A comparison of the effects of aspirin on bleeding time measured using the Simplate® method and closure time measured using the PFA-100®, in healthy volunteers

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Aims: The aim of this study was to compare the effects of aspirin on platelet function as measured by the ‘classical’ template bleeding time with a new ex vivo method measuring closure times using the PFA-100® machine. Platelet aggregation in response to arachidonic acid was also measured ex vivo.

Methods: The trial was a randomized, double-blind, placebo-controlled crossover design, with each volunteer taking 750 mg aspirin (BP) or placebo, three times a day for 5 days, with an 18 day wash-out period between treatments. Bleeding times and closure times were measured before the first dose on the first day and 0.5 h after the last dose on the fifth day of each treatment period. They were also measured 2 weeks after the last day of the trial.

Results: Baseline bleeding times (pre-placebo) were 415 s using the Simplate®, whilst baseline closure times were 115 s using the PFA-100®. Aspirin treatment caused an increase of both the template bleeding time (61%) and the closure time of the PFA-100® (79%) when compared with the effects of placebo. The platelet aggregatory response to arachidonic acid was completely inhibited following aspirin treatment and was unaffected following placebo. Two weeks after the end of the trial, all values had returned to pre-treatment levels. The template bleeding time was unaltered in 1 of the 12 volunteers during aspirin treatment and was significantly prolonged in 3 of the 12 volunteers during placebo treatment. The PFA-100® closure time was unaltered in 1 of the 12 volunteers during aspirin treatment and was prolonged in 1 subject during placebo treatment.

Conclusions: The change in closure time using the PFA-100® is as sensitive and reproducible to the effects of aspirin on platelet function as is the template bleeding time test. However, the PFA-100® produced less variable effects with fewer false positive results.

Keywords: bleeding time, simplate, PFA-100, aspirin, volunteers

Introduction
Measurement of the consequence of platelet dysfunction or of the effects of drug treatment on the normal haemostatic mechanism in patients and volunteers has been the subject of much experimentation over many decades. All techniques have involved some form of cutaneous incision and an estimation of the time taken for the bleeding to cease. The original method of Duke [1] was changed from the ear-lobe to the forearm by Ivy [2], and was then standardized with the introduction into routine practice of the Simplate® device, which produced a uniform size of cut by virtue of a spring-loaded blade [3]. Such techniques have been regularly used to help diagnose platelet dysfunction or disorders [4, 5], as well as to evaluate the effects of drugs which may attenuate haemostasis in the treatment of, for example, unstable angina [6, 7]. Whilst such methods are capable of detecting such activity, they are known to show poor reliability and reproducibility. Furthermore, they involve an invasive procedure which produces scarring of varying degrees.

The bleeding time is determined by the formation of a platelet plug at the site of injury. Drugs and diseases that affect platelet aggregation prolong bleeding time, whereas factors affecting coagulation of blood, such as haemophilia, do not. Recently, an automated method (PFA-100®) has been described [8, 9] which, it is suggested, can be used to evaluate the effects of platelet disorders and drugs on platelet function by making an ex vivo measurement based upon the formation of a platelet plug. This device requires only a small sample of venous blood to be taken from the subject into an anticoagulant. The sample is then placed into the PFA-100® where it is drawn through a capillary tube and comes into contact with a membrane. This membrane is coated with collagen and (in the present experiments) adrenaline to activate the platelets. The blood is then aspirated through a precision aperture (147 μm diameter) in the membrane and platelets begin to adhere to the circumference of the aperture. A stable platelet plug then
forms that occludes the aperture and the time taken for blood to stop passing through the aperture is registered. This time is described as the closure time and is an ex vivo assessment of platelet function. The purpose of the present study was to compare platelet function in healthy, male volunteers by measuring bleeding time using the Simplate method and closure times using the PFA-100, and to assess the effects of aspirin on platelet function, we also evaluated the effects of treatment on platelet aggregation, ex vivo, using arachidonic acid to stimulate aggregation of platelet-rich plasma.

Methods

Subjects

Twelve, healthy, male volunteers (age range 27–50 years) were recruited from the Zeneca Pharmaceuticals Volunteer panel to take part in this trial. Health was confirmed on the basis of a normal medical, which included history and examination, clinical chemistry, haematology and urinalysis, respiratory function tests (FEV1 and FVC), 12-lead ECG and 24 h ambulatory cardiac monitoring. A negative test for hepatitis B surface antigen was also a pre-requisite for this study. Volunteers were required to abstain from the use of any aspirin-like drugs for 14 days prior to the first study day and throughout the duration of the study. All volunteers gave fully informed written consent prior to participation in the study, which was approved by the independent Zeneca Pharmaceuticals Research Ethics Committee.

Study design

This was a double-blind, placebo-controlled cross-over study with the volunteers being randomly assigned to take either 750 mg aspirin, three times per day for 5 days, or matching placebo. There was an 18-day washout period between the two arms of the study and the volunteers also received a post-trial medical examination 14 days after the end of the dosing period.

On the first study day, the volunteers were required to remain supine for 30 min, prior to a measurement of bleeding time using the Simplate technique [3]. Briefly, a blood pressure cuff was placed around the upper arm and inflated to 40 mm Hg. An area on the volar surface of the forearm was swabbed with alcohol and allowed to air dry. A single incision was made in line with the forearm with the Simplate device and the blood blotted with a filter paper every 30 s until bleeding had stopped. Care was taken during the blotting not to touch the edge of the clot and wound, and the same individual conducted all the bleeding time measurements throughout the study. The time taken from the incision being made to the end of bleeding was measured. The same operator made the bleeding time measurements in the same volunteer throughout the trial period. From a 16g cannula inserted into an antecubital vein on the other arm, a 2 ml blood sample was drawn and gently expelled into a citrate-coated tube, capped and inverted to ensure mixing. An 800 μl sample of this was placed into the PFA-100 analyser for measurement of the closure time. The cartridges used were collagen/adrenaline, as these are reported to be sensitive to the platelet-inhibitory effects of aspirin [8]. Each blood sample was tested in duplicate and the mean closure time calculated.

A further 20 ml blood sample was taken into a 50 ml syringe containing 2 ml of 3.2% (w/v) trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 200 g for 15 min (25 °C) and 0.25 ml aliquots dispensed into silicocussed glass tubes. These were stirred at 900 rev min⁻¹ and pre-incubated at 37 °C for 60 s in a Biodata aggregometer. Aggregation was stimulated by the addition of arachidonic acid and measured as an increase of light transmission (minimum and maximum transmission having been set previously using PRP and platelet-poor plasma, respectively). Each aggregation response was allowed to proceed to its maximum and the ECAff for a full concentration-response curve calculated.

The volunteers were required to take their treatment at 08.00, 16.00 and 24.00 h each day, with normal food intake. On the fifth day, volunteers were required to take their last treatment (08.00 h) and then a repeat of the bleeding time, closure time and platelet aggregation measurements were made 0.5 h after the final dose of aspirin or placebo. After 18 days, each volunteer returned and the process was repeated with the alternate drug treatment. Two weeks after the last treatment day, all volunteers received a post-trial medical examination and a final assessment of the bleeding time and closure time.

Drug supply

Aspirin was supplied as aspirin BP, 300 mg, from Unichem. The placebo tablets were provided by the Pharmaceutical Department of Zeneca Pharmaceuticals.

Data analysis

The data were analysed by analysis of variance, the treatment effect being estimated from the difference between the placebo and the aspirin least squares means. The existence of differential carry-over was assessed by fitting an analysis of covariance model to the pre-dose value in the second period of the crossover, allowing for the baseline in the first period and a treatment sequence effect. To assess the reproducibility of each measurement within a volunteer, the lowest and highest values of bleeding time and closure time were compared using both pre-dose periods and the values at the post-trial medical (i.e. the three baseline assessments). The differences between these values within each individual were expressed as a percent difference.

Results

All volunteers completed the trial successfully with only minor adverse events reported. An analysis of covariance showed there was no carryover between treatments.

Simplate method

Before aspirin treatment, the mean bleeding time was 443 s (range = 270–870), whilst on the placebo arm of the trial,
Figure 2. developed by Kratzer & Born [11]. The Thrombostat was placebo treatment, whilst a fourth volunteer did not show The PFA-100 © 1997 Blackwell Science Ltd Br J Clin Pharmacol with both the Simplate [12] and a lancet [13] method. Closure time, PFA-100 method using the Simplate device (a) and closure times using the (Figure 1a) as well as the occurrence of the occasional Range of bleeding times obtained in 12 volunteers the typical wide variability obtained with this method Figure 1. The mean di The effect of aspirin was highly significant when compared against placebo (P=0.001), the increase being 61%. At the pre-dose value was 415 s (range = 300–660). Following 5 days treatment with aspirin, the bleeding time rose to a mean of 855 s (range = 480–2190), the corresponding bleeding time after placebo being 330 s (range = 330–1740). The mean difference between the two baseline and post-trial measurements of closure time was 21%. In one volunteer, the closure time did not increase following either treatment period and in another volunteer it increased by a similar time following both treatments (29 s following aspirin and 24 s following placebo. Figure 2). In three volunteers, the blood sample ran out before registering a closure time following aspirin treatment.

Platelet aggregation
Aspirin treatment abolished the aggregatory response to arachidonic acid in all 12 volunteers, such that the mean pre-aspirin E(50) was 337 µmol (range = 146–690) whilst post-aspirin, no aggregation was observed following the highest concentration used (2 mm). The mean E(50) pre-placebo was 310 µmol (range = 155–632) and post-placebo was 362 µmol (range = 142–689).

Discussion
The purpose of the present study was to compare platelet function by measuring the bleeding times using the standard template method (Simplate device) with the closure times obtained using a new machine, the PFA-100®, and to determine the effects of aspirin on these times in a placebo controlled, cross-over study in healthy, male volunteers.

In our volunteers, the template bleeding times using the Simplate device were within the ranges previously described in a number of other studies [3, 10]. The data also show the typical wide variability obtained with this method (Figure 1a) as well as the occurrence of the occasional ‘outlier’. The presence of an individual result which is greatly outside the normal range might reflect an abnormal haemostatic response of that individual and, as part of a haematological examination, would warrant further detailed examination of that individual’s platelet function. In the present trial, however, there was no consistency in the bleeding times which were outside the normal range; thus, at the post-trial medical all the bleeding times were very close, suggesting that the very long bleeding times in the post-dose placebo and the post-dose aspirin groups were, indeed, ‘outliers’ rather than individuals with an intrinsic clotting deficiency. Overall, however, this method has demonstrated the known effects of aspirin treatment to prolong bleeding time, in the present case by 61% when compared with the effect of placebo.

The PFA-100® is a newly-available machine which has been developed from the Thrombostat 4000, originally developed by Kramzer & Born [11]. The Thrombostat was evaluated in a number of studies and shown to be at least as sensitive to the effects of aspirin on bleeding time compared with both the Simplate [12] and a lancet [13] method. However, its widespread use was hampered by problems of reproducibility in the preparation of the collagen-coated filters used. The PFA-100® has overcome these difficulties and has also made use of more modern micro-chip technology to facilitate user-friendliness. In our experience, the PFA-100® was quick and simple to use and very robust. Preliminary tests confirmed the reported difference in

Closure times seen between the two sample wells available [9] and we chose to use just the first well and repeat the samples twice. However, further evaluation during the course of the present study suggested that this did not provide any advantage over the recommended method of testing in duplicate using the two wells provided.

The normal range of closure times obtained in our volunteers corresponds well with those reported from other trials, the range of pre-drug values being between 83 and 161 s in the present study and from 77–133 s in the study by Manninen et al. [9]. The PFA-100 values were also less variable than the bleeding time values, with coefficients of variation of 12% for the PFA-100 and 23% for the Simplate method. The increase of closure time following aspirin treatment was 79% when compared with the placebo effect, using the PFA-100® a similar value to that obtained with the Simplate device (61%). Thus, both methods appear equally capable of detecting the effect of aspirin on platelet plugging.

Both methods failed to identify one of the twelve volunteers during aspirin treatment, the PFA-100® closure time not being prolonged at all, whilst the Simplate bleeding time was only increased by 30 s from a pre-dose value of 7.5 min. However, these results were obtained from different volunteers. The results of the platelet aggregation studies confirmed that platelet cyclo-oxygenase was completely inhibited in all volunteers. The fact that in two subjects haemostasis was not affected using either method of measurement would suggest either an intrinsic deficit in the techniques used or, that in these individuals, platelet aggregation/plugging did not rely upon the production of cyclo-oxygenase products.

The PFA-100® produced one false positive effect, the closure times being prolonged equally following both placebo and aspirin treatment. The template bleeding times, however, were significantly prolonged in three of the twelve subjects following both placebo and aspirin treatment. In one subject, bleeding time was increased by 22 min following aspirin and 18 min following placebo. However, at the post-trial examination, both methods produced a similar range of values to the two pre-dose times, with no significant outliers. Thus, the PFA-100 appears to be less prone to produce false positive results than the Simplate method.

Whilst both methods have demonstrated the known effects of aspirin on the normal haemostatic mechanism in healthy male volunteers, the PFA-100® is not necessarily a direct replacement for the Simplate bleeding time. Rather, it can be used as a technique to provide rapid information on global platelet function and, in this respect, it offers distinct advantages over the Simplate method. The closure times are objective and less variable and, of particular concern when evaluating the effects of drugs on haemostasis, it requires only a venous blood sample to be provided, thus eliminating the need for an incision to be made to the subject.

References
1. Duke WW. The relation of blood platelets to hemorrhagic disease: Description of a method for determining the bleeding time and coagulation time and report of three cases of hemorrhagic disease relieved by transfusion. JAMA 1910; 55: 1185–1192.
Platelet function and bleeding times


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