Aspirin Responsiveness in Healthy Volunteers Measured with Multiple Assay Platforms

Brad S. Karon,1* Amy Wockenfus,1 Renee Scott,1 Stacy J. Hartman,1 Joseph P. McConnell,1 Paula J. Santrach,1 and Allan S. Jaffe1

BACKGROUND: We evaluated the sensitivity, precision, and concordance of 4 assays designed to detect aspirin responsiveness or resistance.

METHODS: Twenty-nine healthy laboratory volunteers took 80 mg aspirin for 7 days, and a subset of volunteers took 325 mg aspirin for an additional 7 days. We measured platelet function by light transmission aggregometry with arachidonic acid, PFA-100, and VerifyNow. PFA-100 and VerifyNow assays were performed in duplicate to assess method imprecision. Some volunteers had samples taken within 2–4 h of the final dose of aspirin and again within 20–24 h of the final dose. We measured urinary 11-dehydro-thromboxane B2 at baseline and after 80 or 325 mg aspirin.

RESULTS: No volunteers were nonresponsive to aspirin therapy as measured by the PFA-100. One of 29 participants demonstrated lack of response to aspirin as measured by VerifyNow and urinary 11-dehydro-thromboxane B2; 2 of 29 demonstrated lack of response as measured by light transmission aggregometry. Imprecision was &lt;10% for the PFA-100 and VerifyNow. Concordance was high (&gt;90%) between all assays. Neither aspirin dose (80 vs 325 mg) nor timing between final dose of aspirin and blood draw (2–4 vs 20–24 h) affected any of the assays.

CONCLUSIONS: Light transmission aggregometry, PFA-100, VerifyNow, and urinary 11-dehydro-thromboxane B2 are all sensitive to the effects of aspirin in healthy individuals. Variables such as aspirin dose, timing between final dose of aspirin and blood collection, and imprecision do not affect the ability of the assays to detect aspirin effect on platelet function.

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Platelet inhibition induced by treatment with aspirin is a cornerstone of preventive therapy for arterial thrombotic events, reducing the risk of acute arterial thrombotic events by at least 25% in high-risk patients (1, 2). In patients with acute myocardial infarction, it also reduces mortality (3). The mechanism of the aspirin effect on platelet function is well defined. Aspirin acts through irreversible inhibition of cyclooxygenase enzyme activity in platelets and therefore reduces levels of the platelet activator thromboxane A2 (1, 2). Many patients, however, still have acute arterial thrombotic events despite treatment with aspirin, a situation that some consider to be due to “aspirin resistance” or “aspirin nonresponsiveness.” Several definitions of aspirin resistance have been proposed, including (1) a clinical definition of aspirin resistance applied to a patient that has an acute arterial thrombotic event despite aspirin therapy; (2) a definition based on ex vivo assessment of platelet activation, i.e., failure of platelets to be inhibited after aspirin therapy as measured by various platelet function tests in the clinical laboratory; and (3) a definition predicated on the failure of aspirin to inhibit thromboxane A2 production in vivo (1, 2).

Over the past decade, multiple efforts have been made to develop clinical laboratory tests that provide more meaningful measurements of aspirin effects on platelet function and thromboxane generation to study and define aspirin resistance or nonresponsiveness. The most commonly used tests include light transmission aggregometry in response to arachidonic acid (AA LTA), the Dade Behring PFA-100 platelet function analyzer, and the VerifyNow Rapid Platelet Function Assay (Accumetrics Inc.). The most common measurements of the aspirin effect on thromboxane generation are serum thromboxane B2 and urinary 11-dehydro-thromboxane B2 (d-TxB2),2 both metabolites of thromboxane A2 (1, 2). Numerous studies on both

1 Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN.

* Address correspondence to this author at: Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First St. SW, Rochester, MN 55905. Fax: 507-538-7060; e-mail karon.bradley@mayo.edu.

2 Nonstandard abbreviations: d-TxB2, 11-dehydro-thromboxane B2; AA LTA, light transmission aggregometry in response to arachidonic acid; ARU, aspirin response unit.

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normal individuals and various at-risk patient populations have led to vastly different estimates of the prevalence of aspirin nonresponsiveness, in part depending on what laboratory measure is used to define the syndrome. However, no study has evaluated all 4 of the commonly used tests for aspirin effect under well-controlled conditions so that all laboratory and clinical variables affecting test performance could be accounted for. The lack of consistency among studies has impeded the ability to define a gold standard test or method for assessing aspirin response, or even to understand whether laboratory assessment of aspirin effect is warranted (1, 2).

Understanding the effects of aspirin on ex vivo platelet function tests in normal healthy volunteers is the first step toward understanding how to apply the tests to patients at risk for arterial thrombosis. Two previous studies found that both AA LTA and VerifyNow are capable of detecting the effects of aspirin in the vast majority of individuals taking 100 mg aspirin, whereas the PFA-100 did not detect aspirin effect in approximately 30% of these individuals (4, 5). One of these studies also measured urinary d-TxB₂ and found it very sensitive in detecting aspirin inhibition of thromboxane production (5). In contrast, another study of healthy volunteers found that after 100 mg aspirin for 12 days, PFA-100 values increased markedly and remained increased thereafter, suggesting that the PFA-100 is very sensitive to the effects of aspirin (6). Comparison of results between these studies of healthy volunteers is difficult due to different definitions used for resistance in each study, differences in duration of aspirin therapy, different citrate concentrations used for blood collection, and different intervals between dosing of aspirin and blood collection.

In contrast to normal healthy populations, studies of at-risk patient populations have found that the different assays lead to vastly different estimates of aspirin resistance and that correlation between AA LTA, thromboxane metabolites, and VerifyNow is very poor (7–11). In general, these studies have found that the PFA-100 classifies the highest percentage of patients as nonresponders, whereas the VerifyNow classifies the lowest percentage of patients as nonresponsive (7–11). To interpret studies comparing AA LTA, PFA-100, VerifyNow, and urinary d-TxB₂ as tests for aspirin responsiveness, it is important to know whether aspirin dose or duration, timing between final dose of aspirin and blood collection, and/or method imprecision affect the ability of any of these tests to detect the effects of aspirin in healthy volunteers. To date, no study has evaluated the impact of these variables on all 4 commonly used assays for aspirin effect in a population of healthy adults. Accordingly, we measured the effect of both 80 and 325 mg aspirin doses on platelet function in healthy volunteers as measured by AA LTA, VerifyNow, and PFA-100 and on thromboxane generation measured by urinary d-TxB₂. Factors potentially affecting assay results were carefully controlled so that accurate comparisons could be made between the various measurement platforms.

**Materials and Methods**

**PARTICIPANTS AND SPECIMEN COLLECTION**

Twenty-nine healthy laboratory volunteers consented to take 80 mg nonenteric coated aspirin once per day for 7 days. Volunteers were provided with the pills and asked to document on a log sheet the time that aspirin was taken each day. Participants were screened for any bleeding history and were not taking antithrombotic medication or medication for heart disease. All were asked to refrain from taking any over-the-counter pain medication for 10 days before the study. At baseline (before the first aspirin dose), participants provided a urine sample for urinary d-TxB₂, and had blood drawn into tubes containing 0.105 mol/L (3.2%) sodium citrate for PFA-100 and AA LTA measurement and into collection tubes provided by the manufacturer for VerifyNow measurement. Blood was processed and testing complete (for AA LTA, VerifyNow, and PFA-100) within 2 h of collection. Urine samples were frozen at −70°C for later analysis. The study design was approved by the Mayo Clinic Institutional Review Board.

The 29 volunteers returned within 2–4 h after taking the seventh dose of 80 mg aspirin for repeat blood collection for PFA-100, AA LTA, and VerifyNow and for urine collection. A subset (n = 13) of volunteers also returned 20–24 h after the seventh dose of 80 mg aspirin for an additional blood collection for PFA-100, AA LTA, and VerifyNow. Ten volunteers went on to take 325 mg of nonenteric coated aspirin for an additional 7 days after completion of the 80 mg study; these volunteers had blood and urine collected for PFA-100, VerifyNow, AA LTA, and d-TxB₂ within 0–2 h of the final dose of 325 mg aspirin and again (blood collection only) within 20–24 h of the final dose.

**LABORATORY TESTING**

LTA was performed with a Biodata aggregometer (Bio/Data Corp.) using platelet-rich plasma adjusted to a platelet concentration between 200 and 300 × 10⁹/L and using 1.6 mmol/L (0.5 g/L) arachidonic acid as described (7). Two dedicated technicians did all of the testing to reduce variability. PFA-100 using collagen and epinephrine (CEPI) cartridge, VerifyNow (Accumetrics), and urinary d-TxB₂ analyses were performed as described (7). PFA-100 and VerifyNow measure-
ments were made in duplicate and the average value used for data analysis. Definitions of aspirin resistance used were AA LTA ≥20%, PFA-100 <193 s, and VerifyNow ≥550 aspirin response units (ARU) as defined previously with these techniques (7).

Urinary d-TxB2 measurements were performed using the AspirinWorks® Test kit; a competitive enzyme immunoassay produced by Corgenix Inc., according to manufacturer suggestions. Urinary d-TxB2 values (normalized to creatinine) of >1500 pg/mg creatinine were used to define resistance as recommended by the manufacturer (12). Intraassay precision at concentrations of 453 ng/L, 796 ng/L, and 2603 ng/L d-TxB2 demonstrated CVs of 2.5%, 10.8%, and 7.3%, respectively, when 4 to 5 replicates were measured at each concentration during a single run. To minimize imprecision, study samples were analyzed on a single run of testing.

Recovery studies using urine samples with measured d-TxB2 concentrations of 1850 and 438 ng/L demonstrated 70%–80% recovery of expected values when the 2 samples were mixed together at varying ratios. At higher d-TxB2 concentrations (>4000 ng/L), recovery was suboptimal, with 50%–70% recovery of expected values, indicating limitations in the ability of the assay to accurately measure high concentrations of d-TxB2. We collected 55 urine samples from healthy volunteers (not on aspirin) to compare ELISA d-TxB2 results to an assay under development using mass spectrometry. The relationship between the 2 assays was ELISA d-TxB2 = mass spectrometric d-TxB2 × 4.20 – 332, with an r² of 0.9282, indicating good correlation of ELISA results to more definitive methods using mass spectrometry.

**Table 1.** PFA-100 values for participants at baseline (before aspirin treatment) and after 80 or 325 mg aspirin.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>80 mg</th>
<th>20–24 h</th>
<th>325 mg</th>
<th>20–24 h</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>2–4 h</td>
<td></td>
<td></td>
<td>2–4 h</td>
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<tr>
<td>Mean (SD), s</td>
<td>135 (27)</td>
<td>281 (19)</td>
<td>281 (19)</td>
<td>286 (11)</td>
<td>282 (25)</td>
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<tr>
<td>Median, s</td>
<td>127</td>
<td>284</td>
<td>285</td>
<td>280</td>
<td>295</td>
</tr>
<tr>
<td>CV, %</td>
<td>7.5</td>
<td>3.6</td>
<td>5.0</td>
<td>4.2</td>
<td>3.5</td>
</tr>
<tr>
<td>No. nonresponsive</td>
<td>NA</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. tested</td>
<td>29</td>
<td>29</td>
<td>13</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

CV was calculated from duplicate testing. Some participants were tested both within 2–4 h of the final dose of aspirin and again 20–24 h after the final dose. NA, not applicable.

Baseline PFA-100 measurements on the 29 healthy volunteers yielded a mean (SD) value of 135 (27) s, median 127 s (range 103–218 s). Samples collected within 2–4 h of the seventh daily dose of 80 mg aspirin (n = 29) demonstrated a mean value of 281 (19) s, median 284 s (Table 1). Mean and median PFA values did not vary significantly (P > 0.05) when participants were tested 20–24 h after the seventh daily dose of 80 mg aspirin (n = 13) or when samples were collected after an additional 7 days of 325 mg aspirin in selected volunteers. We collected 62 PFA-100 measurements on 29 participants during aspirin treatment; none were treated as values of 300. A 2-sided P value of <0.05 was considered significant for evaluating mean and median differences between treatment groups, using InStat version 3 for Windows 2000 (GraphPad Software). Concordance between tests was determined by categorizing all results as either responsive or nonresponsive to aspirin as described above and then calculating the percent concordance as the number of tests in same category divided by all tests performed.

**Results**

Baseline VerifyNow results for the 29 individuals demonstrated a mean (SD) value of 621 (38) ARU, median 636 ARU (range 497–649 ARU). Samples collected within 2–4 h of the seventh daily dose of 80 mg aspirin demonstrated a mean value of 281 (19) s, median 284 s (Table 1). Mean and median PFA values did not vary significantly (P > 0.05) when samples were collected within 20–24 h after the seventh daily dose of 80 mg aspirin (n = 13) or when individuals took 325 mg aspirin for an additional 7 days and were tested either 2–4 h or 20–24 h after the final dose of

**STATISTICAL ANALYSIS**

Continuous variables are presented as mean, SD, and median. For data analysis, PFA-100 values ≥300 s were treated as values of 300. A 2-sided P value of <0.05 was considered significant for evaluating mean and median differences between treatment groups, using InStat version 3 for Windows 2000 (GraphPad Software). Concordance between tests was determined by categorizing all results as either responsive or nonresponsive to aspirin as described above and then calculating the percent concordance as the number of tests in same category divided by all tests performed.
325 mg aspirin (Table 2). Only 1 of 60 VerifyNow measurements made on 29 participants during aspirin treatment was $\geq 550$ ARU (Table 2). All measurements were made in duplicate, and the CV from duplicate testing was $<10\%$ in all treatment groups (Table 2).

Normal individuals at baseline had AA LTA results of 67$\%$ (20$\%$), median 70$\%$ aggregation. Blood samples collected within 2–4 h of the seventh dose of 80 mg aspirin showed a mean of 9$\%$ (7$\%$), median 6$\%$ aggregation (Table 3). Neither mean or median percent aggregation differed significantly when participants were tested within 20–24 h after the final dose aspirin or when participants took 325 mg aspirin for 7 days ($P > 0.05$) (Table 3). Only 2 of 60 LTA measurements made on the 29 participants during aspirin treatment showed aggregation $\geq 20\%$ (Table 3).

Urine was collected from the normal volunteers once at baseline (before aspirin treatment), a second time after the final dose of 80 mg aspirin, and a third time after the final dose of 325 mg aspirin. Baseline d-TxB$_2$ levels, normalized to mg creatinine, ranged from 1136 to 5920 pg d-TxB$_2$ per mg creatinine. Many of the baseline urine samples contained high (>4000 ng/L) concentrations of d-TxB$_2$, for which assay performance was suboptimal. For this reason, d-TxB$_2$ results are reported as percent inhibition comparing 80- and 325-mg doses (since both calculations rely on the same baseline number) and number of samples with postaspirin levels of $>1500$ pg d-TxB$_2$ per mg creatinine (a cutoff that reflects d-TxB$_2$ levels in the optimal measurement range).

After 80 mg aspirin treatment for 7 days, mean (SD) percent inhibition of d-TxB$_2$ was 70$\%$ (19$\%$), median 75$\%$. Mean (SD) percent inhibition of d-TxB$_2$ was 69$\%$ (28$\%$), median 78$\%$, for the subset of 10 participants who went on to take 325 mg aspirin for an additional 7 days ($P > 0.05$, no difference from mean/median at 80 mg) (Table 4). One of 39 d-TxB$_2$ measurements made on the aspirin-treated participants demonstrated $>1500$ pg d-TxB$_2$ per mg creatinine, the cutoff recommended by the manufacturer for determining aspirin response.

### Table 2. VerifyNow values for participants at baseline (before aspirin treatment) and after 80 or 325 mg aspirin.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>80 mg</th>
<th>325 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2–4 h</td>
<td>20–24 h</td>
<td>2–4 h</td>
</tr>
<tr>
<td>Mean (SD), ARU</td>
<td>621 (38)</td>
<td>419 (52)</td>
<td>426 (41)</td>
</tr>
<tr>
<td>95th CI of mean</td>
<td>607–636</td>
<td>399–439</td>
<td>400–452</td>
</tr>
<tr>
<td>Median, ARU</td>
<td>636</td>
<td>401</td>
<td>418</td>
</tr>
<tr>
<td>CV, %</td>
<td>3.2</td>
<td>5.0</td>
<td>4.5</td>
</tr>
<tr>
<td>No. nonresponsive</td>
<td>NA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>No. tested</td>
<td>29</td>
<td>29</td>
<td>12</td>
</tr>
</tbody>
</table>

CV was calculated from duplicate testing. Some participants were tested both within 2–4 h of the final dose of aspirin and again 20–24 h after the final dose. NA, not applicable.

### Table 3. Percent aggregation by AA LTA for participants at baseline (before aspirin treatment) and after 80 or 325 mg aspirin.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>80 mg</th>
<th>325 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2–4 h</td>
<td>20–24 h</td>
<td>2–4 h</td>
</tr>
<tr>
<td>Mean (SD), %</td>
<td>67 (20)</td>
<td>9 (7)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>95th CI of mean</td>
<td>59–74</td>
<td>5–11</td>
<td>5–8</td>
</tr>
<tr>
<td>Median, %</td>
<td>70</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>No. nonresponsive</td>
<td>NA</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>No. tested</td>
<td>29</td>
<td>29</td>
<td>13</td>
</tr>
</tbody>
</table>

CV was calculated from duplicate testing. Some participants were tested both within 2–4 h of the final dose of aspirin and again 20–24 h after the final dose. NA, not applicable.
Aspirin Response Measured by Multiple Assays

A total of 39 results for PFA-100, VerifyNow, AA LTA, and d-TxB₂ could be directly compared. Concordance (percent results in same category) between VerifyNow and AA LTA was 92.3% (36 of 39). Concordance between VerifyNow and d-TxB₂ was 94.8 (37 of 39); all other assay pairs demonstrated 97.4% (38 of 39) concordance.

### Discussion

Our data suggest that healthy volunteers are rarely nonresponsive to the effect of aspirin irrespective of aspirin dosage or measurement technique. All participants had platelet function assessed 2–4 h after the final dose of 80 mg aspirin; repeat measurement 20–24 h after the final dose in a subset of volunteers demonstrated that LTA, PFA-100, and VerifyNow can reproducibly detect the aspirin effect regardless of timing after the final dose and blood draw. Concordance between the 4 tests was >90%, suggesting that each test is capable of measuring the effect of aspirin on platelet function and thromboxane production in healthy individuals. Our findings that AA LTA, VerifyNow, and urinary d-TxB₂ are sensitive to the effects of aspirin on platelet function in normal healthy volunteers is consistent with earlier reports (4, 5), but our findings with the PFA-100 differ significantly from those studies.

Two previous studies found that approximately 30% of healthy individuals taking 100 mg aspirin for either 2 days (5) or 7 days (4) were nonresponsive to aspirin by PFA-100 according to each author’s definition. One study (5) used a different definition for aspirin nonresponse (PFA-100 result <300 s) and also collected blood in 0.129 mol/L (3.8%) citrate as opposed to 0.105 mol/L (3.2%). Both of these factors may have contributed to the higher rate of aspirin nonresponse found in that study compared with ours. Closure times on the collagen/epinephrine cartridge are lower when blood is collected in 0.105 mol/L citrate as opposed to 0.129 mol/L (13). The lower closure times observed with 0.105 mol/L citrate may allow better detection of the aspirin effect, as evidenced in our study.

A second study (4) was more similar to ours with regard to study design and duration of aspirin therapy. Measurements by PFA-100 were made in 96 healthy volunteers taking 100 mg aspirin for 7 days. Blood was collected in 0.105 mol/L citrate, and PFA-100 measurements were made the morning after the seventh dose of aspirin, similar to our measurements made 20–24 h after the final dose of 80 mg aspirin. Baseline mean (SD) PFA-100 results in that study were 128 (31) s (4), similar to the 135 (27) s mean closure time observed in our study. The cutoff used for aspirin nonresponse (190 s) was also similar to our cutoff (193 s). Yet those authors found that 29 of 96 healthy individuals (30.2%) were nonresponders by PFA-100; we found that all 42 measurements (29 within 2–4 h of final dose and 13 within 20–24 h of final dose) made on 29 participants taking 80 mg aspirin demonstrated closure times >193 s. We have no data-driven explanation for the differences we have observed.

Similar to previous studies (4, 5), we find that AA LTA and VerifyNow are sensitive assays for the effect of aspirin on platelet function in healthy individuals. Our observations differ from those published previously (4, 5) in demonstrating that the PFA-100 is equally sensitive to the effects of either 80 or 325 mg aspirin (compared to AA LTA, VerifyNow, or d-TxB₂) and extend those observations by demonstrating that these techniques can reproducibly detect the aspirin effect regardless of aspirin dose or timing between final dose and blood collection. Our study also showed a 70%–80% reduction in urinary d-TxB₂ levels by aspirin therapy, consistent with previous studies (5, 14).

Method imprecision across practice sites could contribute to differences observed between studies for many of the assays, although data on interlaboratory precision is available only for the PFA-100. A study conducted by the College of American Pathologists on proficiency testing results across approximately 600 laboratory sites indicated that levels of precision with either normal or tirofiban-treated samples were low to very low, with CVs of 21.1% and 49.7% for normal and tirofiban-treated samples, respectively (15). Imprecision across sites for samples with abnormal platelet function could relate to technical factors such as time between blood draw and sample analysis (almost all studies claim analysis within 2 h of draw) and/or number and expertise of persons doing the testing (in our study, 2 dedicated laboratory technologists did all testing). Biological variables known to affect the PFA-100 results include abnormal hematocrit and fibrinogen, as well as levels of factor VIII and von Willebrand factor (13, 16). Thus, although within-laboratory precision was very good for the PFA-100 as assessed by duplicate testing, method imprecision across testing laboratories or biologic differences in study populations could con-
tribute to differences in study results. Because the test is performed within the cartridge (rather than the instrument) for the PFA-100, we also cannot rule out changes in cartridge performance over time as an explanation for differences between our study and others.

In contrast to results on healthy volunteers, studies using AA LTA, PFA-100, VerifyNow, or d-TxB₂ to measure aspirin effect in patients at risk for arterial thrombosis have found that agreement between any combination of these tests is poor (7–11). Estimates of aspirin nonresponse by AA LTA (4%–30%), d-TxB₂ (approximately 20%), and VerifyNow (14%–23%) in patient populations are much higher than those determined for healthy volunteers (7–11, 17). Our study demonstrates that preanalytic variables (timing between final dose of aspirin and blood draw), method imprecision (with the possible exception of interlaboratory precision for PFA-100), aspirin dose, and/or inherent differences in the sensitivity of the various assays to aspirin effect cannot account for the variable estimates of aspirin nonresponse in studies of patient populations at risk for arterial thrombosis. This suggests that these populations have pathophysiologies that evoke different (most likely thromboxane-independent) mechanisms to stimulate platelets, and that the various assays have differential sensitivity to these platelet activation mechanisms. One such example is the situation of acute plaque rupture, where increases in tissue factor are thought to lead to platelet activation via stimulation of the prothombinase complex and thrombin activation (18).

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References