Sandwich Immunoassay for Bioactive Plasma Adrenomedullin

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Background: Adrenomedullin (ADM) is a circulating peptide known to regulate vasodilation and vascular integrity. Increased plasma ADM concentrations have been described for several life-threatening conditions, including cardiovascular diseases and septic shock. Reliable methods for the simple quantification of bioactive ADM (bio-ADM) are lacking.

Methods: Monoclonal antibodies against the amidated C-terminus and middle portion of bio-ADM were generated and used for the development of a 1-step immunometric assay for the specific quantification of bio-ADM in plasma. The assay was developed in a microtiter plate/chemiluminescence label format with a significantly reduced incubation time. Precision, linearity, specimen stability, and distribution of results in healthy subjects were evaluated.

Results: The use of monoclonal antibodies against predetermined epitopes of bio-ADM enabled the development of an assay for the determination of bio-ADM directly in EDTA plasma. Plasma samples were stable for up to 24 h at ambient temperature and over multiple freeze–thaw cycles without loss of immunoreactivity. The assay had a limit of detection of 3 pg/mL and a limit of quantification of 11 pg/mL. The assay exhibited acceptable linearity characteristics and was not influenced by complement factor H, a putative ADM-binding protein. In healthy subjects, bio-ADM concentrations were all above the limit of detection, and approximately half of them were above the limit of quantification.

Conclusions: By using monoclonal antibodies with defined epitope specificities, we have developed a simple, rapid, accurate, and sensitive sandwich immunoassay for bio-ADM. The assay is a potentially novel tool to support patient management, particularly in acute care in the field of sepsis and other indications, which are currently being investigated, such as acute heart failure.

IMPACT STATEMENT

The assay technology described enables for the first time, to the best of our knowledge, a rapid direct measurement of the biologically active form of plasma adrenomedullin. Because adrenomedullin is a key player in acute cardiac diseases and septic shock, the use of this assay could affect physician decision-making in these fields.

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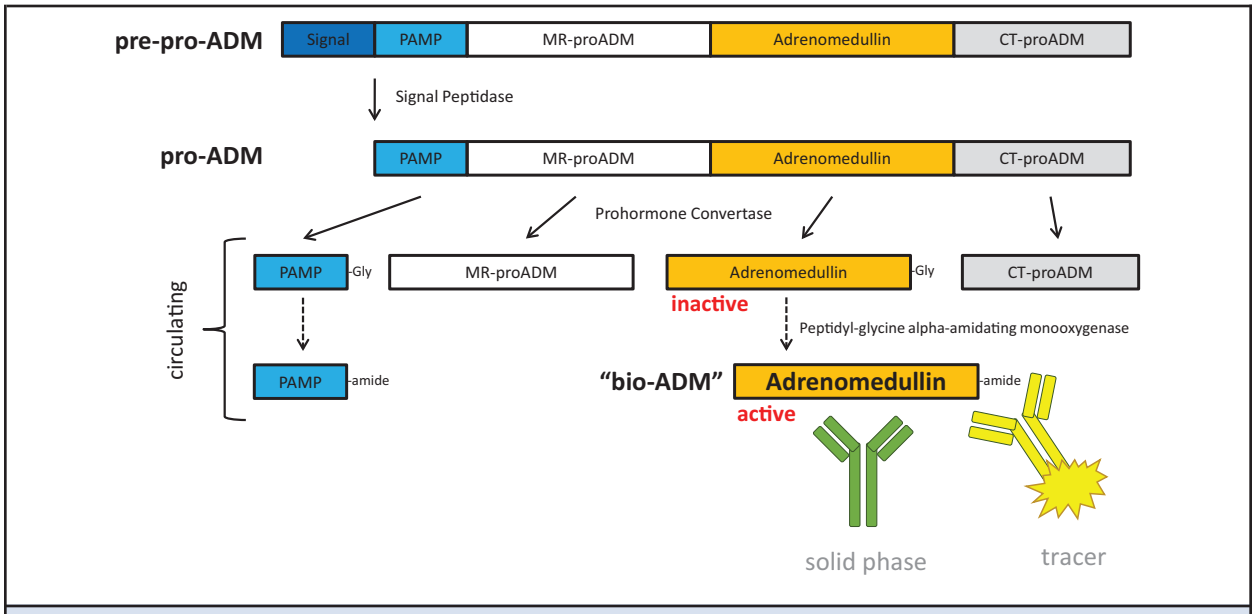

Fig. 1. Scheme for biogenesis and measurement of bio-ADM.
The scheme depicts the various processing steps, which lead from the primary translational gene product pre-pro-ADM to biologically active ADM (bio-ADM) and has been adapted from previously published figures (*2*, *22*, *23*, *39*). First in the maturation process, the signal sequence is clipped off. The resulting pro-ADM is then proteolytically cleaved in 4 fragments (PAMP, proadrenomedullin NH_2-terminal 20 peptide; MR-proADM, midregional proadrenomedullin; Adrenomedullin-Gly, C-terminally glycine-extended adrenomedullin; CT-proADM, C-terminal proadrenomedullin, also known as adrenotensin). The resulting C-terminally glycinated ADM is biologically inactive and is subsequently (but only partially) converted into the biologically active C-terminally amidated ADM (bio-ADM). Using highly specific monoclonal antibodies directed against the middle portion of ADM and the amidated C-terminus, bio-ADM can be detected with an immunometric assay.

Adrenomedullin (ADM) is a circulating peptide comprising 52 amino acids and it was first isolated from pheochromocytoma (*1*). A plethora of biological functions has been described for ADM, with the most prominent being its vasodilatory activity (*2*). Another important function of ADM is the stabilization of the endothelial barrier and the prevention of vascular leakage (*3*, *4*). Plasma concentrations of ADM are increased in various pathologies including cardiac diseases (*5*–*9*), chronic renal failure (*10*, *11*), and—the most pronounced—in septic shock (*9*, *12*–*18*).

Several assay systems for measuring ADM have been described, but they are unreliable (*19*), as they may be influenced by the ADM-binding protein (complement factor H) (*20*) and suffer from instability of ADM (*21*). In addition, several of the described assays require a large sample volume, a preanalytical sample extraction, and a long incubation time to achieve sufficient analytical sensitivity.

To circumvent the reported problems associated with the measurement of ADM, midregional pro-adrenomedullin (MR-proADM) has been introduced as an alternative surrogate marker (*22*, *23*). Although MR-proADM and ADM stem from the same precursor peptide, the stoichiometric relationship between the two is imperfect: Following the proteolytic fragmentation of the precursor peptide, ADM is subject to a crucial maturation step, the C-terminal amidation (Fig. 1), which occurs only

[3] **Nonstandard abbreviations:** ADM, adrenomedullin; ADM-Gly, C-terminally glycine-extended ADM; bio-ADM, bioactive adrenomedullin; LOD, limit of detection; LOQ, limit of quantification; MR-proADM, midregional proadrenomedullin.

partially and to a varying extent (*24*). Only the amidated-ADM variant (termed bio-ADM) can bind to its receptor with high affinity and efficiently induce cAMP formation (*25*). Moreover, nothing is known about how clearance kinetics compare between bio-ADM and MR-proADM, and it must be assumed that they differ. Taken together, conceptually it would be preferable to measure bio-ADM rather than other derivatives of the ADM precursor peptide because bio-ADM is likely to have the closest association with the clinical status of patients.

A prototype bio-ADM assay with similar reagent components as the bio-ADM assay described here was recently reported, but was not suitable for routine use (*26*, *27*). This prototype assay used tubes instead of a microtiter plate as solid phase and had a long incubation time. Here we set out to develop and technically validate a simple and reliable immunoassay with a considerably shorter incubation time for the sensitive and specific detection of bio-ADM.

MATERIALS AND METHODS

Peptides

ADM-related peptides were synthesized for immunization, screening of hybridoma cell lines, and/or epitope mapping (JPT Technologies). If no cysteine was present in the selected naturally occurring amino acid sequence, an additional N-terminal cysteine residue was introduced for conjugation. Peptides used for epitope mapping were biotinylated at their N-terminus. The following peptides were (name, amino acid sequence, position within ADM) chosen: P21–32, CTVQKLAHQIYQ-OH, 21–32; P42–52, CAPRSKISPQGY-NH_2, 42–52 (plus N-terminal cysteine); P33–52+G, biotin-FT-DKDKDNVAPRSKISPQGYG-OH, 33–52+G; P33-52COOH, biotin-FTDKDKDNVAPRSKISPQGY-OH, 33–52; P33-52NH_2, biotin-FTDKDKDNVAPRSKISPQGY-NH_2, 33–52; P33-51, biotin-FTDKDKDNVAPRSKISPQG-OH, 33–51; P33-50, biotin-FTDKDKDNVAPRSKISPQ-OH, 33–50. Full-length, C-terminally amidated human ADM 1–52 was purchased (American Peptide Company) and used for screening and as calibrator. The following peptides were purchased from Bachem AG: human calcitonin gene-related peptide (α CGRP), human ADM-2 (intermedin), human calcitonin, and human amylin.

Antibodies

Monoclonal antibodies directed against peptides P21–32 and P42–52 were generated by standard procedures (*28*, *29*). In brief, peptides were conjugated to BSA using sulfo-MBS (m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester). Balb/c mice were immunized and boostered with these conjugates; spleen cells were fused with SP2/0 myeloma cells to generate hybridoma cell lines. From the immunization with peptide P42-52, we screened cell lines for their ability to secrete antibodies that would bind to full-length ADM but negligibly to peptide P33-52+G. With this approach, we selected a hybridoma cell line secreting monoclonal antibody HAM2302 (particularly directed against the amidated C-terminus of ADM). From the immunization with peptide P21-32, cell lines were screened for their ability to secrete antibodies that would bind to full-length ADM. With this approach, we selected a hybridoma cell line secreting monoclonal antibody HAM2203 (against the middle portion of ADM). A third monoclonal antibody, which is directed against the N-terminal moiety of ADM, termed HAM1101, has been described previously (*30*).

Antibodies were produced by standard procedures and purified via protein A chromatography to obtain >95% purity as judged by capillary gel electrophoresis.

The affinity of the antibodies to bio-ADM was determined with label-free surface plasmon resonance using a Biacore 2000 system (GE Healthcare Europe).

The epitope specificity of the antibody HAM2302 was assessed as follows: purified antibodies were

Table 1. Epitope mapping of the anti-C-terminal ADM monoclonal antibody HAM2302 used in the bio-ADM assay.

Peptide #	Amino acid sequence	Position in ADM	$B^a/B_{P33\text{-}52NH2}$
P33-52+G	biotin-FTDKDKDNVAPRSKISPQGYG-OH	33-52+G	0.09%
P33-52COOH	biotin-FTDKDKDNVAPRSKISPQGY-OH	33-52	0.03%
P33-52NH_2	biotin-FTDKDKDNVAPRSKISPQGY-NH_2	33-52	100.00%
P33-51	biotin-FTDKDKDNVAPRSKISPQG-OH	33-51	0.02%
P33-50	biotin-FTDKDKDNVAPRSKISPQ-OH	33-50	0.02%

[a] The binding (B) of labeled antibody on tubes coated with the indicated peptides was analyzed. The peptides were N-terminally biotinylated and immobilized on tubes coated with streptavidin

labeled by incubation with MACN-acridinium-NHS (N-hydroxysuccinimide)-ester (1 g/L; InVent) in a 1:5 mol/mol ratio for 30 min at 22 °C. The reaction was stopped by addition of 1/20 volume of 1 mol/L Tris-HCl, pH 8.0, for 10 min at 22 °C. Labeled antibodies were separated from free label by size-exclusion chromatography on a CentriPure P5 column (emp Biotech) and a Bio-Silect® SEC 400-5 column (Bio-Rad Laboratories) for HPLC. Polystyrene microtiter plates (Greiner) were coated with streptavidin (Sigma-Aldrich) as follows: 250 µL of a 10 µg/mL streptavidin solution in 50 mmol/L Tris and 100 mmol/L NaCl, with pH 7.8, was pipetted into each well. After 20 h incubation at 22 °C, solutions were aspirated, and wells were washed 3 times with 300 µL PBS buffer containing 5 g/L BSA per well. Biotinylated peptides, as listed in Table 1, were immobilized on microtiter plates as follows: peptides were diluted to a final concentration of 1 mg/L in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) containing 5 g/L BSA, and 250 µL were pipetted into each well. After 3 h incubation at 22 °C and orbital agitation at 1.2 g, solutions were aspirated, and the microtiter plates were washed 3 times with 300 µL of 10 mmol/L Na-phosphate, 3% Karion FP, 0.5% BSA, with pH 6.5, and the last washing step incubated for 1 h at 22 °C. The chemiluminescence-labeled antibody was diluted in buffer (300 mmol/L K-phosphate, 100 mmol/L NaCl, 10 mmol/L Na-EDTA, 5 g/L BSA, 1 g/L unspecific mouse and bovine IgG, 50 µmol/L amastatin, 100 µmol/L leupeptin, 0.1% Na-azide, pH 7.0) to a concentration of 10 ng/200 µL per well, and 200 µL of this tracer solution was added and incubated for 18 h at 22 °C with orbital agitation at 1.2 g. Each well of the microtiter plate was washed 5 times with 350 µL of washing solution (20 mmol/L PBS, 1 g/L Triton X-100, pH 7.4) and the remaining chemiluminescence was measured for 1 s per well with a Centro LB 960 microtiter plate luminescence reader (Berthold Technologies).

Chemiluminescent Immunoassay for the quantification of bio-ADM

Labeled Compound (Tracer). Purified monoclonal anti-P42–52 antibody (HAM2302, 1 g/L) was labeled by incubation in 10% labeling buffer (500 mmol/L sodium phosphate, pH 8.0) with 1:4.5 mol/L ratio of MACN-acridinium-NHS-ester (1 g/L, InVent GmbH) for 20 min at 22 °C. After adding 5% 1 mol/L Tris-HCl, pH 8.0, for 10 min, HAM2302 was separated from free label via CentriPure P10 columns (emp Biotech GmbH) and by size-exclusion HPLC on Protein KW-803 (Shodex, Showa Denko Europe). The purified HAM2302 was lyophilized as a 9-fold concentrate in 50 mmol/L HEPES, 4.5% BSA, 0.9% bovine IgG, 0.18% mouse IgG, 450 µmol/L amastatin, 900 µmol/L leupeptin. The product was reconstituted in buffer before use (150 mmol/L HEPES, 10 mmol/L Na-EDTA, pH 6.8). The final concentration was approximately 20 ng of labeled antibody per 150 µL.

Solid phase

White polystyrene microtiter plates (Greiner Bio-One International AG) were coated (18 h at 20 °C) with monoclonal anti-P21–32 antibody (HAM2203, 1 µg/0.2 mL per well 50 mmol/L Tris-HCl, 100 mmol/L NaCl, pH 7.8). After blocking with 30 g/L Karion, 5 g/L BSA (protease free), 6.5 mmol/L monopotassium phosphate, 3.5 mmol/L sodium dihydrogen phosphate (pH 6.5), the plates were vacuum-dried.

Calibration

The assay was calibrated using dilutions of synthetic human bio-ADM (American Peptide Company). The lowest calibrator did not contain bio-ADM, but a concentration of 2 pg/mL was assigned to facilitate logarithmic evaluation. The calibrators were lyophilized in 20 mmol K_2PO_4, 6 mmol/L Na-EDTA, 5 g/L BSA, 100 µmol/L leupeptin, 50 µmol/L amastatin, 10 µg/mL of anti-N-terminal antibody HAM1101 (*30*, *31*), pH 8.0, and reconstituted in H_2O before use.

Assay parameters

To start the assay, 100 µL of samples/calibrators were pipetted into coated microtiter plates. After adding 150 µL of labeled HAM2302, the microtiter plates were incubated for 1 h at 22 °C under agitation. Unbound tracer was removed by washing 5 times (each 350 µL per well) with washing solution (20 mmol/L PBS, 1 g/L Triton X-100, pH 7.4). Well-bound chemiluminescence was measured for 1 s per well by using the Centro LB 960 microtiter plate luminescence reader (Berthold Technologies).

Interassay precision

The interassay precision profile was determined from 12 independent runs of native patient samples containing a spectrum of different endogenous bio-ADM concentrations. Before the measurements, the samples were divided into 12 aliquots each, which were stored at −80 °C until measurement. The 12 runs were performed by 12 different operators on 2 consecutive days. For each run, 1 microtiter plate was used for calibrators and samples. Measurements were performed in duplicate. Out of this analysis, the limit of quantification (LOQ, functional assay sensitivity), defined as the bio-ADM concentration quantifiable with a CV of 20%, was determined. The limit of detection (LOD) was determined as the upper 2 SD of the lowest (i.e., zero) calibrator.

Interference study

Complement factor H, purified from human plasma (>97% pure by SDS-PAGE; #BP10-15-1106), was purchased from BIOPUR AG. Serial dilutions of complement factor H were incubated for 1 h at 22 °C with a constant concentration of synthetic human bio-ADM (200 pg/mL) in PBS buffer, pH 7.4, containing 0.5% BSA, 50 µmol/L amastatin, and 100 µmol/L leupeptin, and bio-ADM was measured thereafter with the assay described in the immunoassay section. Recovery calculations were corrected for the effect of the buffer background coming from the complement factor H preparation. The influence of complement factor H on bio-ADM recovery was tested up to an almost 400 000-fold molar excess of complement factor H over ADM.

Samples

Residual, anonymized EDTA plasma samples initially collected for routine laboratory and clinical studies conducted in the Gemeinschaftslabor Cottbus, Germany, were provided. These were from patients with normal and increased bio-ADM concentrations. Normal-range samples were collected from 88 apparently healthy subjects without clinical evidence of acute disease or history of chronic illness [57 females, 31 males, mean age (SD): 42.2 (12.7) years]. Written consent was obtained from all participants.

Statistical analysis

All statistical analyses were conducted with GraphPad Prism 7.0.

RESULTS

Selection of antibodies

For the sandwich immunoassay, we developed monoclonal antibodies directed against the middle portion (HAM2203) and the amidated C-terminal moiety of bio-ADM (HAM2302). HAM2203 and HAM2302 were of the IgG1 subtype and had affinity constants of 2×10^{-9} M and 1.1×10^{-9} M, respectively, as determined by surface plasmon resonance spectroscopy (data not shown). The epitope specificity of the antibody against the C-terminal moiety (HAM2302) was characterized in detail (Table 1) and showed that the antibody was highly specific for the free amidated C-terminus, as it exists in bio-ADM. Further <0.1% binding was observed against closely related peptides, i.e., a carboxylated instead of amidated peptide, a glycine-extended variant, and several C-terminally truncated variants.

Immunoassay

We developed a microtiter plate-based chemiluminescence immunometric assay for bio-ADM using the HAM2203 antibody directed against the middle portion of bio-ADM as solid-phase antibody and the HAM2302 antibody directed against the amidated C-terminus of ADM as tracer antibody. Dilutions of synthetic bio-ADM peptide served as calibrators, covering a concentration range up to 1094 pg/mL. It turned out that addition of the anti-N-terminal ADM antibody HAM1101 described previously (*30*, *31*) did not interfere with recovery of bio-ADM in the assay. This finding had two implications. First, this antibody could be added in the preparation of the calibrators to avoid loss of bio-ADM peptide, which would otherwise stick to surfaces (*19*). Second, the assay could be used to not only measure free bio-ADM but also complexed bio-ADM when HAM1101 antibody (or the humanized derivative HAM8101) is bound to the N-terminus of bio-ADM, which can form after in vivo administration (*30*). This is of interest, as HAM8101, also known as Adrecizumab, is currently being clinically developed as a drug candidate, and the bio-ADM assay can be used in this development to assess changes of total bio-ADM concentrations after administration of HAM8101.

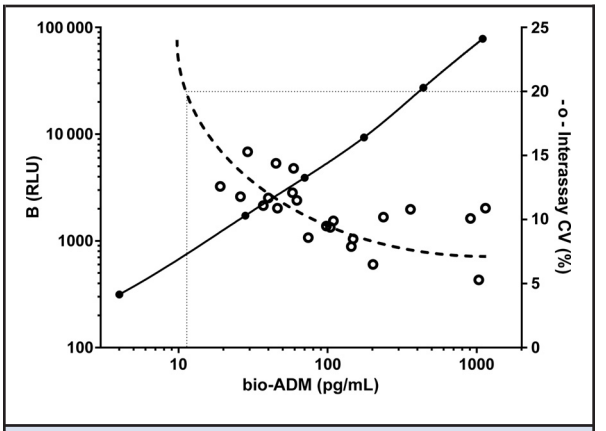

Fig. 2. Typical dose–response curve and interassay precision profile of the bio-ADM assay. The bio-ADM concentration corresponding to 20% CV is indicated (LOQ; functional assay sensitivity).

Plasma (100 µL) was incubated in a 1-step procedure with 150-µL labeled antibody on the microtiter plate for 1 h at 22 °C. A typical dose–response curve along with the obtained interassay precision profile is shown in Fig. 2. From the interassay precision profile, the quantitative measuring range of the assay (concentrations measurable with an interassay CV of 20% or lower) was deduced to be 11–1094 pg/mL. The LOQ, functional assay sensitivity, was 11 pg/mL. The LOD of the assay was determined at 3 pg/mL.

ADM belongs to a family of peptides including calcitonin gene-related peptide (CGRP), ADM-2 (intermedin), calcitonin, and amylin. A common structural feature of these peptides is the presence of a

Table 2. Linearity of the bio-ADM immunoassay as determined by dilution experiments.

Panel A[a]			Panel B[b]	
Sample A bio-ADM (pg/mL)	Sample B bio-ADM (pg/mL)	Recovery of expected for 1 + 1 dilution of sample A with sample B (%)	bio-ADM (pg/mL) in undiluted sample	Mean recovery of expected for 3 serial dilutions of samples (dilution factors 2, 4, and 8) (%)
84.4	21.4	90.5	794.2	97.9
110.3	11.4	93.5	676.5	100.1
73.6	56.2	102.2	312.3	97.9
68.7	25.9	90.4	321.6	97.9
80.9	22.3	100.2	290	94.6
88.2	21.4	112.5	439.7	101.5
122.2	21.4	88.5	254.6	98.1
84.4	11.4	104.6	252.5	103.8
110.3	56.2	96.9	287.9	102.2
73.6	25.9	107.2	283.5	101.9
68.7	22.3	93.7		mean: 99.9
80.9	11.9	99.4		
257.4	21.4	86.8		
88.2	25.9	100.9		
		mean: 97.7		

[a] Native EDTA plasma samples were measured in the bio-ADM assay individually; pairs of these samples were mixed 1 + 1, and experimentally measured bio-ADM values were related to theoretically expected values.
[b] Other native EDTA plasma samples with high bio-ADM concentrations were serially diluted with human plasma with a known low bio-ADM concentration (6 pg/mL) by a factor of 2, 4, and 8, and the measured bio-ADM concentrations were related to theoretically expected values (means of expected values of undiluted and 1:2, 1:4, and 1:8 diluted samples were used as reference values).

disulfide bond at or near the N-terminus, formed by 2 cysteine residues, which are separated by 4 or 5 amino acid residues from another. However, the amino acid sequences of the peptides are very different. As expected, none of the peptides cross-reacted in the bio-ADM assay [signals were below the LOD for all concentrations tested (up to 100 000 pg/mL); data not shown].

The linearity of the assay was assessed by the following two methods: (a) by dilution of native plasma samples containing supranormal concentrations of bio-ADM with a native plasma sample containing a very low bio-ADM concentration (as previously determined in the bio-ADM assay), and (b) by mixing various native plasma samples containing different concentrations of endogenous bio-ADM and, finally, by testing the possible interference of a putative ADM-binding protein, complement factor H. These methods showed acceptable linearity (Table 2). In addition, we found that complement factor H, even at an almost 400 000-fold molar excess over bio-ADM, did not interfere with recovery of bio-ADM (Fig. 3).

The ex vivo stability of bio-ADM in 4 native EDTA plasma samples containing high bio-ADM concentrations was determined. On storage at 22 °C, recoveries were as follows [mean (SD)]: at 4 h: 92.5 (6.2) %, at 24 h: 95.3 (14.6) %, at 48 h: 90.5 (20.4) %, and at 144 h: 72.2 (23.0) %. Up to 4 freeze–thaw cycles (with overnight freezing periods) did not alter the recovery of bio-ADM in native EDTA plasma samples (data not shown).

Finally, we measured bio-ADM in 88 healthy subjects (Fig. 4). For all samples, bio-ADM concentrations were higher than the LOD of the assay. The median interquartile range, (IQR), was 13.7 (9.6–18.7) pg/mL, and mean (SD) was 15.6 (9.2) pg/mL.

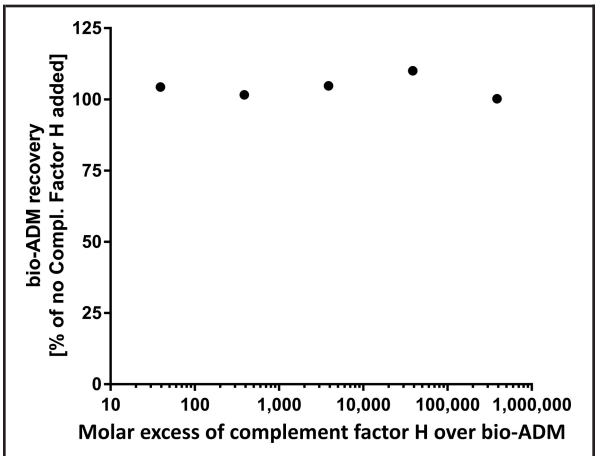

Fig. 3. Influence of complement factor H on the bio-ADM assay.
ADM at a fixed concentration was preincubated with various concentrations of complement factor H and then measured in the bio-ADM assay. Results were calculated as recovery compared with the ADM concentration measured in the absence of complement factor H.

As the LOQ of the assay is 11 pg/mL, individual concentrations below this value must—by definition—not be considered quantitative. Thus, the aforementioned low-end IQR and SD values can serve as orientation-only values. In any case, only supranormal bio-ADM concentrations are clinically relevant, and these are detected with high precision by the assay.

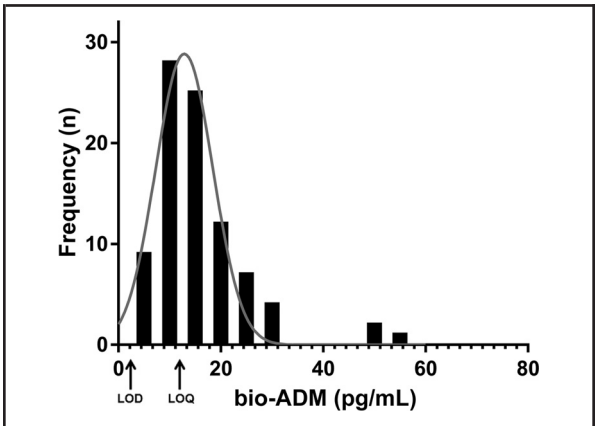

Fig. 4. Normal distribution of bio-ADM.
EDTA plasma samples obtained from 88 healthy subjects were measured in the bio-ADM assay. The frequency distribution of measured concentrations is shown. LOD and LOQ are indicated.

Besides its application in human samples, the assay can also be used for the measurement of bio-ADM in various animal species. This is of particular interest for the preclinical development of drug candidates that affect the ADM system, such as an anti-ADM antibody. The amino acid sequence of bio-ADM is evolutionarily highly conserved, and we determined 100 (15) % cross-reactivity of the assay with bio-ADM from mice, rats, dogs, pigs, and cynomolgus monkeys (tested with synthetic peptides in buffer; data not shown).

DISCUSSION

In this study, we describe an immunoassay for the sensitive, accurate, and specific quantification of bio-ADM in plasma under conditions suitable for routine use.

As for several different peptide hormones, C-terminal amidation of ADM by peptidyl-glycine α-amidating monooxygenase (PAM) is essential for its biological activity (*25*). Both the inactive glycine-extended ADM variant (ADM-Gly) and the biologically active amidated form of ADM circulate in the bloodstream (*32*). The ratio of both ADM variants in plasma may differ depending on conditions. For instance, different ratios have been noticed for arterial and venous blood samples obtained from patients with acute myocardial infarction (*33*). A changing ratio of the ADM variants has also been observed in experimental cardiac hypertrophy (*34*). Most strikingly, a >3-fold different ratio has been reported for patients with sepsis compared with healthy subjects (*24*). The amount of synthesized MR-proADM, another fragment of the ADM precursor peptide, represents the sum of synthesized inactive ADM-Gly and active amidated ADM. As the ratio between the two can differ, MR-proADM cannot always reflect the actual amount

of active ADM. Thus, the specific measurement of bio-ADM could be a better reflection of the clinical status of patients than measurement of immature ADM or other peptides derived from the ADM precursor. In line with this concept, bio-ADM concentrations in patients with sepsis have been found to be more strongly associated with clinical outcome relative to MR-proADM concentrations (*26*).

For our bio-ADM immunoassay, we developed a monoclonal antibody, which specifically binds to the amidated C-terminus of bio-ADM. It exhibits <0.1% cross-reactivity with the inactive glycine-extended ADM variant. The same low cross-reactivity was observed with truncated ADM variants.

ADM has been reported to be prone to proteolytic degradation at the N-terminal part of the peptide (*35*) and thus is considered unstable (*21*). For detectability by an immunoassay, analyte stability is not a fixed physical variable per se, but is largely a function of epitopes chosen for antibody binding.

Consequently, we selected a region in the middle of the ADM peptide, immediately downstream from the disulfide ring structure, as epitope for the second antibody of the assay. When combining this antibody with the anti-C-terminal antibody in our immunometric assay, we observed high stability of native bio-ADM in EDTA plasma. The selection of antibodies with defined epitope specificities as used here should thus facilitate reliable measurement of bio-ADM under standard sample handling and transport procedures used in hospital routine.

Another immunometric assay for the specific detection of mature ADM has been described previously (*36*). There, the epitope of one of the antibodies was located further toward the N-terminal region. Acceptable analyte stability was reported, when aprotinin was added to the EDTA–blood draw. Other preanalytical storage conditions were not investigated. This assay required 200 μL of sample volume and an overnight incubation. Even under these conditions, the intraassay CV at the mean of the investigated normal population was around 20%. The possible influence of complement factor H was not investigated.

In contrast, the bio-ADM assay described here uses less sample volume, has a considerably shorter incubation time, and has a better analytical sensitivity, all features compatible with a routine application of the assay. Our assay is sensitive enough to quantitatively distinguish normal from pathologically increased bio-ADM levels.

We addressed an important analytical issue relating to the accuracy of the assay. In the literature, complement factor H has been described as an ADM-binding protein (*20*). Normal plasma concentrations are in the range of 250 μg/mL. It has been observed and intensely discussed that such binding protein can interfere with the detection of ADM by immunoassay (*20*, *37*). We found that our bio-ADM assay was not affected by complement factor H: Recovery of ADM was not influenced after preincubation with complement factor H, even if it was used in an up to an almost 400 000-fold molar excess over ADM. This molar ratio is higher than naturally occurring in plasma, physiologically and pathophysiologically.

The assay is highly specific for bio-ADM, as no cross-reactivity with ADM-related peptides (ADM-2, CGRP, calcitonin, amylin), even when applied in very high concentrations far exceeding those described to occur in plasma, was detected.

The measuring range of the assay (11 to >1000 pg/mL) covers the entire spectrum of supranormal ADM concentrations that have been reported to occur in pathological conditions and in pregnancy (*38*). The highest known ADM concentrations are known for septic shock (*14*, *15*, *26*), but even there, concentrations are typically well below 1000 pg/mL.

Taken together, we have developed an immunometric assay for bio-ADM, which is suitable for routine use. The design used is easily adaptable on established automated immunoassay analyzers and point-of-care test systems. Recently, a prototype bio-ADM assay with the same key design features as the bio-ADM assay described here was

used to assess the possible clinical utility of bio-ADM measurement in patients with sepsis and septic shock (*26*, *27*). The prototype assay used tubes instead of a microtiter plate as solid phase and less sample volume at the expense of a longer incubation time. Increased bio-ADM concentrations identified patients with sepsis at an increased risk for adverse outcomes and their need for vasopressor therapy. It was concluded that bio-ADM may help individualizing hemodynamic support therapy, while avoiding harmful effects (*27*). Guidance in this field is not or not sufficiently provided by other biomarkers established in intensive care. For instance, markers such as C-reactive protein, procalcitonin, or leukocytes address the infectious/inflammatory status but do not indicate progression from sepsis into septic shock.

Another important possible indication for measurement of bio-ADM arises from the known biological role of ADM in the regulation of vascular integrity (*3*). This may allow for the monitoring of tissue decongestion in acute heart failure, which is currently being investigated in a large, multicenter observational study. In the future, the assay may also be useful for the stratification of patients, who shall be treated with innovative drugs that target the ADM system, such as a monoclonal anti-ADM antibody (*30*). Thus, measurement of bio-ADM in critically ill patients can be a helpful tool to guide patient therapy and management.

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REFERENCES

1. Kitamura K, Kangawa K, Kawamoto M, Ichiki Y, Nakamura S, Matsuo H, Eto T. Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. Biochem Biophys Res Commun. 1993;192:553–60.
2. Hinson JP, Kapas S, Smith DM. Adrenomedullin, a multifunctional regulatory peptide. Endocr Rev. 2000;21:138–67.
3. Hippenstiel S, Witzenrath M, Schmeck B, Hocke A, Krisp M, Krull M, et al. Adrenomedullin reduces endothelial hyperpermeability. Circ Res. 2002;91:618–25.
4. Temmesfeld-Wollbruck B, Brell B, David I, Dorenberg M, Adolphs J, Schmeck B, et al. Adrenomedullin reduces vascular hyperpermeability and improves survival in rat septic shock. Intensive Care Med. 2007;33:703–10.
5. Jougasaki M, Wei CM, McKinley LJ, Burnett JC, Jr. Elevation of circulating and ventricular adrenomedullin in human congestive heart failure. Circulation 1995;92:286–9.
6. Nishikimi T, Saito Y, Kitamura K, Ishimitsu T, Eto T, Kangawa K, et al. Increased plasma levels of adrenomedullin in patients with heart failure. J Am Coll Cardiol. 1995;26:1424–31.
7. Kobayashi K, Kitamura K, Etoh T, Nagatomo Y, Takenaga M, Ishikawa T, et al. Increased plasma adrenomedullin levels in chronic congestive heart failure. Am Heart J. 1996;131:994–8.
8. Kobayashi K, Kitamura K, Hirayama N, Date H, Kashiwagi T, Ikushima I, et al. Increased plasma adrenomedullin in acute myocardial infarction. Am Heart J. 1996;131:676–80.
9. Pousset F, Masson F, Chavirovskaia O, Isnard R, Carayon A, Golmard JL, et al. Plasma adrenomedullin, a new independent predictor of prognosis in patients with chronic heart failure. Eur Heart J. 2000;21:1009–14.
10. Sato K, Hirata Y, Imai T, Iwashina M, Marumo F. Characterization of immunoreactive adrenomedullin in human plasma and urine. Life Sci. 1995;57:189–94.
11. Ishimitsu T, Nishikimi T, Saito Y, Kitamura K, Eto T, Kangawa K, et al. Plasma levels of adrenomedullin, a

newly identified hypotensive peptide, in patients with hypertension and renal failure. J Clin Invest. 1994;94:2158–61.
12. Wang P, Zhou M, Ba ZF, Cioffi WG, Chaudry IH. Up-regulation of a novel potent vasodilatory peptide adrenomedullin during polymicrobial sepsis. Shock 1998;10:118–22.
13. Hirata Y, Mitaka C, Sato K, Nagura T, Tsunoda Y, Amaha K, Marumo F. Increased circulating adrenomedullin, a novel vasodilatory peptide, in sepsis. J Clin Endocrinol Metab. 1996;81:1449–53.
14. Ueda S, Nishio K, Minamino N, Kubo A, Akai Y, Kangawa K, et al. Increased plasma levels of adrenomedullin in patients with systemic inflammatory response syndrome. Am J Respir Crit Care Med. 1999;160:132–6.
15. Ehlenz K, Koch B, Preuss P, Simon B, Koop I, Lang RE. High levels of circulating adrenomedullin in severe illness: correlation with C-reactive protein and evidence against the adrenal medulla as site of origin. Exp Clin Endocrinol Diabetes. 1997;105:156–62.
16. Nagaya N, Nishikimi T, Uematsu M, Yoshitomi Y, Miyao Y, Miyazaki S, et al. Plasma adrenomedullin as an indicator of prognosis after acute myocardial infarction. Heart 1999;81:483–7.
17. Richards AM, Nicholls MG, Yandle TG, Frampton C, Espiner EA, Turner JG, et al. Plasma N-terminal pro-brain natriuretic peptide and adrenomedullin: new neurohormonal predictors of left ventricular function and prognosis after myocardial infarction. Circulation 1998;97:1921–9.
18. Richards AM, Doughty R, Nicholls MG, MacMahon S, Sharpe N, Murphy J, et al. Plasma N-terminal pro-brain natriuretic peptide and adrenomedullin: prognostic utility and prediction of benefit from carvedilol in chronic ischemic left ventricular dysfunction. J Am Coll Cardiol. 2001;37:1781–7.
19. Lewis LK, Smith MW, Yandle TG, Richards AM, Nicholls MG. Adrenomedullin (1–52) measured in human plasma by radioimmunoassay: plasma concentration, adsorption, and storage. Clin Chem. 1998;44:571–7.
20. Pio R, Martinez A, Unsworth EJ, Kowalak JA, Bengoechea JA, Zipfel PF, et al. Complement factor H is a serum-binding protein for adrenomedullin, and the resulting complex modulates the bioactivities of both partners. J of Biol Chem. 2001;276:12292–300.
21. Meeran K, O'Shea D, Upton PD, Small CJ, Ghatei MA, Byfield PH, Bloom SR. Circulating adrenomedullin does not regulate systemic blood pressure but increases plasma prolactin after intravenous infusion in humans: a pharmacokinetic study. J Clin Endocrinol and Metab. 1997;82:95–100.
22. Struck J, Tao C, Morgenthaler NG, Bergmann A. Identification of an adrenomedullin precursor fragment in plasma of sepsis patients. Peptides 2004;25:1369–72.
23. Morgenthaler NG, Struck J, Alonso C, Bergmann A. Measurement of midregional proadrenomedullin in plasma with an immunoluminometric assay. Clin Chem. 2005;51:1823–9.
24. Ohta H, Tsuji T, Asai S, Tanizaki S, Sasakura K, Teraoka H, et al. A simple immunoradiometric assay for measuring the entire molecules of adrenomedullin in human plasma. Clin Chim Acta. 1999;287:131–43.
25. Eguchi S, Hirata Y, Iwasaki H, Sato K, Watanabe TX, Inui T, et al. Structure-activity relationship of adrenomedullin, a novel vasodilatory peptide, in cultured rat vascular smooth muscle cells. Endocrinology 1994;135:2454–8.
26. Marino R, Struck J, Maisel AS, Magrini L, Bergmann A, Di Somma S. Plasma adrenomedullin is associated with short-term mortality and vasopressor requirement in patients admitted with sepsis. Crit Care 2014;18:R34.
27. Caironi P, Latini R, Struck J, Hartmann O, Bergmann A, Maggio G, et al. Circulating biologically active adrenomedullin (bio-ADM) predicts hemodynamic support requirement and mortality during sepsis. Chest 2017.
28. Lane RD. A short-duration polyethylene glycol fusion technique for increasing production of monoclonal antibody-secreting hybridomas. J Immun Methods 1985; 81:223–8.
29. Harlow E, Lane D. Antibodies: a laboratory manual. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press, 1988.
30. Wagner K, Wachter U, Vogt JA, Scheuerle A, McCook O, Weber S, et al. Adrenomedullin binding improves catecholamine responsiveness and kidney function in resuscitated murine septic shock. Intensive Care Med Exp. 2013;1:21.
31. Struck J, Hein F, Karasch S, Bergmann A. Epitope specificity of anti-adrenomedullin antibodies determines efficacy of mortality reduction in a cecal ligation and puncture mouse model. Intensive Care Med Exp. 2013;1: 22.
32. Kitamura K, Kato J, Kawamoto M, Tanaka M, Chino N, Kangawa K, Eto T. The intermediate form of glycine-extended adrenomedullin is the major circulating molecular form in human plasma. Biochem Biophys Res Commun. 1998;244:551–5.
33. Asakawa H, Nishikimi T, Suzuki T, Hara S, Tsubokou Y, Yagi H, et al. Elevation of two molecular forms of adrenomedullin in plasma and urine in patients with acute myocardial infarction treated with early coronary angioplasty. Clin Sci. 2001;100:117–26.
34. Wang X, Nishikimi T, Akimoto K, Tadokoro K, Mori Y, Minamino N. Upregulation of ligand, receptor system, and amidating activity of adrenomedullin in left ventricular hypertrophy of severely hypertensive rats: effects of angiotensin-converting enzyme inhibitors and diuretic. J Hypertens. 2003;21:1171–81.
35. Lewis LK, Smith MW, Brennan SO, Yandle TG, Richards AM, Nicholls MG. Degradation of human adrenomedullin (1–52) by plasma membrane enzymes and identification of metabolites. Peptides 1997;18:733–9.
36. Ohta H, Tsuji T, Asai S, Sasakura K, Teraoka H, Kitamura K, Kangawa K. One-step direct assay for mature-type

adrenomedullin with monoclonal antibodies. Clin Chem. 1999;45:244–51.

37. Pio R, Elsasser TH, Martinez A, Cuttitta F. Identification, characterization, and physiological actions of factor H as an adrenomedullin binding protein present in human plasma. Microsc Res Tech. 2002;57:23–7.

38. Gibbons C, Dackor R, Dunworth W, Fritz-Six K, Caron KM. Receptor activity-modifying proteins: ramping up adrenomedullin signaling. Mol Endocrinol. 2007;21:783–96.

39. Beltowski J, Jamroz A. Adrenomedullin–what do we know 10 years since its discovery? Pol J Pharmacol. 2004;56:5–27.