 11%) for MN and 277 (22.1) pmol/L (CV, 8%) for NMN. After the addition of 507 pmol/L MN and 546 pmol/L NMN, we obtained between-assay CVs (n = 5) of 6.4% and 6.8%, respectively. Mean (SD) recoveries of these additions to this control sample and to four different plasma samples from healthy volunteers (basal values, 107–176 pmol/L MN and 179–297 pmol/L NMN) were 99.5 (6.8)% for MN and 98.8 (4.6)% for NMN. Addition to the control plasma of 3803 pmol/L MN and 4094 pmol/L NMN yielded mean (SE) recoveries 98.3 (1.5)% (n = 10) for MN and 99.1 (1.6)% for NMN. Because these analytical recoveries were virtually equal to 100%, the procedural recovery of the internal standard EHPEA of 88.5 (3.5)% (n = 76) also held for MN and NMN.

We evaluated the linearity of the assay by assaying mixtures of two samples from healthy controls with relatively high (351 pmol/L MN and 350 pmol/L NMN) and low (98 pmol/L MN and 129 pmol/L NMN) concentrations in fixed proportions (0 + 1, 1 + 3, 1 + 1, 3 + 1, and 1 + 0 parts) *(5)*. We observed no significant nonlinearity. The scatter about the regression line was 5.8 pmol/L for MN and 16.2 pmol/L for NMN.

For testing the short-term stability of metanephrines in whole blood, we collected 40 mL of blood by venipuncture from six healthy volunteers in the sitting position into 10 heparin-containing tubes, 2 of which were immediately centrifuged at 1500*g*: 1 at room temperature, and the other at 4 °C. The other tubes were kept for 1, 2, 4, and 6 h at either room temperature or at 4 °C (refrigerator) before centrifugation. After centrifugation, 1.1 mL of plasma was immediately transferred to tubes containing 10 μL of a solution (EGTA/GL) of EGTA (625 mmol/L) and glutathione (500 mmol/L), after which the samples were stored at −80 °C until being assayed. From the same volunteers, we collected another 30 mL of blood into heparin-containing tubes. This blood was divided into two portions that were centrifuged immediately: one at room temperature and one at 4 °C. These temperatures were maintained during subsequent separation and aliquoting of plasma as above and when kept for 4, 24, 48, and 72 h before final storage at −80 °C.

We collected 100 mL of blood from each of another five healthy volunteers by venipuncture, in the sitting position, into 10 precooled heparin-containing 10-mL tubes on melting ice. The blood was centrifuged (10 min at 1500*g* and 4 °C) immediately after collection, and the plasmas from all tubes from each volunteer were combined. Of this combined plasma, we stored 1.1-mL portions in tubes containing EGTA/GL for 0, 1, 2, 4, 8, 16, and 32 days at 4 or −20 °C until final storage at −80 °C. The remaining material from all volunteers was mixed to obtain a pooled plasma. This pooled plasma was divided into two portions, one of which was stored in 1.1-mL aliquots in tubes containing 10 μL of EGTA/GL, whereas the other portion was distributed likewise into tubes containing only 10 μL of 625 mmol/L EGTA in distilled water (pH 7.4). Both pooled plasmas finally were stored at −80 °C after 0, 1, 2, 4, 8, 16, and 32 days at 4 or −20 °C.

Tests for stability were evaluated by means of repeated-measures ANOVA using SPSS, Ver. 10. A possible effect of the addition of glutathione was tested by ANOVA.

The top panel in Fig. 1 shows that in whole blood, a short period of 15 min at room temperature before centrifugation did not seem harmful for either MN or NMN. Subsequently, the concentrations NMN increased rapidly, the increase being significant after 1 h, and decreased thereafter, whereas MN decreased significantly after 2 h to values approximately one-half of the original concentrations within 6 h after venipuncture. In contrast, at 4 °C, both analytes remained stable for the whole 6-h period.

After centrifugation (middle panel in Fig. 1), NMN

could be kept at least for 1 day at room temperature, whereas MN decreased significantly after 4 h, with concentrations decreasing to ~30% of the original value after 1 day. At 4 °C, both NMN and MN were significantly decreased after 2 days, although by only 4% and 5%, respectively. We detected no further decrease for up to 3 days. After 16 days of storage at 4 °C (Fig. 1, bottom panel), NMN concentrations had decreased significantly by 10%, whereas MN showed a decrease at 4 days after which the concentrations were largely restored at 8 days. We detected no further significant decrease for up to 32 days. Measurements in samples that had been kept at −20 °C showed no detectable change during the whole 32-day period.

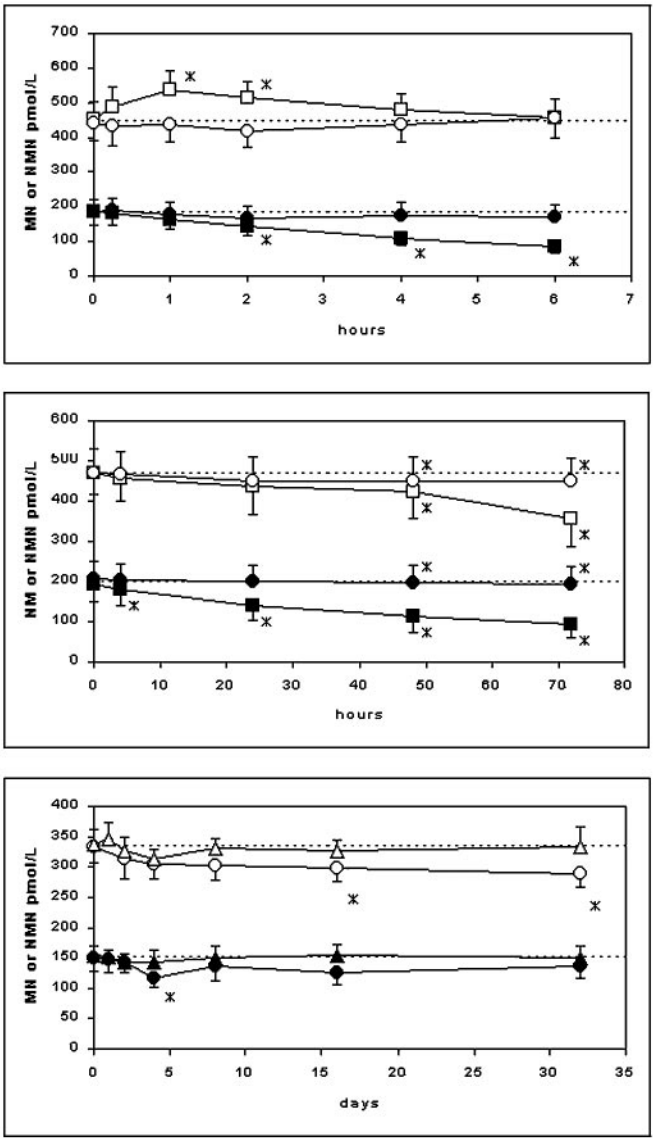

Fig. 1. Effect of collection and storage conditions on concentrations of metanephrine.

Squares indicate storage at room temperature, *circles* indicate storage at 4 °C, and *triangles* indicate storage at −20 °C. *Closed symbols* indicate MN; *open symbols* indicate NMN. *Dashed lines* indicate the mean initial concentrations; ∗, significant differences ($P < 0.05$) with respect to initial concentrations. (*Top*), mean (SE; *error bars*) concentrations of metanephrines vs time before centrifugation, reflecting (in)stability in whole blood at room temperature and 4 °C. (*Middle*), mean (SE; *error bars*) concentrations of metanephrines vs time after centrifugation, for storage at room temperature and 4 °C. (*Bottom*), mean (SE; *error bars*) concentrations of metanephrines vs time after centrifugation, for prolonged storage at 4 and −20 °C.

The overall within-run CV, as estimated from triplicate measurements in fresh samples, was 5.6% and 3.6% for MN and NMN, respectively. No effect of addition of the reducing agent glutathione to pooled plasma was detected that could not be accounted for by this within-run variation.

The use of ammonium acetate as a medium for introduction of the sample on both cation-exchange and HPLC columns improved the procedural recovery, which is the major determinant of assay precision, by a factor of 1.4 with respect to the original method.

The stability of catecholamines in whole blood and plasma has been studied by Boomsma et al. *(6)*, but to date, the stability of metanephrines has not been studied systematically. Apparently MN and NMN do not always show the same pattern. In whole blood, at room temperature NMN increases first, whereas MN begins to decrease within the first 2 h. Remarkably, such an initial increase also was reported by Boomsma et al. *(6)* for norepinephrine and was ascribed to release of the hormone from red blood cells. This seems to hold as well for its primary metabolite, NMN, which might be generated by catechol-*O*-methyltransferase present in red blood cells. Interestingly, the transient increase in norepinephrine occurred at 4 °C in that report, whereas at room temperature only degradation was observed. The increase in NMN in the present study, however, was observed at room temperature. This might be explained by a lower degradation rate of NMN than of norepinephrine at room temperature, whereas red cell catechol-*O*-methyltransferase may be less active at 4 °C. Apparently, spuriously increased values are obtained under those conditions (maximum increase observed, 42%) that might lead to misinterpretation, so that handling of whole blood at room temperature would better be avoided, especially because both metanephrines appear to be stable at 4 °C in whole blood for at least 6 h before centrifugation. This may be advantageous in situations where blood samples have to be collected when refrigeration is possible but centrifugation services are not immediately accessible. After separation of plasma, both MN and NMN degrade at room temperature (Fig. 1, middle panel). The decrease in MN (9%) after the first 4 h is significant, and the concentration decreases to less than one-half of its original value after 3 days. At 4 °C, some decrease is detectable after 2 days, although the actual decreases are only 4% for MN and 5% for NMN after 3 days. Indeed, in the longer-term stability study (Fig. 1, bottom panel) this decrease was not detected. After 2 weeks, NMN had decreased significantly by 10%, and MN showed a peculiar but highly significant decrease at 4 days for which no explanation could be given. One may conclude that cooled plasma samples may be shipped without the obvious need for addition of a reducing agent if this does not take more than 3 days. This is of practical relevance because at present the assay is not widely available. Finally,

both MN and NMN are stable for at least 1 month when frozen at −20 °C, also in the absence of a reducing agent.

On the basis of the present data, we give the following recommendations for handling, storage, and shipment: Blood should be kept at 4 °C and must be centrifuged within 6 h. Even in the absence of a reducing agent, plasma can be kept at 4 °C for 3 days without appreciable degradation and shipped if this does not take more than the same time span. Storage or shipment of longer duration must be at −20 °C or lower.

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Use of Magnetic Beads for Plasma Cell-free DNA Extraction: Toward Automation of Plasma DNA Analysis for Molecular Diagnostics, *Christine Stemmer,[1*] Michèle Beau-Faller,[1] Erwan Pencreac'h,[1] Eric Guerin,[1] Anne Schneider,[1] Didier Jaqmin,[2] Elizabeth Quoix,[3] Marie-Pierre Gaub,[1] and Pierre Oudet[1]* ([1] Service de Biochimie et Biologie Moléculaire, Hôpital de Hautepierre Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France; [2] Service de Chirugie Urologique and [3] Service de Pneumologie Lyautey, Hôpitaux Universitaires de Strasbourg, 67000 Strasbourg, France; *address correspondence to this author at: Service de Biochimie et Biologie Moléculaire, Hôpital de Hautepierre, Hôpitaux Universitaires de Strasbourg, 67098 Strasbourg cedex, France; fax 33-388127539, e-mail christine.stemmer@wanadoo.fr)

Urine, breast milk, plasma, and serum have been shown to contain cell-free DNA *(1–7)*. For plasma DNA detection, several recent studies addressed the need for careful evaluation and standardization of preanalytical processes *(8–12)*. Key problems appear, such as possible contamination of plasma by white blood cells; the generally low and variable amount of circulating DNA, making extraction/quantification difficult and time-consuming; poor DNA quality; and the presence of PCR inhibitors. In any case, automation of DNA extraction, which is a prerequisite for introduction of these diagnostic approaches in clinical laboratories, is difficult to achieve because of the volumes of plasma necessary to get sufficient DNA.

In this study (summarized in Fig. 1), we propose a new semiautomated, time-saving process for extraction of plasma cell-free DNA that provides high yields and is suitable for PCR amplification.

Two 5-mL blood samples from each of 23 patients being monitored for lung (n = 19) or colon cancer (n = 4) and 20 healthy controls were collected in EDTA-containing tubes. Informed consent was obtained for each. Blood was centrifuged at 800*g* for 10 min, and the collected plasma was transferred to a 15-mL BD Falcon™ polypropylene tube for an additional centrifugation step of 10 min at 1500*g* to remove any remaining leukocytes and platelets. An equal volume of 2× concentrated proteolytic buffer (2×: 20 mmol/L Tris-HCl, 50 mmol/L EDTA, 200 mmol/L NaCl, 10 g/L sodium dodecyl sulfate, and 400 mg/L proteinase K) was added to the plasma, mixed, and incubated 1 h at 37 °C. After digestion, plasma samples were concentrated by centrifugation at room temperature (to avoid sodium dodecyl sulfate precipitation) for 20 min at 2600*g* in Amicon Ultra-15 filtration devices (Millipore). The concentrated plasmas were stored at −20 °C until DNA extraction (Fig. 1, step 1).

DNA was extracted with use of KingFisher silicate magnetic beads and a KingFisher ML robotic magnetic particle processor (ThermoLifeSciences) according to the manufacturer's protocol. DNA was quantified by fluorometry (Fluoroskan; ThermoLifeSciences) with Picogreen reagent (Molecular Probes) as recommended by the manufacturer. For plasma DNA extraction, slight modifications were introduced in the protocol: up to 300 μL of concentrated plasma was mixed with 950 μL of lysis buffer together with 80 μL of magnetic beads, and DNA was eluted in Tris-EDTA for 20 min. For comparison purposes, plasma DNA was extracted with use of the QIAamp DNA Midi reagent set (Qiagen), according to the

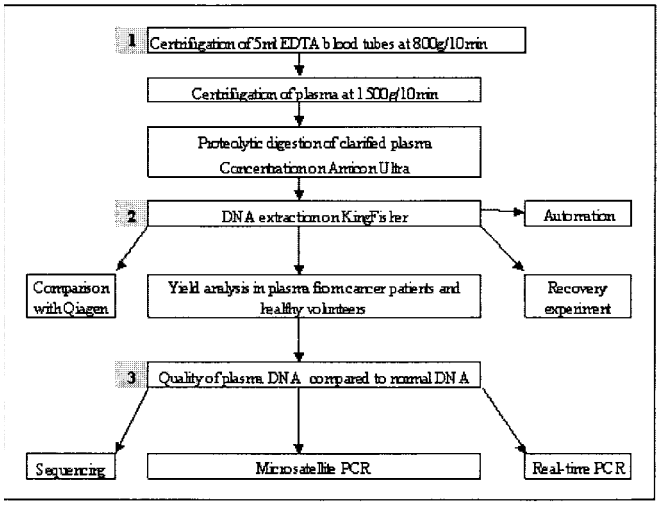

Fig. 1. Experimental design.

Step 1, plasma treatment; *step 2*, DNA extraction; *step 3*, analysis of plasma DNA quality by PCR.