Use of Single-Value Protein Compensation of the Jaffe Creatinine Assay Contributes to Clinically Significant Inaccuracy in Results

To the Editor:

Effective use of equations for calculating estimated glomerular filtration rate (eGFR) requires reliable plasma creatinine measurements (1). Considerable effort has been made to standardize calibration (2, 3), but standardization does not correct for analytical interferences, such as protein interference in Jaffe creatinine assays.

A single-value correction factor is often used to correct for non-specific protein interference in Jaffe assays, but this approach does not take into account differences in plasma albumin concentrations among samples. Because of the current interest in low creatinine results for calculation of eGFR, we evaluated the validity and clinical impact of using a single compensation value.

We determined the dependence of creatinine results on albumin concentration by adding increasing amounts of human albumin powder to plasma specimens containing low albumin and creatinine concentrations of 50 and 340 μmol/L, respectively. We then measured, in triplicate, creatinine and albumin (by the Roche bromocresol purple method) on a Modular Analytics system that uses a constant $-26 \mu\text{mol/L}$ adjustment of creatinine results. As expected, the reported creatinine results decreased with decreasing albumin concentrations.

We assumed that when protein concentration is within the adult reference interval, the Roche factor of $-26 \mu\text{mol/L}$ provides reliable compensation. Hence, the “true” creatinine results of these samples were estimated at an albumin concentration of 40 g/L. The differences between these true creatinine results and the measured reportable results are attributable to miscompensation at the respective albumin concentrations that differed from 40 g/L. More accurate compensation values specific for each albumin concentration were then calculated and used to derive the following protein compensation formula by regression analysis:

$$\text{Compensation (μmol/L)} = 0.544 \times \frac{\text{creatinine (mg/dL)}}{\text{albumin (g/L)}} + 4.075 \text{ μmol/L}$$

It is noteworthy that at 0 g albumin/L the intercept is only 4.075 μmol/L, which represents interference from nonalbumin protein. This result is consistent with albumin per se being the main component of nonspecific protein interference in the Jaffe assay.

To evaluate the clinical impact of miscompensation on creatinine results, the laboratory information system database for our health region was searched for a 6-month period (January 1 to June 30, 2006) to obtain 49,824 records of specimens (from 17,347 patients) for which both creatinine and albumin were measured. Test results and patient data were downloaded into Microsoft Excel and the above equation applied to compensate creatinine results for measured albumin concentrations (after adding back the Roche adjustment of 26 μmol/L). The difference between the 2 creatinine results (i.e., single-value compensated vs formula compensated) represents the analytical error introduced by not adjusting the protein compensation factor for variations in albumin concentration.

The dataset was segregated into categories based on patient age and sex, and the reported creatinine results were compared with

<table>
<thead>
<tr>
<th>Patient category</th>
<th>URDL</th>
<th>Total patients (samples)</th>
<th>Total patients (samples) with creatinine &gt; URDL</th>
<th>Error in patients with creatinine &gt; URDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total range, %</td>
</tr>
<tr>
<td>Group</td>
<td>Age/Sex</td>
<td>μmol/L</td>
<td>mg/dL</td>
<td>Total patients (samples)</td>
</tr>
<tr>
<td>1</td>
<td>0–1 month</td>
<td>88</td>
<td>1.0</td>
<td>333 (1072)</td>
</tr>
<tr>
<td>2</td>
<td>1 month–5 y</td>
<td>35</td>
<td>0.4</td>
<td>680 (1851)</td>
</tr>
<tr>
<td>3</td>
<td>6–12 y</td>
<td>62</td>
<td>0.7</td>
<td>530 (1477)</td>
</tr>
<tr>
<td>4</td>
<td>13–18 y</td>
<td>88</td>
<td>1.0</td>
<td>831 (1964)</td>
</tr>
<tr>
<td>5</td>
<td>19–60 y/F</td>
<td>97</td>
<td>1.1</td>
<td>4969 (11,991)</td>
</tr>
<tr>
<td>6</td>
<td>19–60 y/M</td>
<td>106</td>
<td>1.2</td>
<td>4309 (14,259)</td>
</tr>
<tr>
<td>7</td>
<td>&gt;60 y/F</td>
<td>88</td>
<td>1.0</td>
<td>2827 (8439)</td>
</tr>
<tr>
<td>8</td>
<td>&gt;60 y/M</td>
<td>114</td>
<td>1.3</td>
<td>2868 (8771)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td>17,347 (49,824)</td>
</tr>
</tbody>
</table>
the upper reference decision limits (URDL) specific for each category used in our laboratory (Table 1). Of the 49,824 creatinine results reported, 15,686 (or 31.5%) exceeded their respective URDLs. The range of analytical error attributable to miscompensation was greatest in the pediatric age categories 2 (0–1 month) and 3 (1 month to 5 years) (e.g., errors of −29% to +24% in group 2) for which the URDLs are lowest, and albumin concentrations are usually lower than adult values. This magnitude of error invalidates use of the single-value protein compensation method for these age groups. In patient categories 1 and 4, in which URDLs were higher, the range of error observed was still unacceptably high, as indicated by the large proportions of error >3.4% (namely 97% and 38%). In the adult categories 5, 6, 7, and 8 the proportion of error >3.4% were lowest, but the range of error encountered was still too high for ensuring reliable creatinine results for clinical practice.

The data presented here indicate unacceptable inaccuracy in creatinine results due to variations in albumin concentrations. Hence, the single-value compensated creatinine Jaffe assay cannot achieve the current desired performance goal of 3.4% bias as recommended by the Laboratory Working Group of the National Kidney Disease Program (1), nor the recommended optimum goal of 1.7%. Awareness of this limitation may be helpful in setting clinical guidelines for result interpretation and in making future choices on methodology.

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References


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Stability of Thiopurine Metabolites: A Potential Analytical Bias

To the Editor:

In a report recently published in this journal (1), Reinshagen et al., who investigated 6-thioguanine nucleotide (6-TGN) concentrations in patients with inflammatory bowel disease (IBD), concluded that standard and adapted dosing of azathioprine led to identical 6-TGN concentrations and remission rates and that therapeu-

cric drug monitoring of thiopurine therapy was of no clinical benefit in patients with a wild-type thiopurine S-methyltransferase (TPMT) genotype.

As Reinshagen et al. point out, monitoring thiopurine metabolites is a more or less accepted method for assessing pharmacotherapeutic compliance. The use of monitoring to assess the achievement of therapeutic goals is still a matter of ongoing discussion, however, and the study conducted by Reinshagen et al. provides valuable data to inform this debate.

Unfortunately, critical analytical problems still plague thiopurine research. Important differences exist in analytical procedures and reference values (2), and the stability of the thiopurine metabolites themselves is an issue as well. Few studies on stability at 5–7 days are available, although some results have been reported by Pike et al. (3) and Sauviat et al. (4). Decreases in 6-TGN concentrations of 2%–4% per day at ambient temperature (3) and up to 75% on day 4 and <35% on day 7 for samples stored at 20 °C and 4 °C (4) were reported. These results indicate that the stability of 6-TGN in blood samples is limited, a pivotal concern in the many countries where blood samples must be shipped to central laboratories for measurement of thiopurine metabolites. In these situations thiopurine metabolite concentrations can be expected to decrease during transport and/or sample storage.

We prospectively investigated the stability of 6-TGN and 6-methylmercaptopurine (6-MMP) metabolites in blood samples from IBD patients visiting our hospital. Blood was sampled in lithium-heparin tubes, homogenized, and immediately divided into equal portions for storage at controlled conditions (room temperature at 22 °C or refrigeration at 4 °C). A validated analytical procedure de-
scribed by Shipkova et al. (2) was used to measure 6-TG and 6-MMP nucleotides. The assay had precision values of 5.7% and 4.9% (within day) and 6.9% and 7.2% (between day) for 6-TGN and 6-MMP, respectively. To report the measured metabolite concentrations, erythrocytes were isolated, washed, and counted in the final suspensions before analysis. Analysis was performed at baseline and on days 1, 4, 5, 6, and 7 after sampling.

We obtained samples from 10 patients. The (pseudo)median 6-TGN concentration at day 7 decreased significantly to 53% during storage at room temperature (V = 0, P = 0.002, 95% CI 48%–70%); under refrigeration, the median 6-TGN concentration at day 7 decreased to 90% (not significant). Inter- and intraday variation in our analytical method seems an explanation for these results. Concerning 6-MMP, (pseudo)median concentrations at day 7 decreased significantly to 55% (V = 0, P = 0.011, 95% CI 40%–69%) and 86% (V = 2, P = 0.011, 95% CI 75%–96%) during storage at 22°C and 4°C, respectively (Table 1; Wilcoxon rank-sum test). In addition, decreases in (pseudo) medians were significantly less for both metabolites from day 4 to day 7 during refrigeration [paired Wilcoxon rank-sum test]. These data have been reported previously (5).

Pike et al. (3) reported less dramatic decreases in nucleotide concentrations (14%–28% decrease at day 7), but our findings are more or less equivalent to the results reported by Sauviat et al. (4). Differences may be largely attributable to the study designs (i.e., the exact storage conditions) and the various analytical methods used. Higher storage temperatures can result in more substantial decreases in 6-TGN and 6-MMP concentrations in blood samples, as is clearly demonstrated by our work. Despite a high correlation shown between various analytical methods, nucleotide hydrolysis techniques vary considerably, mainly in the type of acids, D,L-dithiothreitol, and hydrolysis time used in the analytical procedure (2). These variations can ultimately lead to incomplete hydrolysis and subsequent lower measured 6-TGN concentrations.

Apart from these analytical issues, we have again demonstrated an essential and clinically relevant decrease in both 6-TGN and 6-MMP concentrations attributable to sample storage/shipping conditions, findings that are of pivotal importance for the

### Table 1. Median decreases (±95 CI) in 6-TGN and 6-MMP values (N = 10) during 7 days storage under controlled conditions.

| Day | Room temperature | | | Refrigeration | | |
|-----|------------------|---|---|----------------|---|
|     | Content, % | 95% CI | Content, % | 95% CI |
| 0   | 100              |       | 100         |       |
| 1   | 88,91 84,62 98,98 |       | 92,19 88,13 101,32 |       |
| 4   | 69,36 65,10 82,01 |       | 89,46 76,08 100,36 |       |
| 5   | 64,17 59,16 71,93 |       | 84,59 79,75 95,35 |       |
| 6   | 54,74 52,81 65,08 |       | 86,58 78,88 97,60 |       |
| 7   | 53,33 48,77 70,00 |       | 90,27 83,34 103,12 |       |

| Day | Room temperature | | | Refrigeration | | |
|-----|------------------|---|---|----------------|---|
|     | Content, % | 95% CI | Content, % | 95% CI |
| 0   | 100              |       | 100         |       |
| 1   | 84,64 76,24 92,74 |       | 91,98 71,69 99,90 |       |
| 4   | 72,67 65,62 79,83 |       | 87,82 78,50 100,02 |       |
| 5   | 72,46 59,46 83,54 |       | 87,16 74,42 104,86 |       |
| 6   | 62,77 51,37 73,59 |       | 83,19 72,86 95,31 |       |
| 7   | 54,62 40,08 68,34 |       | 86,30 75,06 96,36 |       |
use of therapeutic monitoring in (future) multicenter studies and for interpretation of pharmacological data in clinical practice in patients on thiopurine therapy. In addition, exact storage conditions are often not mentioned in published reports; their omission may partly explain the current controversy concerning thiopurine metabolite research.

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References


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Drug Monitoring and Toxicology (DMT)

To the Editor:

Tazocin is an injectable antibiotic preparation with broad-spectrum activity against aerobic and anaerobic gram-positive and gram-negative bacteria (1). Reported side effects that might prompt metabolic disease investigations include change in consciousness and encephalopathy (2, 3). We report 2 patients in whom interpretation of organic acid analysis was complicated by administration of tazocin.

Case 1 was a 56-year-old man admitted for routine aortobifemo­ral bypass graft surgery. Past medical history included peripheral vascular disease, hypercholesterolemia, and epilepsy. Postoperatively the patient developed an acute ab­domen and at laparotomy required extensive small bowel resection to treat a mesenteric infarction. His subsequent course was complicated by heparin-induced thrombocytopenia. The patient further deteriorated, with a reduced level of consciousness (Glasgow Coma Scale 12/15), and was noted to have metabolic acidosis, with an increased anion gap of 27 mmol/L (reference interval 14–18 mmol/L). To exclude pyroglutamic acidosis as a cause of the metabolic de­rangement, a urine sample was sent for organic acid analysis by GC-MS (4). The results showed the presence of 4-ethyl 2,3 dioxo-1-piperazine, which was identified from a standard library (Fig. 1). This GC-MS peak was attributed to drug consumption, but its exact nature was not immediately recognized by the analyzing laboratory or other specialist laboratories consulted.

The 2nd case was a 67-year-old man admitted with a myocardial infarction. This patient underwent angioplasty, and subsequently developed left ventricular failure, hy­potension, and acute renal failure necessitating intensive care. This patient had known severe peripher­al vascular disease and developed osteomyelitis of his right foot, requiring right below-knee am­putation. He developed a high an­ion gap metabolic acidosis (32 mmol/L) and a urine sample was sent for organic acid analysis. The presence of 4-ethyl 2,3 dioxo-1-piperazine was detected.

Each patient had been pre­scribed a number of drugs, but only tazocin was being given to both individuals. Tazocin is a combination of piperacillin sodium and the lactamase inhibitor ta­zobactam sodium. The structure of piperacillin is sodium (2S,5R,6R)-6-[(R)-2-(4-ethyl-2,3-dioxo-1-piperazine-carboxamido)-2-phenylacetamido]-3,3-dimethyl-7-oxo-4-thia-1-aza­ bicyclo[3,2,0]heptane-2-carboxylic acid, and it was this substance that was detected in the urine.

Tazocin has previously been reported to produce a peak in the B region in capillary zone electro­phoresis, potentially simulating a small monoclonal protein (5). We observed that organic acid analysis by GC-MS of intravenously ad­ministered tazocin produced a peak. Although the presence of this tazocin peak is unlikely to result in
an incorrect diagnosis, describing our finding may enable others noting such a peak to realize its identity more rapidly.

References


3. Tong MKH, Sui Y-P, Yung C-Y, Kwan T-H. Pip-

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False-Positive Rates for the Qualitative Analysis of Urine Benzodiazepines and Metabolites with the Reformulated Abbott Multigent™ Reagents

To the Editor:

In May, 2007, Abbott notified customers of the implementation of a new polyclonal antibody pool in the Multigent™ benzodiazepine reagent (supplied by Seradyn, Inc.) for use on their Architect™ chemistry analyzer. The new antibody pool demonstrated an increased analytical sensitivity for this screening method, which allowed detection of lower concentrations of individual benzodiazepines and their metabolites. Unfortunately, by increasing the sensitivity, Abbott decreased the specificity of this assay, resulting in an increased number of false-positive benzodiazepine results.

For the past 15 years, the clinical chemistry laboratory of the VA Boston Healthcare System has performed screening for benzodiazepines in urine by immunoassay. Before 2001 the toxicology laboratory confirmed every screen-positive benzodiazepine result by HPLC. Table 1 summarizes the historical benzodiazepine data in 2000, which shows that 94.6% of the screen-positive results were confirmed as positive. Because of the high confirmation rate, the laboratory altered its policy of automatically confirming every screen-positive result to a policy of confirming screen-positive benzodiazepine by clinician request only (1). This protocol resulted in a dramatic decrease of 95% in the number and costs of confirmation testing (1).

Table 1 also summarizes the recent VA Boston Healthcare System experience of using the reformulated Abbott Multigent benzodiazepine reagent (List no. 3L 39–20; Lot No. 49274M200 and higher). There was a significant increase to 25.1% in the screen-positive rate for patient samples tested for benzodiazepines in June and July 2007, compared to the historical screen-positive rate of 13.1%. Because the laboratory suspected that the new Abbott reagents were yielding false-positive benzodiazepine results, the toxicology laboratory performed HPLC confirmation testing for all screen-positive benzodiazepine results over the 2-month period and found a positive confirmation rate of 74.3%. The false-positive rate was 25.7%, which is 5 times the 5.4% false-positive rate obtained with the SYVA™ reagent used in 2000 (Table 1). During the 6-month period before the introduction of the reformulated benzodiazepine reagent, we had observed a screen-positive rate of 15.1% (1126/7476 screens) with the Abbott reagents, not unlike our historical rate of 13.1%.

We reviewed the medical records of patients (n = 37) from whom a total 50 randomly selected urine specimens were obtained that yielded positive screening results for benzodiazepines with the Abbott Multigent, but were negative with HPLC (false-positive results). Of the 50 false-positive results, 16 (32%), from 9 of the 37 patients, were found in patients on the medication sertraline (Zoloft), a drug commonly used for depression, social anxiety disorders, posttraumatic stress and panic disorders, and obsessive-compulsive disorders. Sertraline is listed by Abbott in their package insert as a substance that commonly cross-reacts with and yields false-positive benzodiazepine screening results. Abbott also cites the possibility of high concentrations of oxaprozin yielding a false-positive benzodiazepine result, but none of the 37 patients were being prescribed this medication.

Because of the apparent high false-positive rate observed with the Abbott Multigent reagent, we proposed returning to the SYVA Emit benzodiazepine technology. A comparison of the Abbott/SYVA benzodiazepine reagents was conducted in our laboratory. Of the 50 randomly selected samples found positive by Multigent that gave negative results following HPLC confirmation, 47 of 50 screened negative by SYVA (94% concordance) and 3 of 50 gave positive screen results.

From May to July 2007, the toxicology section of the VA Boston Healthcare System, which performs all HPLC confirmation for all VA medical centers in New England, has analyzed a total of 1975 HPLC
benzodiazepine confirmation tests compared to approximately 20 tests during a similar 3-month period in 2006. Because of the unacceptably high false-positive rates for benzodiazepine screening using the Multigent reagents and the significant increase in toxicology workload and costs, the VA Boston Healthcare System elected to switch to the Abbott/SYVA reagents for benzodiazepine screening in August 2007. It is our understanding that Abbott is aware of the problem we have described and is in the process of reformulating its Multigent benzodiazepine reagent to increase its specificity.

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Reference


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Are Clinical Laboratories Prepared for Accurate Testing of 25-Hydroxy Vitamin D?

To the Editor:

Vitamin D deficiency is an important concern, but assays for serum 25-hydroxy vitamin D (25-OH-D), the accepted marker for vitamin D nutritional status, are not standardized. A quick search of Medline yielded 6014 citations on vitamin D deficiency, 51 of which were published in the first 6 mo of 2007 (1). Recognition that vitamin D deficiency may be more prevalent in most patient populations than earlier assumed has resulted in an unexpected and marked increase in the volume of testing for 25-OH-D in clinical laboratories (1). Historically, not many tests in the clinical laboratories have increased at the rate of 80% to 90% per year, as is the case for 25-OH-D in our reference laboratory. Several million 25-OH-D tests are likely to be performed this year in the US by various reference and local hospital laboratories, raising questions about the adequacy of the methods used to make these measurements.

Various methods are available for measuring circulating concentrations of 25-OH-D (until recently, only RIA was available). Current methods include HPLC, RIA with low throughput to high throughput, automated chemiluminescence immunoassays, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). These new methods have already aroused controversy (2–4). Correlation and agreement studies between immunoassays and LC-MS/MS methods for 25-OH-D have been reported by several investigators (2–4). These studies report reasonable correlations but with significant differences, the
reasons for which are not transparent or well understood. Automated or manual competitive immunoassays are known to have less specificity for low-molecular-weight compounds, and immunoassays for 25-OH-D are no exception.

The College of American Pathologists (CAP) and the UK-based DEQAS (Vitamin D external quality assessment scheme) surveys provide independent approaches to monitor the performance of laboratories that use various methods for testing of 25-OH-D. The survey feedback does not assess the accuracy of 25-OH-D measurements by laboratories, but scores laboratories for agreement within the group using a particular method. Recent CAP data (CAP survey, 2006 Ligands Special) indicate that clinical laboratories using chemiluminescence immunoassays can report a result ranging from 41 to 96 μg/L for a survey sample with a value of 31 μg/L determined by LC-MS/MS (BGS-04 in Fig. 1). There could be many reasons for these variations, including drifts in the reagents being manufactured, but there is a clear and urgent need for harmonization and standardization.

NIST is developing additional quality control materials (human serum, SRM 972) that will contain 25-OH-D2, 25-OH-D3, and the metabolite 3-epi-25-OH-D at 4 different concentrations as characterized by LC-MS/MS. Although the biological significance of the metabolites remains to be elucidated, the preparation of this SRM is especially important for assays for which the cross-reactivity with these metabolites is not well defined (5).

LC-MS/MS is becoming the technique of choice for various reference laboratories. Laboratories that use in-house LC-MS/MS have responsibility for many steps of the assay. The LC-MS/MS technology for testing of human samples is not approved by the FDA, and manufacturers of LC-MS/MS instrumentation are not responsible for troubleshooting the assays. Laboratories performing 25-OH-D testing by LC-MS/MS technology have differences in their standard operating procedures, and thus interlaboratory CVs are in the range of 20%. The preparation of the reagents required for in-house LC-MS/MS assays is conducted by individual laboratories under their institutionally regulated standard procedures. The complexity of the LC-MS/MS technology in its present form demands a robust, fully automated platform that can meet the need for throughput, precision, and accurate testing of vitamin D and metabolites. Multiplexed immunoassays may have the potential of achieving accuracy and precision for multiple vitamin D metabolites. For better patient care, the goal should be not only to have an accurate 25-OH-D value but also precision for 25-OH-D testing, with a CV <1%.

Currently there are no guidelines or agreement among clinical laboratories on the optimum reference intervals for 25-OH-D to classify patients with moderate to severe vitamin D deficiency. Before it is too late, it is in the interest of clinical laboratories and diagnostic companies to work together to standardize the reagents and reference intervals to achieve the quality for 25-OH-D tests that our patients deserve.

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References

Fig. 1. CAP survey data for 2006 on commonly used immunoassays for 25-OH-D.

The ranges of results for survey materials BGS-01 through BGS-04 are shown for laboratories using either RIA (solid lines, n = 16) or automated chemiluminescent immunoassays (broken lines, n = 18). Our LC-MS/MS 25-OH-D test results are shown (closed circles) for comparison. CAP survey data for survey materials BGS-01 and BGS-02 in 2007 have similar trends. CLIA, chemiluminescence immunoassay.
Nonstandard abbreviations: MPO, myeloperoxidase.

1 Myeloperoxidase (MPO) has been shown to play a role in the pathogenesis of coronary artery disease. It is released from activated neutrophils at sites of vascular damage and has been shown to increase in atherosclerotic plaques before the onset of myocardial injury (1). Elevations in MPO occur independently of C-reactive protein (CRP) and other markers of inflammation, and it has been suggested as a marker of risk in patients who have high as well as low cardiac troponin T concentrations and who present with acute coronary syndrome (2).

We recruited 12 apparently healthy individuals, 10 women and 2 men with a mean age of 33 years (range 27–43) for estimation of the biological variation of MPO. None had a history of heart disease or any clinically apparent inflammatory processes. Serum samples were collected between 0800 and 1000 on the same day of the week, weekly for 5 weeks as described by Fraser and Harris (3). Venous blood was collected in plastic serum separator tubes. All samples were centrifuged after clotting and were stored at −70 °C before analysis. All samples were analyzed with the MPO ELISA kit from Immundiagnostik and the model 3550 microplate reader from Bio-Rad.

The statistical methodology of Fraser and Harris was used unless otherwise stated (3). Except for the analytical assessment, all assessments were done on both untransformed and logarithmically transformed (natural logarithm) data. The data, which are presented in Fig. 1, provided 60 estimates of analytical variance and 12 estimates of within-subject variance. The analytical variance plotted against the cumulative percentile ranking followed the expected χ² distribution within good proximity. Cochran’s test of maximum variance identified 1 analytical outlier. The residuals of a 1-way ANOVA of the means of duplicates were used for assessment of within-subject distribution (a deviation from the methodology of Fraser and Harris). Cochran’s test detected 1 within-subject outlier that was not present after log-transformation. The outlier was in a sample from subject 1, from whom the analytical outlier was also obtained. In order not to be biased toward 1 distribution, all values of subject 1 were excluded from further analysis. No other outliers were detected after this exclusion. Nonnormality of the untransformed and log-transformed data (after exclusion of subject 1) was not apparent by Shapiro-Wilk tests (P = 0.58 and P = 0.26, respectively). The within-subject index of heterogeneity was calculated as the ratio of the CV of the individual variances to the theoretical CV calculated as [2/(n − 1)]¹/², where n is the average number of specimens from each subject (3). The absolute difference of this ratio from unity was less than the theoretical SD calculated as 1/(2n)¹/², suggesting that the within-subject variances were homogeneous. Nonnormality of the between-subject distribution as assessed using subject averages and the Shapiro–Wilk test could not be shown for either the untransformed or log-transformed data (P = 0.10 and P = 0.09, respectively). No outliers were found in either of the 2 distributions using a Reed–Dixon ratio criterion of 1/3. Given these statistics, the analytical (CVₐ), within-subject (CVᵢ), and between-subject (CV₉) CVs were estimated with nested ANOVA of the untransformed data only.

The CVₐ, CVᵢ, and CV₉ were 4%, 36%, and 30%, respectively. The design of the biological variation study led to an unrealistically low CVₐ, which is desirable for accurate estimates of the other variance components (3). A more realistic CVₐ of 8.4%, obtained from the literature, was used in further calculations (2). The index of individuality (Ii), calculated as (CVₐ + CVᵢ)/CV₉, was 1.5, which is satisfactory for comparison of an observed value to a reference value (3). Contrary to CRP, which has a low Ii, the high Ii of MPO supports the use of a population-based cutoff for risk stratification (4). A cutoff of 350 μg/L has been proposed for risk stratification and is used here only as an example because the assay has not been stan-

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dardized (2). The interval in which correct classification will not be certain at this concentration can be calculated as

$$MPO_i = 350 \pm \left( z \times \sqrt{\frac{CV_A^2 + CV_i^2}{n}} \right) \times 350$$

where $MPO_i$ is the value to be calculated, 350 is the suggested concentration in ug/L, $n$ is the number of replicate samples, and $z$ is 1.64 for a 1-sided 95% confidence interval (5). With a single-sampling strategy, correct classification of patients with concentrations between 138 and 562 µg/L will not be 95% certain. The reference change value of MPO, calculated as $2.77 \times (CV_A^2 + CV_i^2)^{1/2}$, is 102%. In conclusion, the high li of MPO supports the use of a population-based cutoff but the high CVI leads to a rather large interval in which correct classification will not be certain.

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References


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Mismeasure of C-Type Natriuretic Peptide

To the Editor:

It has been difficult to establish an endocrine role for C-type natriuretic peptide (CNP). Release of CNP from the heart remains to be convincingly demonstrated (1–5), and data concerning CNP in cardiac disease have been conflicting, possibly because of specificity problems in measurement of CNP. All natriuretic peptides (NPs) display strong homology, and antibodies raised against one NP may consequently bind other NPs. With the relatively high plasma concentrations of A- and B-type NPs (ANP and BNP), a CNP assay with even a small degree of ANP or BNP cross-reactivity may produce falsely high CNP concentrations. This is especially relevant in heart failure with augmented cardiac ANP and BNP expression. We examined the selectivity of a widely used CNP RIA.

To evaluate cross-reactivity in the CNP assay, we measured serial dilutions of human CNP-22 (4.5–582 pmol/L), ANP-28 (5–100 000 pmol/L), and BNP-32 (4–81 000 pmol/L) against the calibrators of the CNP assay. We obtained human cardiac tissue samples from a heart failure patient undergoing cardiac transplantation. The local ethics committee approved the use of human tissue, and written informed consent was obtained from the patient before surgery. The extracts were subjected to measurements of CNP, BNP, NT-proBNP, and NT-proCNP using a commercial CNP RIA (Phoenix), a BNP assay (Shionoria), and in-house RIAs directed against the N-terminus of proBNP and proCNP, respectively.

Results are expressed as mean (SE). We used nonlinear regression analysis to fit 4-parameter logistic models to the data from the buffer experiments. Based on the model, we calculated the cross-reactivity as a function of BNP-32 concentration using a 1-phase exponential decay model. Likewise, we calculated cross-reactivity as a function of the BNP concentration by fitting data from the heart extract experiments to a 1-phase exponential decay model. Cross-reactivity was calculated as measured CNP concentration divided by measured BNP concentration.

Serial dilutions of human CNP-22, BNP-32, and ANP-28 in barbital buffer measured with the CNP RIA revealed cross-reactivity for BNP-32 in physiological concentrations (Fig. 1A). The dilution curves for CNP-22 and BNP-32 were not parallel; cross-reactivity increased with decreasing BNP-32 concentrations, being 5.1% at a BNP-32 concentration of 100 pmol/L and 1.6% at 1000 pmol/L (Fig. 1B). As expected, BNP and NT-proBNP concentrations were high in the atria and lower in the ventricles (Fig. 1C). NT-proCNP and particularly CNP concentrations were very low in both atria and ventricles (Fig. 1D). With only trace amounts of NT-proCNP in the extracts, most of the measured CNP represents cross-reacting BNP. Calculating cross-reactivity as CNP concentrations divided by BNP concentrations in the extracts, we fitted a curve parallel to that obtained in the buffer experiment (Fig. 1B). Cross-reactivity was estimated to be 3.2% at a BNP concentration of 100 pmol/L and 1.1% at 1000 pmol/L.

Nearly all reports on CNP measurement in plasma are based on the same RIA. According to the manufacturer, the assay does not exhibit any cross-reactivity with ANP or BNP. Nevertheless, our data indicate substantial BNP cross-reactivity of the CNP assay in buffer with added BNP-32 and in heart extracts (Fig. 1B). Ideally, chromatographic evaluation of the extracts could resolve the BNP and CNP forms, as coelution of CNP and BNP immunoreactivity would have provided further proof of BNP cross-reactivity. However, the CNP concentration in the heart extracts did not exceed 15 pmol/L, rendering chromatography very difficult. The difference in the degree of cross-reactivity between the buffer with added BNP-32 and the unmodified heart extracts may be partly because immunoreactive BNP in the extracts comprises both BNP-32 and intact proBNP, which may have another cross-reactivity profile. Alternatively, BNP measured by the Shionoria assay may partly have been cross-reacting ANP, which is highly expressed in the cardiac atria. Because N-terminal proCNP, proANP, and proBNP have virtually no sequence resemblance, cross-reactivity of the NT-proCNP assay with proANP and proBNP is highly unlikely. If intact proCNP(1-103) is present in the heart, the NT-proCNP assay would probably also measure it.

The CNP assay may still be valuable for CNP measurement in samples with low BNP concentrations; however, measurements done in samples with a high BNP concentration—such as heart tissue extracts or plasma samples from patients with heart failure—should be interpreted cautiously.

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References


Fig. 1. Dilution curves for human CNP-22, ANP-28, and BNP-32 measured with Phoenix CNP-22 assay. (A) Vertical broken lines mark ED_{50} of CNP-22 and BNP-32. (B) Fitted curves for cross-reactivity as a function of BNP-32 in the buffer experiment and BNP immunoreactivity in the cardiac tissue extract experiment. (C) BNP and N-terminal proBNP in cardiac extracts. (D) CNP and NT-proCNP in cardiac extracts. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.
We describe a modified multiplex T-ARMS-PCR, the hexaprimed ARMS-PCR (H-ARMS-PCR), which is for when 2 polymorphisms are close in the sequence. H-ARMS-PCR uses only 6 primers and provides direct information about haplotype structure.

The CTLA4 gene (cytotoxic T-lymphocyte–associated protein 4; also known as CD152) is a negative regulator of T-cell function (3). The CTLA4 polymorphisms −318 C>T (rs5742909) and +49 A>G (rs231775) are associated with susceptibility to autoimmune diseases and cancer (3, 4). To genotype these 2 polymorphisms, which are only 365 bp apart in the 5′ region of the CTLA4 gene, we designed an H-ARMS-PCR that combines a single pair of common primers and 2 pairs of allele-specific primers in the same tube (Fig. 1A).

The PCR reaction (25 μL) contained 200 μmol/L deoxyxynucleoside triphosphates, 100 ng of genomic DNA, 1.25 U of HotStarTaq DNA Polymerase with its buffer (Qiagen), and the following primers at the indicated concentrations (deliberate mismatches from the reference sequence, GenBank no. M74363, are in boldface italics): −318fo, 5′-CAATGAATGAATTGACTGGA TG-3′ (0.5 μmol/L); +49ro, 5′-TA CAGAGCCAGCCAAGGCAGATT- 3′ (0.8 μmol/L); −318fi(c), 5′-CTC CACTGTATTCCAGATCGTC-3′ (2.5 μmol/L); −318ri(t), 5′-AC TGAAGCTTACATGCTACTCTA-3′ (0.5 μmol/L); +49fi(a), 5′-GCACA AGGCTCCAGCAGCCAGGTCTATAGC-3′ (0.1 μmol/L); and +49ri(g), 5′-ACAGGAAGGCTGACGGCCAG GTCTTAGC-3′ (0.5 μmol/L). Cycling conditions were 12 min at 95 °C, followed by 5 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s; and a final cycle at 72 °C for 10 min.

We genotyped both the −318 C>T and +49 A>G CTLA4 polymorphisms with this newly designed H-ARMS-PCR (Fig. 1A) for samples from 80 individuals that had previously been characterized with 3 established methods [restriction fragment length polymorphism (RFLP) PCR, direct DNA sequencing, and T-ARMS-PCR] (4) and observed no discrepancies. Moreover, H-ARMS-PCR provides both an additional internal control and direct information about the haplotype structure by generating an amplicon that is specific for 1 of the 4 haplotypes that 2 polymorphisms can theoretically have. In this case, the haplotype-specific amplicon is observed when the alleles −318C and +49G are on the same chromosome (Fig. 1A).

We further tested this approach by implementing H-ARMS-PCR in the genotyping of 2 polymorphisms that are 142 bp apart in the 3′ untranslated region (UTR) of the CYP19A1 gene (cytochrome P450, family 19, subfamily A, polypeptide 1): rs10046 (C>T) and rs4646 (G>T). CYP19A1 encodes aromatase, the enzyme responsible for the final step of estrogen biosynthesis. CYP19A1 polymorphisms have been associated with estrogen concentrations in woman and with susceptibility to breast and prostate cancers (5).

We used the PCR to analyze these 2 CYP19A1 polymorphisms in the 3′ UTR (Fig. 1B; see legend for PCR conditions) in 100 individuals already characterized by RFLP PCR and/or direct DNA sequencing (P.P. and R.N., unpublished data) and observed no discrepancies. A haplotype-specific amplicon is present when alleles rs10046C and rs4646T are on the same chromosome. This H-ARMS-PCR is currently being used in a mul-

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**Letters**

**Hexaprimed Amplification Refractory Mutation System PCR for Simultaneous Single-Tube Genotyping of 2 Close Polymorphisms**

To the Editor:

Tetraprimed amplification refractory mutation system PCR (T-ARMS-PCR) is a simple and inexpensive genotyping method for differentiating both alleles of a polymorphism/mutation (both single-nucleotide polymorphisms and small insertions/deletions) with a single-tube PCR (1). In T-ARMS-PCR, a pair of common (outer) primers produces a non–allele-specific control amplicon and in combination with 2 allele-specific (inner) primers (designed to anneal in the opposite orientation) produces 2 allele-specific amplicons. These allele-specific amplicons have different sizes because the polymorphism/mutation is asymmetrically located with respect to the common primers. Thus, the amplicons can be separated by standard gel electrophoresis. T-ARMS-PCR has also been designed in a multiplex fashion to genotype more than one polymorphism/mutation by a single-tube PCR (2).

We describe a modified multiplex T-ARMS-PCR, the hexaprimed ARMS-PCR (H-ARMS-PCR), which is for when 2 polymorphisms are close in the sequence. H-ARMS-PCR uses only 6 primers and provides direct information about haplotype structure.

The CTLA4 gene (cytotoxic T-lymphocyte–associated protein 4; also known as CD152) is a negative regulator of T-cell function (3). The CTLA4 polymorphisms −318 C>T (rs5742909) and +49 A>G (rs231775) are associated with susceptibility to autoimmune diseases and cancer (3, 4). To genotype these 2 polymorphisms, which are only 365 bp apart in the 5′ region of the CTLA4 gene, we designed an H-ARMS-PCR that combines a single pair of common primers and 2 pairs of allele-specific primers in the same tube (Fig. 1A).

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We genotyped both the −318 C>T and +49 A>G CTLA4 polymorphisms with this newly designed H-ARMS-PCR (Fig. 1A) for samples from 80 individuals that had previously been characterized with 3 established methods [restriction fragment length polymorphism (RFLP) PCR, direct DNA sequencing, and T-ARMS-PCR] (4) and observed no discrepancies. Moreover, H-ARMS-PCR provides both an additional internal control and direct information about the haplotype structure by generating an amplicon that is specific for 1 of the 4 haplotypes that 2 polymorphisms can theoretically have. In this case, the haplotype-specific amplicon is observed when the alleles −318C and +49G are on the same chromosome (Fig. 1A).

We further tested this approach by implementing H-ARMS-PCR in the genotyping of 2 polymorphisms that are 142 bp apart in the 3′ untranslated region (UTR) of the CYP19A1 gene (cytochrome P450, family 19, subfamily A, polypeptide 1): rs10046 (C>T) and rs4646 (G>T). CYP19A1 encodes aromatase, the enzyme responsible for the final step of estrogen biosynthesis. CYP19A1 polymorphisms have been associated with estrogen concentrations in woman and with susceptibility to breast and prostate cancers (5).

We used the PCR to analyze these 2 CYP19A1 polymorphisms in the 3′ UTR (Fig. 1B; see legend for PCR conditions) in 100 individuals already characterized by RFLP PCR and/or direct DNA sequencing (P.P. and R.N., unpublished data) and observed no discrepancies. A haplotype-specific amplicon is present when alleles rs10046C and rs4646T are on the same chromosome. This H-ARMS-PCR is currently being used in a mul-

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ticenter Italian clinical trial (GIM5) to test the possible association of CYP19A1 polymorphisms with the efficacy of adjuvant therapy with an aromatase inhibitor (letrozole) after tamoxifen treatment in postmenopausal patients with early breast cancer.

In addition, we genotyped a series of 40 individuals for both CTLA4 and CYP19A1 polymorphisms by H-ARMS-PCR analysis with different thermal cyclers (iCycler® by Bio-Rad, Px2® by Hybaid, and PTC-100® by MJ Research) and in different laboratories, and we obtained fully concordant results, demonstrating the robustness and reproducibility of this approach. All human samples were collected after informed consent was obtained according to institutional procedure.

We have shown that H-ARMS-PCR is an effective and robust technique for genotyping 2 close polymorphisms (in a range of about 100–400 bp) with only 6 primers. With appropriate long-range Taq DNA polymerase, H-ARMS-PCR may work for genotyping even more distantly separated polymorphisms, likely within a distance of about 5 kb.

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References

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