Differences in the effects of extended-release aspirin and plain-formulated aspirin on prostanoids and nitric oxide in healthy volunteers

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SUMMARY
This study was designed to evaluate the effects of extended-release aspirin on platelet aggregation and the production of prostanoids and nitric oxide. The participants in this double blind, randomized and crossover study were 20 healthy volunteers. Interventions were 150 mg of plain-formulated aspirin (PFASA) and 150 mg of extended-release aspirin (ERASA). Blood samples were collected before and 10, 20, 60, 120, 240, 480 and 1440 min after the first dose; 3, 7 and 14 days after daily administration and 24 h after the last dose. The main measures were platelet aggregometry, thromboxane B₂, 6-keto-prostaglandin (PG) F₁α and nitric oxide in each control. Platelet aggregation was inhibited by 50% with ERASA, and by 77% with PFASA. No differences were found in chronic treatment. Thromboxane B₂ was inhibited more by the latter (51–67%), but 90% inhibition was observed in both groups after 3 days. The levels of 6-keto-PGF₁α was reduced by 20% with ERASA and by 58% with PFASA. Nitric oxide production increased in both groups, but after 24 h, and 7–14 days, elevated concentrations of nitric oxide were found only in the ERASA. The antiplatelet effects of ERASA provide pharmacological advantages (greater prostacyclin synthesis and prolonged increase in nitric oxide production) over those provided by the plain formulation.

INTRODUCTION
Platelets play a fundamental role in the genesis and evolution of occlusive atherosclerotic disease in coronary, cerebral or peripheral vessels. Drugs that inhibit platelet function have shown a clear effect in the secondary prevention of ischaemic arterial accidents and the early and late complications of revascularization procedures such as angioplasty, bypass and stent implantation [1–5]. Aspirin, the most widely used antiplatelet drug, has been shown to be at least as effective as other platelet anti-aggregants in preventing these disorders [1–3].

Aspirin works by blocking the enzyme cyclo-oxygenase, although the mechanism of action differs in platelets (inhibition of thromboxane A₂ synthesis) and in the vessel wall (inhibition of prostacyclin production) [6]. This difference results in the so-called aspirin dose dilemma, i.e. how to establish the range of doses which produce minimal interference with prostacyclin synthesis while providing maximal inhibition of thromboxane synthesis and prevention of platelet aggregation [7]. It is currently accepted that the solution to this dilemma lies in pharmacokinetics. After oral intake, acetylsalicylic acid (ASA) is metabolized in the liver to salicylic acid (SA). Platelet cyclo-oxygenase is almost entirely inhibited in the pre-systemic and systemic circulation. Prostacyclin synthesis in peripheral vessels is influenced by the balance between ASA and SA: the deacetylated metabolite binds to cyclo-oxygenase (but does not inhibit) this
enzyme, and competes with ASA in blocking this enzyme; moreover, the effect of repeated doses of ASA is cumulative, because of irreversible cyclo-oxygenase inhibition and the absence of platelet nucleus [8–11].

On the basis of these premises, the ideal dose of aspirin is assumed to be the one which produces the lowest amount of ASA in the bloodstream that will effectively block thromboxane synthesis and inhibit platelet aggregation while yielding the greatest possible amount of SA for blocking the inhibition of vascular prostacyclin synthesis [12–14]. To satisfy these conflicting demands, a new formulation of aspirin [extended-release aspirin (ERASA)] was developed.

The present study was designed to compare the antiplatelet effects of extended-release and plain formulation aspirin in healthy volunteers. The two formulations were compared with regard to anti-aggregant effect in whole blood, thromboxane/prostacyclin balance, nitric oxide (NO) production and plasma concentration of SA and ASA.

METHODOLOGY

Study design
The participants in this randomized, double blind crossover study were 20 healthy volunteers (13 men, seven women, mean age 31 ± 1.4 years) who had not taken any medication during the 15 days before their inclusion. When informed consent to participate was given, each volunteer was randomly assigned (with the help of a table of random numbers) to receive one of the two formulations of aspirin (plain formulation, PF, or extended release, ER) as the first treatment. During the crossover phase of the study, the subject received the other formulation. The wash-out period between the two treatments was 30 days. The study was approved by the Clinical Assays Committee of the Hospital Clínico Universitario Virgen de la Victoria in Malaga, Spain (file TROM-99/MAD, approved April 20, 1999).

The participants were divided into two groups. Volunteers in one group first received 150-mg dose of plain formulation aspirin (PFASA), and those in the other group received 150-mg dose of ERASA (Madaus SA, Barcelona, Spain). In both cases, drug was administered in a daily dose (30 min before breakfast).

Blood samples were obtained from each volunteer before the drug was taken (basal), 10, 20, 60, 120, 240 (4 h), 480 (8 h) and 1440 min (24 h) after the first dose; 3, 7 and 14 days after repeated doses (2 h after the dose) and 24 h after the last dose. Blood samples were obtained in the morning (between 8.00 h and 8.30 h) before the participants had had anything to eat or drink, and the subjects abstained from eating or drinking (except for up to 200 mL water) until after the 2-h basal blood sample was obtained.

Material
Capsules of PFASA and ERASA were manufactured by Madaus SA (Barcelona, Spain). All reagents used for the in vitro studies, including ASA and SA, were from Sigma Chemical Co (St Louis, MO, USA) unless otherwise stated.

The PFASA capsules contained 150 mg ASA and fulfilled the product specifications of Laboratorios Bayer España (Barcelona, Spain). The ERASA capsules contained 182 mg of micropellets, which consisted of 150 mg ASA and an inert starch core surrounded by layers of ASA and binders (cellulose derivatives and inert acrylics). Both formulations were prepared in standard hard gelatin capsules. The cumulative rate of release of ASA from the ER formulation, determined in vitro, was 15% after the first hour (pH 1), 25% after the second hour (pH 1), 50% after the third hour (pH 6.8), 80% after the fourth hour (pH 6.8) and 100% during the fifth and sixth hours (pH 6.8).

Laboratory analyses

Platelet function parameters
The main indicator of platelet function was maximal aggregation intensity induced by collagen or by arachidonic acid in whole blood, as measured by electrical impedance [15] with a Chrono-Log 540S aggregometer (Chrono-Log Corp., Haverton, PA, USA). Sodium citrate (3.8%) at a proportion of 1:10 was used as the anticoagulant for samples of whole blood. The samples were incubated for 5 min at 37°C, then 1 lM of the calcium ionophore A23187 was added and the samples were shaken for 5 min. The samples were then centrifuged at 10 000 g for 3 min, and the supernatant was frozen at

Nitric oxide production

An electrochemical method was used to quantify NO production [16] in neutrophil concentrates.

Leucocytes were separated on a Ficoll density gradient (Hystopaque 1077 and Hystopaque 1119), and resuspended (final count: 5.2 ± 0.2 × 10⁹ leucocytes) in Ca²⁺ physiological saline solution that contained 140 mM NaCl, 4.6 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose and 10 mM HEPES, pH 7.4. The sample was resuspended in Ca²⁺ saline solution (95% neutrophils, 95% viability as judged by trypan blue exclusion), to a final neutrophil count of 3.4 ± 0.3 × 10⁹ neutrophils/L. The sample was then divided into aliquots and an ISO-NO electrode for NO determination (World Precision Instruments, Aston, Stevenage, Hertfordshire, UK). All measurements were made at 37°C. Basal NO production was recorded, then 1 μM of the calcium ionophore A23187 was added, and the increase in NO production induced by activation of constitutive or calcium-dependent NO synthase was noted.

Plasma concentrations of ASA and SA

Plasma was obtained by centrifuging samples of whole blood with 5 mg/mL potassium fluoride at 2500 g for 15 min at 1°C. Potassium fluoride (5 mg/mL) was added to the resulting plasma to prevent hydrolysis of the ASA, and the samples were stored at −80°C [17]. High-performance liquid chromatography (Hewlett-Packard model 1100 integrated chromatography system; Agilent Technologies, Madrid, Spain) was used to measure ASA and SA concentrations according to the method described by Rumble and Roberts [18], with some modifications. Perchloric acid and methanol were used for precipitation and extraction. Chromatographs were obtained with an SB-C18 column (4.6 mm × 25 mm, particle size 5 μm). The mobile phase consisted of 0.03% acetonitrile, methanol and orthophosphoric acid (50:10:40), and ASA and SA were measured spectrophotometrically at 237 nm.

Statistical methods

All data in the text, tables and figures are the mean ± SEM of the results in each group. The data were analysed with version 9.0 of the SPSSx program for Windows (SPSS Co., Chicago, IL, USA). Data for both the initial and crossover phases of the study were transformed into percentage values of change with respect to the basal values. Once normal distribution of the data had been confirmed, the percentage values for the two treatment groups were compared with Student's t-test for paired samples. Differences for which P < 0.05 were considered significant.

RESULTS

Absolute values for each laboratory test in the initial and crossover phases are shown in Table I. There were no significant differences between the data for the two phases, or between the groups of volunteers assigned initially to one treatment or the other. Therefore, some results are expressed as the per cent change with respect to the basal value for each formulation.

Results up to 24 h

Both formulations of ASA significantly inhibit platelet aggregation induced in whole blood by collagen (Figure 1) and arachidonic acid (Figure 2), and the changes were evident but not statistically significant 10 min after administration. Maximal inhibition of aggregation was observed 8 h after administration: inhibition was 67% with collagen and 77% with arachidonic acid after PFASA, and 53% and 59%, respectively after ERASA. After 24 h, aggregation showed a tendency to return to basal values, and inhibition decreased by 14% in the PFASA group and 20% in the ERASA group.

Platelet production of thromboxane B₂ showed a time-dependent decrease in both treatment groups (Figure 3). After 60 min, the inhibitory effect of PFASA was significantly higher than that of ERASA and after 6 h, inhibition was 67% in the former group and 51% in the latter. There was no significant difference in recovery of
thromboxane B₂ production between the groups after 24 h.

Plasma levels of 6-keto-prostaglandin F₁α (Figure 4) showed a different response to each treatment. In the PFASA group, synthesis of this prostanoid was inhibited in a time-dependent manner and maximal inhibition (58%) was observed 2 h after administration. However, maximal inhibition in the ERASA group was only 20% and this value was observed 4 h after administration. The difference between groups was statistically significant at all sampling times.

When neutrophil NO production was induced via a calcium-dependent pathway (Figure 5), we found a significant increase in NO production in both groups. After 20 min, maximal increase was 144% in the PFASA group and 138% in the ERASA group, with respect to pretreatment value. After 24 h, NO production in the two groups differed significantly, returning to basal values in the PFASA group but remaining 58% above basal values in the ERASA group.

Plasma concentrations of ASA and SA (Figure 6) differed in the two treatment groups. Mean calculated pharmacokinetic parameters are shown in Table II.

### Results after chronic treatment

Both formulations of ASA significantly inhibited platelet aggregation induced in whole blood by collagen (Figure 1) and arachidonic acid (Figure 2). There were no statistical differences between PFASA and ERASA. Maximal inhibition of aggregation was 90–92% after 3 days of treatment.

Platelet production of thromboxane B₂ showed a similar inhibition (90–92%) in both treatment groups, after 3 days (Figure 3). However, plasma levels of 6-keto-prostaglandin F₁α were different according to the treatment: after PFASA administration, an inhibition of 61% was observed, while ERASA only inhibited 6-keto-prostaglandin F₁α production by 38% (Figure 4).

<table>
<thead>
<tr>
<th>Test</th>
<th>Data before randomization (N = 20)</th>
<th>Data before crossover period (N = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells (×10¹²/L)</td>
<td>5.07 ± 0.14 (4.76–5.38)</td>
<td>5.06 ± 0.18 (4.82–5.50)</td>
</tr>
<tr>
<td>Leucocytes (×10⁹/L)</td>
<td>6.19 ± 0.33 (5.50–6.88)</td>
<td>6.23 ± 0.35 (5.78–7.27)</td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>285 ± 15 (254–316)</td>
<td>292 ± 13 (264–320)</td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>13.83 ± 0.45 (12.88–14.78)</td>
<td>13.87 ± 0.44 (12.95–14.79)</td>
</tr>
<tr>
<td>Haematocrit (L/L)</td>
<td>47.04 ± 1.51 (43.88–50.20)</td>
<td>47.78 ± 1.55 (44.54–51.01)</td>
</tr>
<tr>
<td>Mean platelet volume (fL)</td>
<td>7.55 ± 0.12 (7.29–7.81)</td>
<td>7.53 ± 0.16 (7.28–7.89)</td>
</tr>
<tr>
<td>Iₘₙₙₐₓ† aggregation (Ω) induced by 1 µg/mL collagen</td>
<td>13.84 ± 0.64 (12.50–15.19)</td>
<td>13.94 ± 0.68 (12.52–15.17)</td>
</tr>
<tr>
<td>Iₘₙₙₐₓ† aggregation (Ω) induced by 0.4 mM arachidonic acid</td>
<td>14.09 ± 0.46 (13.13–15.04)</td>
<td>14.67 ± 0.69 (13.23–15.11)</td>
</tr>
<tr>
<td>Platelet thromboxane B₂ (nmol/10⁹ platelets)</td>
<td>3.26 ± 0.33 (2.65–3.69)</td>
<td>3.26 ± 0.29 (2.63–3.78)</td>
</tr>
<tr>
<td>Plasma 6-keto-prostaglandin F₁α (nmol/l)</td>
<td>18.15 ± 1.23 (16.40–28.06)</td>
<td>17.64 ± 1.81 (15.39–29.67)</td>
</tr>
<tr>
<td>Calcium-induced NO in neutrophils (µmol/10² neutrophils)</td>
<td>0.86 ± 0.06 (0.61–1.01)</td>
<td>0.85 ± 0.07 (0.59–0.96)</td>
</tr>
<tr>
<td>Plasma nitrites/nitrates (µM)</td>
<td>8.55 ± 0.67 (6.08–10.21)</td>
<td>8.51 ± 0.76 (6.11–9.99)</td>
</tr>
</tbody>
</table>

*Confidence intervals (95% CI) are indicated in brackets. † Iₘₙₙₐₓ: maximum intensity of platelet aggregation, NO: nitric oxide.
Aspirin treatment increased leucocyte NO production. One day after the last dose, NO production was still increased in ERASA group and it returned to the pretreatment values in PFASA group (Figure 5).

**DISCUSSION**

Our findings show that the ER formulation of ASA had a significant anti-aggregant affect, inhibited thromboxane synthesis, and interfered relatively little with prostacyclin synthesis. In addition, this formulation increased NO production, and the increase was maintained for up to 24 h after a single or 15 days of 150 mg/day doses.

Platelet aggregation in whole blood was inhibited by both formulations of ASA. The anti-aggregant effect of ASA is also known to be enhanced after aggregation is induced with collagen or arachidonic acid [19,20] and in the presence of leucocytes [20]. Acetylsalicylic acid was later shown to inhibit aggregation induced by thrombin when platelet samples were incubated with neutrophils [21].

The ability of single 150-mg dose of ERASA to inhibit platelet aggregation might have been expected to be lower...
than the effect of PFASA; however, our findings did not bear out this assumption. The ER formulation inhibited aggregation by 53–59%, which indicates that the rate of ASA release achieved adequate presystemic acetylation of cyclo-oxygenase. This finding was supported by the aggregometric data and by the inhibition of platelet thromboxane synthesis, which showed a time-dependent profile practically identical to that of the inhibition of platelet aggregation induced with arachidonic acid. After 14 days of treatment, these inhibitions were almost complete and equal in both groups of treatment. In addition, maximum ASA concentration after ERASA administration reached approximately 1.5 μg/mL (5 μM), a value equivalent to the 50% inhibitory concentration reported for ASA in whole blood after collagen induction [19]. Despite this effect, it should be remembered that in acute thrombotic accidents and acute myocardial infarction, the initial dose needed to decrease platelet aggregation quickly and efficiently would most probably need to be higher.

**Figure 3** Upper panel: time course of platelet thromboxane B₂ induced by 1 μM calcium ionophore A23187, after a single dose of 150 mg plain formulation (PF) aspirin (●) or extended-release (ER) aspirin (○). Each point represents mean ± standard error of the mean from 20 healthy volunteers. *P < 0.05, **P < 0.001, with respect to ER aspirin. Lower panel: platelet thromboxane B₂ production induced by 1 μM calcium ionophore A23187, after 3, 7 and 14 days of daily treatment, and 24 h after the last dose (separated by a dotted line) with 150 mg PF aspirin (dotted bars) or ER aspirin (open bars). Each bar represents mean ± standard error of the mean from 20 healthy volunteers. *P < 0.05 with respect to all determinations, **P < 0.05 with respect to PF aspirin.

**Figure 4** Upper panel: time course of plasma 6-keto-prostaglandin (PG) F₁α, after a single dose of 150 mg plain formulation (PF) aspirin (●) or extended-release (ER) aspirin (○). Each point represents mean ± standard error of the mean from 20 healthy volunteers. *P < 0.05. **P < 0.001, with respect to ER aspirin. Lower panel: plasma 6-keto-PGF₁α, after 3, 7 and 14 days of daily treatment, and 24 h after the last dose (separated by a dotted line) with 150 mg PF aspirin (dotted bars) or ER aspirin (open bars). Each bar represents mean ± standard error of the mean from 20 healthy volunteers. *P < 0.05 with respect to all determinations, **P < 0.05 with respect to PF aspirin.
dose of aspirin. Low doses of ASA lead to acetylation of cyclo-oxygenase in nearly 100% of the circulating platelets in the portal venous system before first-pass metabolism of ASA in the liver [8–10]. This would explain the similarity in the time course of the inhibition of thromboxane synthesis and of platelet aggregation in whole blood. Our finding that these effects were also observed with ERASA shows that the amount of ASA that reached the presystemic circulation from the ER formulation was sufficient to inhibit both thromboxane synthesis and platelet aggregation.

The main goal of ER formulations of ASA is to ensure that after first-pass metabolism, enough SA reaches the bloodstream to compete with ASA for vascular cyclo-oxygenase [12–14]. This effect prevents prostacyclin synthesis from being inhibited, an important factor in any agent that is expected to have two main actions, i.e. an endogenous antithrombotic effect, and the ability to inhibit gastric secretions and increase gastric mucus production in order to counteract the harmful effects of ASA on the gastric mucosa [22–24].

The ER formulation allowed prostacyclin synthesis to continue at 65–80% of the normal rate, a finding that clearly sets this formulation apart from plain aspirin. Clinical trials will be needed to determine whether the biochemical differences we found between the plain and ER forms are translated into a lower incidence of gastric injury with the ERASA formulation. However,
Table II  Mean calculated pharmacokinetic values after a single dose of 150 mg of plain formulation aspirin or extended-release aspirin, for acetylsalicylic acid (ASA) and salicylic acid (SA).

<table>
<thead>
<tr>
<th></th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (µg/mL)</th>
<th>AUC (µg/mL h)</th>
<th>$K_a$</th>
<th>$K_e$</th>
<th>$V_d$ (l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plain formulation aspirin</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ASA</td>
<td>0.56 ± 0.07</td>
<td>1.42 ± 0.16</td>
<td>4.13 ± 0.46</td>
<td>4.58 ± 0.50</td>
<td>0.44 ± 0.06</td>
<td>82.03 ± 8.89</td>
</tr>
<tr>
<td>SA</td>
<td>1.35 ± 0.15</td>
<td>4.64 ± 0.35</td>
<td>31.91 ± 0.34</td>
<td>1.90 ± 0.22</td>
<td>0.18 ± 0.04</td>
<td>25.08 ± 3.27</td>
</tr>
<tr>
<td><strong>Extended-release aspirin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASA</td>
<td>3.16 ± 0.33</td>
<td>1.19 ± 0.13</td>
<td>11.54 ± 1.11</td>
<td>0.50 ± 0.04</td>
<td>0.18 ± 0.02</td>
<td>70.00 ± 6.55</td>
</tr>
<tr>
<td>SA</td>
<td>4.51 ± 0.50</td>
<td>3.18 ± 0.35</td>
<td>44.30 ± 4.85</td>
<td>0.36 ± 0.05</td>
<td>0.11 ± 0.02</td>
<td>29.20 ± 3.05</td>
</tr>
</tbody>
</table>

$T_{\text{max}}$: peak time, $C_{\text{max}}$: peak concentration, AUC: area under curve, $K_a$: absorption constant, $K_e$: elimination constant, $V_d$: distribution volume.

we can assume a lower inhibition of prostacyclin synthesis, which is widely accepted as an important target of the antithrombotic drugs that lack inhibition of the endogenous antiplatelet substances, such as prostacyclin.

A study by Brown et al. [25] showed that the inhibition of platelet aggregation after the administration of a microencapsulated form of ASA (Caspac XL) was not entirely dependent on the inhibition of platelet thromboxane synthesis. This finding showed that an additional mechanism may be involved in the differences in pharmacokinetic profile between ER and PF ASA. The findings we report here show that unlike PF ASA, the ER formulation did not compromise prostacyclin synthesis. This difference may mean that the ER formulation has antithrombotic effects in addition to the antiplatelet effect of the ASA which results from the inhibition of platelet thromboxane synthesis.

However, the two formulations also differed in their effect on NO production. Calcium-dependent NO activity (related with constitutive NO-synthase) tended to return to basal values in the PFASA group, but remained elevated for longer in the ERASA group, both after the first dose and in the chronic treatment. The time-dependent profile of NO production was similar to that for SA concentration with both types of ASA. Because of this, SA may play an important role in the effect of ASA on NO production.

Earlier studies showed that the anti-aggregant effect of ASA in vitro is greater in whole blood than in platelet-enriched plasma, and that the effect is potentiated in the presence of leucocytes [19,20]. Acetylsalicylic acid has also been shown to increase calcium-dependent NO production by human neutrophils [21,26] and rat aorta [27], although the role of SA in this effect has not been explained. The mechanism for the stimulatory effect of ASA and/or SA on NO production remains still unclear, however, several authors have demonstrated that ASA and SA inhibit the induction of the inducible NOS [28–30] through an inhibition of the transcripotional factor NF-κB [31] or a blockade of glutamate release [32], that increase NO production in ischaemic tissues. Moreover, SA is a scavenger of free radicals, that react with NO and reduce its mean life [33]. For that reason, the ERASA treatment could maintain its action on the NO production through a control of the NO inactivating factors, such as free radicals. The amount of NO-stimulated ex vivo by aspirin could inhibit platelet aggregation, ca. 30%, according to an extrapolation from the results of Radomski et al. [34] in an in vitro study. This amount of NO could act as a modulator of platelet aggregation in whole blood in our experimental conditions.

The present results suggest that the better platelet function profile of ER formulations of ASA in comparison with PF may be influenced by the role of SA and the relative lack of interference with prostacyclin synthesis. Moreover, our findings support the hypothesis put forward by Brown et al. [25] that the anti-aggregant effect of microencapsulated ASA is not entirely dependent on the inhibition of platelet thromboxane synthesis.

CONCLUSION

In conclusion, the administration of 150 mg/day ERASA inhibited platelet aggregation in whole blood, and its pharmacodynamic and pharmacokinetic behaviour was mostly similar to those of a PF of ASA except for the weaker inhibition of prostacyclin biosynthesis and the greater production of NO. These effects are potentially useful adjuncts to the well-known effects of ASA in the secondary prevention of thrombotic accidents.
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