The International Standard for Atrial Natriuretic Factor Calibration by an International Collaborative Study

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SUMMARY An ampouled preparation of human atrial natriuretic factor, ANF-(99-126), was evaluated by 23 laboratories in 10 countries for its suitability to serve as the international standard for ANF. The preparation was calibrated by radioimmunoassay, radioreceptor binding assay, and bioassay and was shown to have satisfactory stability and biological activity. Estimates of the ANF content of a set of specimens of plasma in terms of the standard showed agreement in ranking order when the ANF was extracted prior to assay. However, estimates of the ANF content of the plasmas in terms of either the international standard or the various local standards varied widely among laboratories. On the basis of the results reported here, with the agreement of the participants in the study and with the authorization of the Expert Committee on Biological Standardization of the World Health Organization, the preparation coded 85/669 was established in 1987 as the international standard for ANF, with a defined potency of 2.5 international units per ampoule. (Hypertension 12: 629-634, 1988)

KEY WORDS • atrial natriuretic factor • international standard • collaborative assay

THE atria of mammalian hearts synthesize and secrete a peptide (or peptides) with potent natriuretic and vasoactive properties that is known as atrial natriuretic factor (ANF). Human and rat atria predominantly secrete a peptide of 28 amino acid residues, ANF-(99-126), which represents the C-terminus of a precursor sequence of 126 amino acid residues. Plasma immunoreactive ANF-(99-126) concentration increases in normal rats after volume expansion, while infusion of the peptide lowers blood pressure in several animal models of experimental hypertension. Studies in humans confirm many of the biological actions of ANF-(99-126). It is likely that human ANF-(99-126) and its analogues will prove to be important physiologically and may have a role in the clinical diagnosis and treatment of various cardiovascular diseases, especially hypertension.

At a meeting in Cleveland, Ohio, USA, in September 1985, a Joint Committee of the American Heart Association, the International Society of Hypertension, and the National Institute for Biological Standards and Control agreed that an international standard for human ANF-(99-126) would facilitate research on this important peptide. At its 35th meeting in Geneva in November 1985, the Expert Committee on Biological Standardization of the World Health Organization requested that an international collaborative trial of a suitable material be arranged. This report describes the results of the trial, which was conducted under the joint aegis of the American Heart Association, the International Society of Hypertension, and the World Health Organization.

The aims of the study were

1. To assess in at least 20 laboratories the suitability of the ampouled preparation to serve as the international standard for the assay of human ANF-(99-126).
2. To assess the stability of the ampouled preparation of the proposed standard when subjected to accelerated thermal degradation.
3. To compare the proposed standard with the various local standards in use in the participating laboratories.
Materials and Methods

Proposed International Standard for Human ANF-(99–126)

Human ANF-(99–126), 22 mg, synthesized and highly purified at Bachem Inc., was contributed to the World Health Organization through the good offices of Dr. Rao Machinini of Bachem Inc. (Torrance, CA, USA) and Professor Klaus D. Kohler of Bissendorf Peptide GmbH (Bissendorf, Wedemark, FRG).

The proposed international standard was ampouled under conditions recommended by the Expert Committee on Biological Standardization of the World Health Organization.4 Human ANF-(99–126), 10 mg, was dissolved in 2 L of pyrogen-free 100 mM acetic acid containing 0.1% (wt/vol) human serum albumin (Elstree Blood Products Laboratory, Hertfordshire, UK) and 0.5% (wt/vol) trehalose (Fluka Chemicals, Glossop, Derbyshire, UK). This solution was passed through a 0.45-μm APD membrane filter (Sartorius, Surrey, UK) and distributed in 0.5-ml aliquots into 4000 ampoules coded 85/669. The ampouled solution was lyophilized, and after secondary desiccation, the ampoules were sealed under dry nitrogen by heat fusion of the glass and stored at −20 °C in the dark. The results of preliminary chemical and biological tests on the proposed standard have been described previously.5

Accelerated Degradation of Samples of the Proposed Standard

Ampoules were stored at elevated temperatures to permit assessment of the stability of the ampouled material on accelerated thermal degradation. Ampoules that had been stored for 3 months at 20, 37, 45, and 56 °C were coded E, B, D, and A, respectively, and ampoules that had been stored for 6 months at 37 and 56 °C were coded Q and S, respectively. Ampoules stored continuously at −20 °C were also included for comparison and were coded C. Each participant received an individually selected subset of these samples.

Plasma Samples

Samples were collected from six individuals. Sample J contained pooled plasma from two healthy volunteers with no evidence of cardiovascular or other disease. It was known from previous work that the two donors had low plasma concentrations of ANF-(99–126). Sample G contained plasma from a patient with polycythemia and chronic, obstructive respiratory disease pooled with plasma from a patient with refractory essential hypertension and renal impairment. Sample H contained pooled plasma from two patients with congestive heart failure. All four patients were being treated at the MRC Blood Pressure Unit, Western Infirmary, Glasgow. The plasmas contained final concentrations of potassium EDTA, 2 mg/ml, and aprotinin (Trasylol), 50 kallikrein inhibiting units/ml. The three samples were freeze-dried in ampoules (2 ml/ampoule).

Participants in the Study

Twenty-three laboratories in 10 countries contributed data to this study and are listed at the end of this report. Each laboratory has been identified in the report by a number between 1 and 30 (not all numbers are used) that is not related to the order of participants on the list.

Assays Contributed to the Study

Twenty-two laboratories provided data on radioimmunoassay. In two of these laboratories, assays were carried out using two different antisera; these were regarded as different assay systems (identified by letters A and B) and were therefore considered separately for analysis, thereby bringing the total to 24 assay systems. The majority of participants using radioimmunoassay contributed, as requested, two independent assays, each including all of the study materials.

Two laboratories (9 and 29) carried out radioreceptor binding assays. One other laboratory (22) attempted radioreceptor binding assays using a commercial kit but did not obtain interpretable standard curves.

One laboratory (9) carried out an in vitro assay using the vasorelaxant activity in precontracted rat aortic strips, but the data obtained were insufficient to permit estimation of potency.

Statistical Analysis

For each radioimmunoassay or radioreceptor binding assay, responses were transformed to logits, and log dose–logit response lines were examined for linearity and parallelism.6 Logarithms of estimates of relative potency (immunoreactivity) were obtained as the displacement of parallel log dose–logit response lines and were compared within and among laboratories by using analysis of variance.

Results

Comparison of the Proposed Standard with Ampoules Identical to It

Although it was not known by the participants, ampoules coded C were identical to the proposed standard. Thus it was expected that within an assay the log dose-response lines for the proposed standard and C would be the same and the immunoreactivity of either relative to the other would be 1.0. The observed deviations from this were taken to indicate the minimum deviations that would be achieved in these assay systems and were used to provide a criterion for assessment of other comparisons. Results of the comparison of the proposed standard and C were consistent with the expected finding that these two materials had equivalent immunoreactivity; the overall geometric mean of the immunoreactivity of C relative to that of the proposed standard was 1.02 (95% confidence limits, 0.96–1.09), which was in good agreement with the "known" value of 1.0. The variability of estimates of the immunoreactivity of C relative to that of the
Comparison of the Proposed Standard with Various Local Standards

In the majority of radioimmunoassays, the slopes of the log dose–logit response lines for the proposed standard and C and the various local standards did not differ significantly from one another. Exceptions were the assays in laboratories 19 and 21, in which the log dose–logit response line for the proposed standard was flatter than that for the local standard, and laboratory 11, in which the reverse was true. The calibration of the proposed standard in terms of the various local standards is shown in Figure 1.

Variability of estimates of the immunoreactivity of the proposed standard or C in terms of the various local standards among assays within laboratories was similar to that seen for estimates of the immunoreactivity of C in terms of the proposed standard. However, the variability of estimates among laboratories was significantly greater than that within laboratories (ratio of variances: 10). It is likely that this reflects differences among the various local standards. Estimates of the ampoule content of the proposed standard ranged from approximately 1.3 μg to 5 μg. The geometric mean of 24 laboratory geometric mean estimates (including the reported values) was 2.5 μg per ampoule (95% confidence interval, 2.1–2.9) for the proposed standard and 2.4 μg per ampoule (95% confidence interval, 2.0–2.8) for the identical preparation coded C.

Only two laboratories provided results from radioreceptor binding assays (see Figure 1). These were similar to those from radioimmunoassays; the geometric means of the laboratory mean estimates in terms of laboratory standards were 2.4 μg per ampoule for the proposed standard and 2.5 μg per ampoule for C.

Stability of the Proposed Standard

Slopes of the log dose–logit response lines for the samples subjected to accelerated degradation, coded A, B, D, and E and Q and S, did not differ significantly from those for the proposed standard and C. Geometric mean estimates for the samples subjected to accelerated degradation (including sample C, which was identical to the proposed standard) did not differ significantly from one another and showed no trend that suggested a decrease in activity with increasing temperature or time of storage at elevated temperatures. Estimates by radioreceptor binding assay from a single laboratory were consistent with these results.

Estimation of the ANF Content of Plasma Samples

Log dose–logit response lines for the plasma samples generally were based on four or fewer dilutions, with responses near the extremes of the response range. Consequently, estimates of the slopes of these lines were not reliable. Nevertheless, a tendency for the log dose-response lines for the plasma samples to be flatter than those for the proposed standard and local standards was noted in laboratories 7A, 14, 21, 29, and 30.

Estimates of the ANF content of the three plasma samples in terms of the proposed standard are shown in Figure 2. The most immediately notable feature is the two distinct groups formed by the laboratories that did not extract plasma samples prior to assay (laboratories 9, 14, and 21) and those that did. Estimates from the former group (laboratories 9, 14, and 21) have been treated separately.

Within assays in the laboratories in which plasma samples were extracted before assay, the ranking of estimated ANF concentrations in the three samples was consistent (J < G < H). However, estimates of the ANF concentration in terms of either the proposed standard or the various local standards varied widely among laboratories, although for each sample, estimates in terms of the proposed standard were some 15 to 35% less variable than estimates in terms of local standards. For estimates in terms of the proposed standard, the variance among laboratories for samples G and H was some 20 times greater than that for estimates for C, although the variance within laboratories for G and H was only slightly greater than that for C (approximately 1.5 times). Estimates for sample J, which had a concen-
The proposed standard appears to be suitable to serve as the international standard for human ANF-(99–126). Previous work has shown that it has adequate biological activity. In the present study, the biological activity was confirmed, and the majority of radioimmunoassay systems and two radioreceptor binding assay systems gave dose-response relationships for the proposed standard that were similar to those of the various local standards. Calibration of the proposed standard in terms of the local standards gave a value of 2.5 μg per ampoule, which is consistent with the value calculated from the results of chemical tests. Estimates of ANF content in samples of the proposed standard exposed to elevated temperatures indicated satisfactory stability at the storage temperature of −20 °C.

Estimates of the ANF content of plasma samples in terms of the various local standards were very similar to estimates in terms of the proposed standard (Pearson’s correlation coefficient >0.9 for each sample). Among laboratories the values for esti-

![Figure 2. Frequency distributions of the estimates of the human ANF content of three supplied plasma samples. Estimates are from radioimmunoassays except where otherwise indicated. The numbers in the boxes are laboratory codes. Unfilled boxes denote extracted samples, black-bordered boxes denote nonextracted samples, and boxes with diagonal lines denote radioreceptor binding assays.](image-url)
The ranking of these values was consistent within all laboratories except a limited number of laboratories. The anomalously ordered values were exclusively from laboratories in which samples were not extracted prior to assay. Moreover, estimates for each of these plasma samples, but most noticeably for sample J, were higher than estimates from the laboratories that had performed extraction. No meaningful comparison or estimates from these two groups of laboratories could be obtained.

In terms of local standards, estimates of the ANF content of plasma samples from laboratories in which samples were extracted before assay showed substantial variation. This variation was only slightly reduced when estimates were calculated in terms of the proposed standard. Use of one of the plasma samples, H, as the reference standard substantially reduced variability among laboratories for estimates of the other two samples. This suggests that some of the differences between laboratories resulted from differences in the treatment of samples. It has been reported that extraction of plasma before assay removes interference from platelets and also from an as yet unidentified plasma component that can be demonstrated by gel filtration. Acidification of samples prior to extraction has also been reported to affect the values of estimates. Ten laboratories provided detailed information relating to the pH of assay samples (before extraction), and for these there was no consistent difference between estimates from the three laboratories in which the pH of samples extracted was 4 or less and the seven laboratories in which the pH was more than 7. Even with sample H as a reference standard, the variability of estimates for samples J and G among laboratories was greater than that within laboratories (5 times greater for sample G). This suggests that interactions of plasma samples with the various assay systems also contributed to the variability among laboratories.

Conclusion

At its 37th meeting in Geneva in December 1987, the Expert Committee on Biological Standardization of the World Health Organization established preparation 85/669 as the international standard for human ANF-(99–126) with a unitage of 2.5 international units per ampoule. The standard may be obtained by writing to the Director, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK. A small handling fee will be charged.

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