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Gastrointestinal blood loss after non-steroidal anti-inflammatory drugs. Measurement by selective determination of faecal porphyrins

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1 A method for the detection of gastrointestinal blood loss based upon the selective measurement of faecal porphyrins was tested in two studies in healthy volunteers.

2 In the first study subjects ($n = 6$) received intragastric autologous blood (saline, 2 and 6 ml as a single dose) resulting in a dose dependent increase in faecal porphyrins.

3 In a subsequent placebo controlled cross over study in 12 subjects acetylsalicylic acid (ASA), nabumetone (a new NSAID) or placebo were administered for 5 days with a washout period of 9 days. They were no dietary restrictions.

4 All faeces were collected during the treatment period and both the full faecal homogenate and a random faecal sample were analyzed for deutero- and pemptoporphyrin content by h.p.l.c. Additionally a benzidine reaction was performed.

5 There was a highly significant correlation ($r = 0.95$) between the values obtained from random samples and the full homogenate. ASA increased the faecal porphyrin excretion ($P < 0.001$) compared with placebo in contrast to nabumetone. Complaints of dyspepsia were most common after ASA.

6 Measurement of faecal porphyrins is useful for monitoring NSAID induced upper gastrointestinal blood loss and lacks some of the practical constraints of other methods.

Keywords faecal blood loss NSAID acetylsalicylic acid nabumetone

Introduction

The use of NSAIDs is associated with gastrointestinal toxicity varying from mucosal damage without clinical symptoms to life-threatening bleeding (Carson & Strom, 1988; Henry *et al.*, 1987; Sommerville *et al.*, 1986) especially in elderly patients. Subclinical damage (microbleeding) can be easily detected after most NSAIDs and it appears that there are, at least in the short term, differences between various agents with respect to this (Hunt, 1979; Lanza *et al.*, 1979; Pritchard *et al.*, 1988). It is not precisely known if this type of gastrointestinal damage is predictive for the less common serious effects. The incidence of these effects, however, can only be determined from large epidemiological studies. The association between microbleeding and serious gastrointestinal damage is modified by other factors like age, smoking, preexisting gastrointestinal and rheumatic (Silvoso *et al.*, 1979) disease, and concomitant treatment with anticoagulant drugs. Nevertheless quantification of blood loss during long term treatment is likely to be predictive of the serious gastrotoxicity of different NSAIDs.

Haemoglobin is degraded in the gastrointestinal tract to porphyrins by anaerobic gut bacteria (Beukeveld *et al.*, 1987). The end products of this conversion (which proceeds via the formation of protoporphyrin) are pempto- and deuteroporphyrin. Since this process occurs mainly in the colon, the concentration of these porphyrins mainly reflects blood loss from more proximal sites in the gastrointestinal tract.

We evaluated a new method, based upon the selective measurement by h.p.l.c. of deutero- and pemtoporphyrin, in two studies. In the first study the sensitivity of this test was estimated by the detection of a known amount of autologous blood. The availability of nabumetone, a

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new non-steroidal drug with a supposedly reduced enterotoxic effect (Bianchi Porro *et al.*, 1985; Friedel & Todd, 1988), allowed the comparison of this compound with ASA in the second study. The objectives of this study were to determine if the test was sufficiently sensitive to detect the effects of ASA after subchronic use and able to discriminate between two NSAIDs with a different potential for inducing gastrointestinal damage.

Methods

Eighteen healthy (12 male, 6 female) volunteers (aged 20–26 years) without a history of gastrointestinal disease, were recruited for two studies. Protocols were approved by the medical ethics committee of Leiden University Hospital and subjects gave written informed consent.

Both studies were of a randomised (latin squares) cross over design. In Study I six male subjects received, at weekly intervals, a single administration of saline (6 ml), or two doses of heparinised autologous blood (2 or 6 ml) through a 6F nasogastric tube, infused over 30 min. In Study II 12 subjects received ASA (800 mg three times daily for 5 days), nabumetone (1000 mg at night and 500 mg in the morning for 5 days) or placebo for the same period. The study was blinded using a double dummy technique and drugs were strip packed to ensure correct administration. After each treatment period a 9 day washout period was included.

The subjects were not given any dietary restrictions other than to refrain from eating large amounts of red meat and to drink not more than two glasses of alcohol in the week of drug treatment.

The volunteers were asked to report any side effects and were seen by a research nurse or a physician when they handed in faecal samples.

Subjects started each treatment on a Monday and were asked to collect all faeces in polythene bags until the end of Friday in that week. Faeces were handed in at the clinical research unit as soon as possible and kept at −70° C until analysis.

Analysis

All analyses were performed blind to the administered treatments. A random sample (approx 200 mg wet weight) was taken for measurement of total haemoglobin concentration (colorimetrically with tetramethylbenzidine (Standefer & Vanderjagt, 1977)) and for measurement of porphyrins by high-performance liquid chromatography with spectrofluorometric detection (Beukeveld *et al.*, 1985, 1987). This sample was taken from the unhomogenised stool with a commonly used plastic spoon sampling system, to mimic a clinical outpatient situation. The remaining faeces were homogenized in water (40% w/v) and similarly assayed for total haemoglobin and porphyrins (0.5 ml homogenate). Peaks for deuteroporphyrin and pemtoporphyrin were identified in the chromatogram and peak heights were normalised by division by the peak height of coproporphyrin I. The within-day coefficient of variation (CV) of this assay was 10% and the between-day CV was 11%. The two normalised peak heights were added and summed over all samples received per treatment period. This figure (the summed peak ratio) was used for statistical analysis. The presence of intact heme in the samples was assessed by conversion of the heme by oxalic acid to protoporphyrin and subsequent h.p.l.c. analysis for this breakdown product (Wilson *et al.*, 1985). When no intact heme is present in the samples full conversion to pempto- and deuteroporphyrin can be assumed and this allows a quantitative estimation of the amount of haemoglobin, assuming equal molar fluorescence of pempto- and deuteroporphyrin and using the faecal weight and the molecular weight of haemoglobin. Milligrams haemoglobin per faecal collection were converted to ml blood assuming a haemoglobin value of 15 g 100 ml^{-1} and the cumulative amount excreted per collection period was calculated. In study II this amount was divided by 5 (collection period of 5 days) to give an estimate of average daily blood loss. This was not done for study I since the blood was administered as a bolus on a single day.

Statistical analysis

In the preliminary study I peak ratios after different amounts of blood were compared using a paired *t*-test. In Study II the three treatments were compared using repeated measures analysis of variance. Contrast with placebo and 95% confidence intervals were calculated using Dunnett's *t*-values. This procedure corrects for possible errors associated with repeated comparison of treatments with the placebo (Li,1964). Statistical calculations were carried out using SPSS/PC+ software (SPSS Inc., Chicago, Illinois v3.0). All data are reported as average with the 95% confidence interval of the difference from placebo.

Results

Study I

In study I 2/6 subjects had a positive benzidine test at any time after saline, 3/6 after 2 ml blood and 6/6 after 6 ml blood. The summed peak ratio increased in a dose dependent manner with the amount of blood. The difference between 6 ml and saline was significant ($P < 0.05$) both for the faecal sample and the full homogenate. The average peak ratio after saline was 3.9, after 2 ml blood 7.0 and after 6 ml blood 10.0 (−13.4, 0.08) in the random sample. This corresponded with 5.2 ± 5.3 ml blood/5 days after saline, 7.9 ± 4.8 ml/5 days after 2 ml blood and 11.0 ± 6.9 ml after 6 ml blood. The peak height of coproporphyrin I was not affected by the administration of blood.

Study II

In study II treatment with ASA was associated with the largest number of side effects. Six subjects complained of dyspepsia and one of tiredness. After nabumetone four subjects reported dyspepsia at any time and after placebo there was one complaint of dyspepsia.

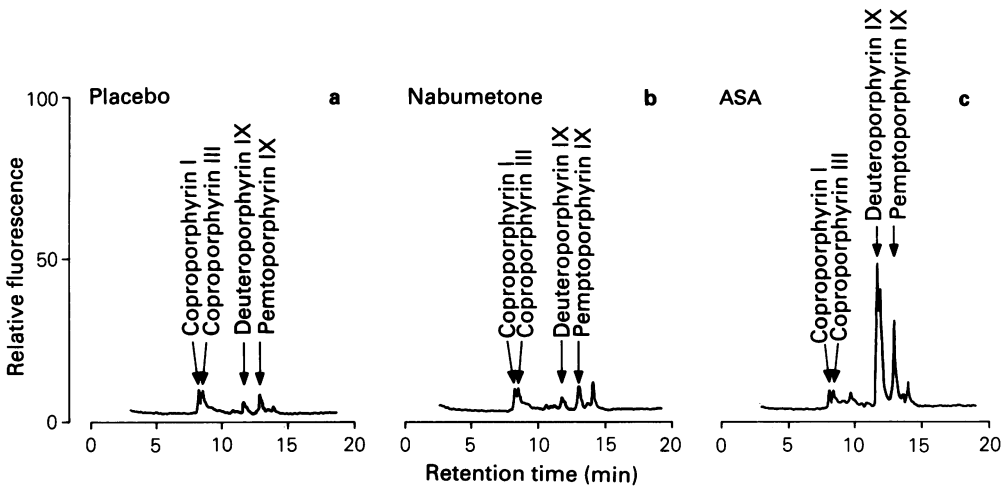

Figure 1 Chromatograms from the random faecal sample obtained from subject 2 at the last day of each of the three treatment periods. Note the constant coproporphyrin I peaks and the increase in deutero- and pemptoporphyrin peaks after ASA. Chromatograms are redrawn for clarity.

Table 1 Sum of the normalized pempto- and deuteroporphyrin peaks over the treatment periods (average ± s.d.)

	Homogenate (peak ratio)	*Random sample (peak ratio)*	*Defaecation frequency (number/5 days)*
ASA	25.2 ± 20.1	24.1 ± 20.0	5.5 ± 1.7
ASA-PLAC	(3.7,33.4)**	(2.7,32.0)*	(−0.8,1.1)
NAB	8.5 ± 7.8	7.7 ± 8.0	5.5 ± 1.4
NAB-PLAC	(−2.0,5.7)	(−3.3,5.2)	(−1.4,1.7)
PLAC	6.7 ± 6.4	6.8 ± 6.0	5.3 ± 1.7

(ASA = acetylsalicylic acid; NAB = Nabumetone; PLAC = placebo). Average and 95% CI for the difference from placebo is given for both the full faecal homogenate and the random sample. When the 95% CI includes zero there is no significant difference from placebo. ($P > 0.05$).
* = $P < 0.05$. ** = $P < 0.01$.

Porphyrin excretion

Chromatograms from the last random sample in each treatment period of subject 2 are shown in Figure 1.

ASA increased the sum of the porphyrin peak ratios in comparison with placebo by 394% and in comparison with nabumetone by 296% in the full faecal homogenate. The increase after ASA in comparison with placebo was statistically significant ($P < 0.05$) both in the homogenate and the random samples. Although small increases in the average peak ratios occurred after nabumetone, these were not significant (Table 1).

No protoporphyrin was detected in any of the samples after conversion by oxalic acid. Therefore an estimate of daily blood loss could be made. The estimated daily blood loss after placebo was 0.84 ± 0.71 ml day^{-1}, after nabumetone 1.02 ± 0.84 ml day^{-1} and after ASA 3.05 ± 2.58 ml day^{-1}. There were good correlations between the individual measurements of the pempto- and deuteroporphyrin peak ratios in the full faecal homogenate and the random samples with correlation coefficients of 0.94 for deuteroporphyrin (n = 197) and 0.960 for pemptoporphyrin (n = 197). Figure 2 shows the average peak ratios for each treatment plotted *vs* sample number. Sample numbers > 5 are not shown since the number of subjects with such a number of samples was too small. All 12 subjects produced four samples per treatment period and eight produced five. The graph demonstrates the development in time of the effect of ASA on the different porphyrins with respect to time in the random samples. Data from the full homogenate were very similar. Defaecation frequency was unchanged by any of the treatments (average 5.5 defaecations per study period).

Benzidine reaction

ASA increased the number of positive samples with a positive benzidine reaction to 80.4% for the homogenate and to 72.6% for the random sample (Table 2). This increase was significant compared with placebo ($P <$ 0.05). By contrast nabumetone did not increase the number of positive samples compared with placebo. There was a considerable percentage of positive samples after placebo (25.5% of the homogenate samples and 16.4% of the random samples).

Discussion

In this study a method for the detection of upper gastrointestinal blood loss induced by non-steroidal anti-inflammatory drugs was evaluated. This method is based

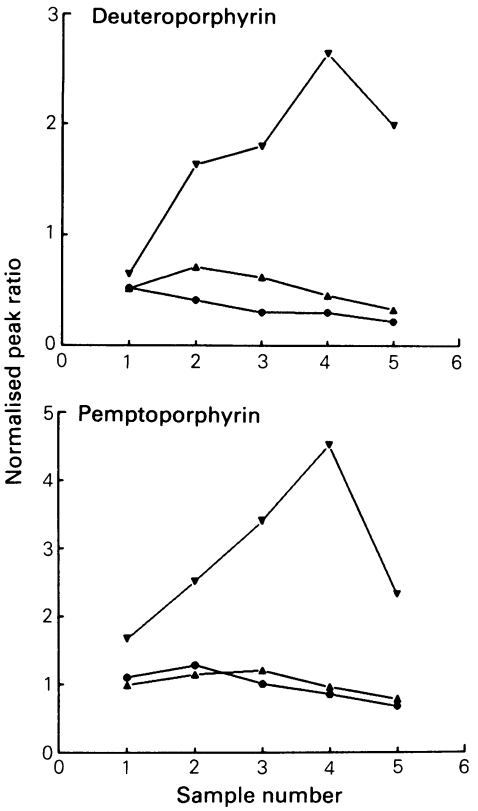

Figure 2 The average normalised peak ratio for the two porphyrins in relation to the sample number after each treatment. ▼ ASA, ▲ nabumetone, ● placebo. Data for the random sample are shown. All subjects produced four samples for each treatment. Only eight subjects produced five samples and any sample numbers beyond this are not shown. Note that the first sample was obtained after start of treatment.

Table 2 Percentage of all samples (both the faecal homogenate and the random sample) with a positive (+ or greater) benzidine test for each of the treatments (average ± s.d.).

	Homogenate (% positive)	*Random sample (% positive)*
ASA	80.4 ± 0.28	72.6 ± 0.30
ASA-PLAC	(29.6,80.0)**	(31.9,80.5)**
NAB	31.9 ± 0.36	17.4 ± 0.22
NAB-PLAC	(−25.7,38.6)	(−23.3,25.3)
PLAC	25.5 ± 0.36	16.4 ± 0.31

(ASA = acetylsalicylic acid;
NAB = Nabumetone; PLAC = placebo).
Average percentages and the 95% confidence interval for the differences from placebo are given. When the confidence interval includes zero the treatments are not significantly different ($P > 0.05$). * = $P < 0.05$. ** = $P < 0.01$.

upon the selective measurement of deutero- and pemptoporphyrin, the two porphyrins that are formed by anaerobic gut bacteria from haemoglobin that has reached the lower gastrointestinal tract intact. This conversion process appears to occur in the small intestine and upper colonic segments, in view of the fact that bleeding in the lower part of the colon does not produce increased deuteroporphyrin excretion (Goldberg & Rimington, 1962; Goldschmiedt *et al.*, 1988). Therefore this method is likely to be more sensitive to blood loss from the upper gastrointestinal tract, when there is more opportunity for bacterial degradation and would only function in the absence of variability in endogenous porphyrin breakdown.

Since the efficiency of the analytical extraction process for porphyrins from faeces can be variable the measurement was normalised against the concentration of coproporphyrin I that is exclusively formed as a breakdown product from endogenous haemoglobin precursors. This study demonstrated again that coproporphyrin I is excreted in the faeces in relatively constant amounts and can therefore be used as an internal standard.

This technique has sufficient sensitivity to be able to detect the effects of ASA and has produced similar results as a study with a slightly higher dose of ASA where ^{51}Cr was used to detect gastrointestinal blood loss (Lussier *et al.*, 1989). A disadvantage of the porphyrin method is that no absolute measure of blood loss can be obtained since conversion of heme may not always be complete. However in this study no protoporphyrin could be detected in the samples, indicating that complete conversion had occurred in these healthy volunteers. Daily blood loss under control conditions amounted to approximately 1 ml day^{-1} in both studies. This is higher than reported with radiochromium (Lussier *et al.*, 1989; Ryan *et al.*, 1987) and may reflect the presence of heme from other sources, since no dietary restrictions were used in this study. The average increase after 2 ml and 6 ml of autologous blood was 2.5 and 5.8 ml respectively, which was in reasonable agreement, although the inter-individual variability was large. In Study II ASA increased blood loss to 3.05 ml day^{-1}, which is in agreement with earlier work (Lussier *et al.*, 1989).

An assay kit for faecal haemoglobin based upon the overall analysis of porphyrins is available (HemoQuant) (Ahlquist *et al.*, 1985) but its capability to detect non-steroidal induced gastrointestinal blood loss has not been evaluated. Additionally the less selective assay would make it more sensitive to artefacts.

There are a number of other methods for the detection of gastrointestinal blood loss. The most commonly used method, involving ^{51}Cr labelled red cells (Holt, 1960) imposes a considerable radiation burden and requires a radionuclide laboratory. In contrast to the ^{51}Cr method no radiation is involved for our method and, in view of the excellent correlation between full faecal homogenates and the random samples from the same faeces, samples (that can be taken by the patient) will be sufficient, which negates the obvious problems connected with full faecal collections. When samples are used, however, no quantitative estimation of blood loss can be made. Although the ^{51}Cr method is likely to be superior in the quantitative assessment of blood loss, measurement of porphyrins may be more widely applicable in relevant patient populations and yield at least equally useful information. Although not fully quantitative, our method allows the estimation of drug effects over time and yields remarkably constant effects after placebo, without extensive dietary measures (Figure 2).

Gastric bleeding can also be assessed by measurement of haemoglobin in gastric washings (Pritchard *et al.*, 1989). This procedure can only be used intermittently and may underestimate damage since measurements are only obtained over 10 min and are limited to the

stomach. Endoscopy gives the most direct impression of gastric damage (Caruso & Bianchi Porro, 1980) but can only be performed acutely or at most infrequently during long term treatment, whereas no information is obtained about possible damage to parts of the small intestine that cannot be visualised (Bjarnason *et al.*, 1984). Immunological detection of intact haemoglobin in the faeces (Frommer *et al.*, 1988) is of limited use for the detection of effects of NSAIDs since the haemoglobin from the upper gastrointestinal tract reaches the faeces mainly as porphyrins, which are not recognised by the antibody.

A highly sensitive benzidine assay (in view of the high false positive rate) produced similar results in this study as the more complicated porphyrin assay, demonstrating that a certain amount of the peroxidase activity from the lost blood was still present. A major drawback of the benzidine assays is that only a qualitative assessment can be made, which does not allow to see changes over time. Additionally there are indications that heme from autologous blood is converted more readily to porphyrins than heme from meat (G. J. J. Beukeveld, personal communication), while samples in both cases would have retained peroxidase activity. The subjects in this study received no special diet, to mimic a clinical outpatient situation as much as possible.

Nabumetone did not increase gastrointestinal blood loss and this appears to confirm its reduced gastrotoxicity. The drug was used in a therapeutic dose but this study does not allow conclusions about its gastrointestinal toxicity in patients, who are likely to have other risk factors. However, it will now be possible to study these populations on an outpatient basis over longer periods of time, using the measurement of porphyrins in stool samples taken by the patients.

In conclusion, we demonstrated the value of the selective measurement of faecal porphyrins for the detection of non-steroidal antiinflammatory drug induced enteropathy. This measurement might, in addition, be useful for the study of the gastrotoxicity of other drugs or dosage forms, for the evaluation of different strategies to reduce gastrotoxicity of drugs (Lanza *et al.*, 1988), or perhaps for the monitoring of the healing rate of gastric ulcers.

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