Plasma Acetylsalicylate and Salicylate and Platelet Cyclooxygenase Activity Following Plain and Enteric-Coated Aspirin

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SUMMARY Compressed and enteric-coated acetylsalicylate (ASA) tablets have been compared in normal healthy subjects. Plasma ASA and salicylate (SA) were measured by high pressure liquid chromatography (HPLC). Platelet cyclooxygenase activity in vitro was studied by a radiometric technique. Following ingestion of 650 mg of ASA in the form of compressed tablets, cyclooxygenase activity was inhibited 95% within 45 min. Enzyme activity was observed to increase within 8 h and reached 10% of control level by 24 h. The pattern suggests that only circulating platelets are affected by ASA ingestion. Following the administration of 650 mg of ASA as enteric-coated tablets comparable inhibition of cyclooxygenase activity was observed, although the effect was delayed, reflecting the delayed appearance of ASA in the plasma. Return to control levels followed a pattern similar to that observed with the compressed tablet.

ACETYLSALICYLATE (ASA) REDUCES the frequency of transient ischemic attacks and lowers the incidence of stroke and death in patients with cerebral vascular disease. A number of aspects of the clinical pharmacology of this commonly used drug require further investigation. The use of enteric-coated ASA may reduce the incidence of gastrointestinal side effects. The efficacy of such a preparation as an antiplatelet agent has not been studied. Since the mechanisms of the antithrombotic effect of ASA is believed to be the inhibition of platelet cyclooxygenase activity, it may be useful to compare the drug preparations with respect to inhibition of this enzyme activity. A large scale clinical trial to establish the efficacy of enteric-coated ASA in patients with cerebral vascular disease is probably unnecessary if an appropriate degree of enzyme inhibition is demonstrated.

The optimal dosage schedule for ASA also is uncertain. Clinical trials in which efficacy of ASA has been demonstrated both in patients with cerebral vascular disease and venous thrombosis have used a q.i.d. regimen. Since the effect of a dose of ASA on platelets is irreversible, it is possible that less frequent administration would constitute equally effective antiplatelet therapy. New platelets enter the circulation at a rate which approximates 10% of the circulating pool per day. If megakaryocytes are affected by ASA in such a way that the platelets formed and released subsequent to a dose of ASA have nonfunctional cyclooxygenase, less frequent dosage may be required than if ASA inhibits only circulating platelets. We have measured the cyclooxygenase activity of platelets over a period which includes the first 24 h after ASA ingestion. We also measured plasma ASA and salicylate (SA) levels following ingestion of plain and enteric-coated ASA to investigate whether plasma
levels of the drug are closely related to antiplatelet effect and are comparable for the 2 drug preparations.

Methods

1. Subjects

Six healthy volunteers of both sexes between the ages of 22 and 37 were studied. The subjects had not ingested ASA or other nonsteroidal anti-inflammatory drugs (NSAID) for at least a week prior to the start of the study, and avoided all such drugs during the study. Each subject received 650 mg ASA (2 × 325 mg ASA compressed tablets, Bayer) after an 8 h fast. Food was not permitted until 2 h following the administration of the drug. After an interval of 5 weeks, 4 of the subjects ingested 650 mg enteric-coated ASA (Frosst, Entrophen). Because of failure to obtain certain blood samples at intervals in the first phase of the study, 4 subjects received one of the drug preparations on a second occasion, separated by an interval of 5 weeks from the first.

Estimation of Plasma ASA and SA by HPLC

Blood samples were drawn into vacuum blood collection tubes containing potassium oxalate (14 mg) and sodium fluoride (17.5 mg). Plasma was separated by centrifugation at 22°C and promptly frozen and stored at −40°C until analysis.

For the analysis, standard solutions of ASA (BDH) and SA (Fisher), and of 2,5-dimethylbenzoic acid [(2,5-DMBA) (Eastman)] and 2-naphthoic acid [(2-NA) (Eastman)] were prepared in analytical grade ether (Mallinckrodt).

To 1 ml of plasma (in 16 × 125 mm culture tubes fitted with Teflon-lined caps) was added the internal standard solution (0.1 ml containing 5 μg of 2,5-DMBA and 4 μg of 2-NA) and 1 ml of N HCl. The mixture was shaken for 3 min with 9 ml of ether. After centrifugation the separated ether was evaporated to dryness under a gentle stream of nitrogen at ambient temperature. After addition of 0.5 ml methanol (Fisher, grade 412) to the residue, the tube was vortexed and 15–20 μl of the resulting solution injected into the high pressure liquid chromatography (HPLC). The peak height ratio of ASA or SA to 2,5-DMBA or 2-NA was calculated and the corresponding concentration found by reference to a calibration curve obtained by similar analysis of blank plasma containing known quantities of ASA or SA.

An Altex pump (model 100) and column (Lichrosorb C12 reverse phase, 3.2 × 250 mm) were used to perform the assay. Separation of ASA, SA, 2,5-DMBA and 2-NA was achieved using acetonitrile (31%, Burdick and Jackson) in phosphoric acid (0.085%, Fisher) as eluent at 1.75 ml/min. Absorbance was monitored using a variable wavelength detector (LDC, Model 1201) set at 227 nm.

3. Measurements of Platelet Cyclooxygenase Activity

The preparation of lysates of washed platelets from Na EDTA platelet rich plasma and the determination of platelet cyclooxygenase activity by incubation with arachidonic acid-"C and isolation of labelled thromboxane B2 (TxB2) and prostaglandin D2 (PGD2) has been described in a previous publication.7

Results

Assay of Plasma ASA and SA by HPLC

Excellent resolution was achieved for a mixture of ASA, SA, 2,5-DMBA and 2-NA with a total retention time as seen in figure 1. Standard curves for ASA (0.5–20 μg/ml) and SA (2–80 μg/ml) were linear within the limits indicated. The use of 2 internal standards, 2,5-DMBA and 2-NA, permitted the simultaneous analysis of ASA and SA at widely separated plasma concentrations. A relatively minor unknown peak was encountered on the trailing edge of
the ASA peak but this contaminant did not interfere with the quantitation of ASA. Thus far over 600 plasma samples have been analysed using one reverse-phase column without any noticeable decrease in performance.

**Plasma Levels of ASA and SA**

As seen in figure 2, ASA and SA were detectable in the plasma of all subjects 45 min after compressed tablet administration. ASA and SA concentrations were undetectable in the plasma until at least 4 h after enteric-coated ASA administration. Also, ASA could be detected at 8 h in several subjects. Both preparations resulted in widely variable ASA and SA levels. Most notably, ASA was undetectable in the plasma during 3 experiments involving enteric-coated ASA.

**Cyclooxygenase Activity**

Greater than 95% inhibition of cyclooxygenase activity was produced by a single 650 mg dose of either plain or enteric-coated ASA. Maximal inhibition was observed 45 min after ingestion of plain ASA (fig. 3). The effect on cyclooxygenase activity after ingestion of the enteric-coated preparation was delayed until 4 h and was maximally observed by about 10 h.

In every subject platelet cyclooxygenase activity increased measurably 8 h after ingestion of the compressed tablets (fig. 3). Activity had returned to approximately 10% of the control level by 24 h ($p < 0.05$, Student's $t$-test, paired comparisons). It should be noted that the scale for the first 24 h period in figures 3 and 4 has been expanded to permit adequate display of the data. A linear increase in activity was observed in the first 24 h and continued for the remainder of the 8-day test period. The time for return to 100% of control activity was estimated by extrapolation to be 9.5 days. Following ingestion of enteric-coated ASA there was a definite return of observed activity by 24 h, i.e., 14 h after the time at which maximal inhibition was detected. The subsequent increase in activity closely paralleled that seen with plain ASA.

**Discussion**

For the 2 preparations tested the plasma SA and ASA levels observed are in keeping with other reports. In searching for a fast, simple and chemically direct method of measuring plasma ASA and SA simultaneously, the described HPLC assay was developed. It is similar to a procedure recently reported. Several HPLC methods have been described for the determination of SA in plasma but these suffer from the disadvantage of the inability to simultaneously determine ASA and SA. Our method is also inherently more reliable than those which measure ASA colorimetrically or fluorometrically as the difference in plasma SA before and after hydrolysis.

The rapid disappearance of ASA following compressed tablet administration is consistent with a previous report. The apparently slower elimination of ASA following the enteric-coated preparation reflects elimination that is rate-limited by absorption. Due

![Figure 2](http://stroke.ahajournals.org/)

**Figure 2.** Mean and range of plasma acetylsalicylic acid (ASA) and salicylic acid levels (μg/ml) observed following ingestion of ASA as compressed (cASA) or enteric-coated (ecASA) tablets. Only measurable levels have been used in the calculation of the mean. *Only a single measurable level observed, **2 identical measurable levels observed.**
FIGURE 3. Platelet cyclooxygenase activity following ingestion of 650 mg compressed ASA tablet. Synthesis of TxB₂ + PGD₂ by platelet lysates in vitro was determined at intervals following ingestion of 650 mg compressed ASA tablet. Activity was expressed as a percent of the control synthesis observed immediately prior to ASA ingestion. Points represent mean value (± SEM) for determinations on 6 subjects.

to the potential for erratic absorption patterns following enteric-coated ASA, detectable plasma ASA levels are dependent on fortuitous blood collection times. This is exemplified in our study by the absence of ASA in all plasma samples of some subjects.

It is of interest that the effect of the 2 ASA preparations on platelet cyclooxygenase activity was about equal in spite of different ASA concentration profiles. Inhibition of platelet cyclooxygenase activity by ASA is believed to be due to irreversible acetylation of the platelet enzyme and requires intact ASA molecules. SA is ineffective. The fact that the inhibitory response seen with the 2 preparations was nearly equal, suggests the following hypotheses: 1. Plasma ASA levels, at least in the portal circulation, attain a bolus threshold required for maximum acetylation for both preparations. The studies of Roth and Marjerus and Burch et al. might support this hypothesis as they report that small doses achieve nearly complete acetylation; 2. Circulating platelets may be gradually acetylated in a titrimetric fashion due to the low but prolonged ASA levels encountered in the portal circulation following enteric-coated ASA. These hypotheses require further investigation.

Whether the results obtained with a single selected enteric-coated ASA preparation can be extrapolated to other products is unknown. Due to the well recognized erratic behaviour of such dosage forms further studies with other products in a large population are necessary to answer the question.

The pattern of return of cyclooxygenase activity to control values following maximal inhibition is similar to that observed by Stuart et al. who studied in vitro lipid peroxidation by platelets following ASA ingestion, and by Roncucci et al. who measured malondialdehyde formation in similar experiments. Our observations suggest that only circulating platelets are acetylated and inhibited by ASA, and that platelets released from megakaryocytes, even within a few hours following the ASA dose, are not affected. This is in contrast with the observations of Majerus and colleagues. The latter workers reported that there is a plateau period of 24 to 48 h following ASA ingestion in which all circulating platelets are completely acetylated and, therefore, presumably inhibited. In our experiments it appears that within 24 h of ingestion of ASA, a measurable proportion of circulating platelets exhibit normal activity. The direct measurements of cyclooxygenase activity may reveal a smaller percentage return of activity more readily than is the case with the acetylation technique in which low levels of acetylation require scintillation counting.

FIGURE 4. Platelet cyclooxygenase activity following ingestion of 650 mg enteric-coated ASA tablet. Synthesis of TxB₂ + PGD₂ by platelet lysates in vitro was determined at intervals following ingestion of 650 mg enteric-coated ASA tablet. Activity was expressed as a percent of the control synthesis observed immediately prior to ASA ingestion. Points represent mean value (± SEM) for determinations on 4 subjects.
close to background. Alternatively, the relationship between degree of acetylation and inhibition of enzyme activity may not be strictly linear at lower levels of acetylation. A third possibility is that our platelet suspensions contain a small leukocyte contaminant which is especially high in cyclooxygenase activity. It is unclear if such a cell were capable of synthesis of new cyclooxygenase protein it could contribute to an early increase in activity in the cell suspensions. Parallel incubations of leukocyte preparations suggest that this is unlikely and that leukocytes account for only approximately 1% of the observed cyclooxygenase activity.

Based on the concept that megakaryocytes are acetylated by ASA, and that platelets released within 24 to 48 h of an ASA dose are inhibited, others have suggested that once daily ASA dosage with doses as low as 20 mg may be effective antiplatelet therapy.\(^1^8\)

Our observations indicate that this suggestion should not be accepted without further evidence. On a once-daily regimen the proportion of new platelets not ex-posed to the inhibitory effect of ASA could rise significantly between doses. This circumstance is potentially undesirable, since unaspirinated platelets can synthesize prostaglandins and thromboxanes and cause intense aggregation and the release reaction in aspirinated platelets.\(^2^4\)\(^-^2^5\) The measurements of platelet cyclooxygenase activity may be used to estimate platelet turnover. The return of cyclooxygenase activity following maximal inhibition by a single ASA dose presumably reflects entrance of new platelets into the circulating pool. The return to 100% of control activity at 9.5 days reflects average platelet lifespan. This figure agrees closely with data obtained by other methods. The measurement of conversion of radio-labelled arachidonate to TxB\(_2\) and PGI\(_2\) has an advantage over the measurement of malondialdehyde as employed in the "aspirin method" described by Stuart et al.\(^2^2\) in that quantification appears to be more precise and is easily automated. Washed platelet suspensions prepared during the study period may be stored frozen and incubated with labelled arachidonate in a single batch. This permits all analytic work to be performed in a single day.

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References

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