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Benorylate hydrolysis by human plasma and human liver

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1 Benorylate (4-acetamido phenyl-*O*-acetylsalicylate) hydrolysis *in vitro* by human plasma and by human liver microsomes and cytosol has been investigated.
2 Benorylate was hydrolysed by a route involving initial hydrolysis of the acetyl group to yield phenetsal followed by hydrolysis to paracetamol and salicylate. Hydrolysis via acetylsalicylate was minor.
3 Benorylate was more actively hydrolysed by liver cytosol than microsomes and about 10 times faster than plasma.
4 Following a single oral dose benorylate (4 g) to volunteers only salicylate and paracetamol were detected in the plasma.
5 The therapeutic effects of benorylate appear to be mediated by salicylate and paracetamol.

Keywords benorylate paracetamol salicylate plasma human liver

Introduction

The anti-inflammatory compound benorylate (4-acetamido phenyl-*O*-acetylsalicylate), a paracetamol ester of acetylsalicylic acid is used to treat pain and inflammation particularly in musculoskeletal disorders. The anti-inflammatory efficacy of benorylate is similar to that of aspirin (Beales *et al.*, 1972; Mayhew, 1978) but benorylate provides limited pain relief following third molar surgery (Moore *et al.*, 1989). The pharmacological actions of benorylate are due to the effects of both aspirin and paracetamol released as the result of hydrolysis, although only paracetamol and salicylate were detected in the blood following an oral dose of benorylate (Robertson *et al.*, 1972). Hamalainen *et al.* (1973) detected unchanged benorylate in sinovial fluid. Following oral administration, benorylate absorbed from the gastrointestinal tract would be a target for hydrolysis by esterases in the gut wall, the liver and the plasma (Williams, 1987).

In this study hydrolysis of benorylate *in vitro* by human plasma and by human liver microsomes and cytosol has been investigated. This has been related to the *in vivo* fate of a single oral dose of benorylate in man.

Methods

Chemicals

Benorylate and phenetsal (4-acetamidophenyl-*O*-salicylate) were gifts from Sterling Research Europe. Acetylsalicylate and salicylate were obtained from Sigma.

Subjects

Human liver samples (wedge biopsies) were obtained from four patients (two female) undergoing cholecystectomy or vagotomy and pyloroplasty. Liver tissue was immediately placed in liquid nitrogen before storage at −80° C until analysis. All liver samples were later found to be histologically normal.

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Human plasma samples Control plasma samples were collected from nine patients (four female) participating in a clinical study of the efficacy of benorylate in postoperative dental pain (Moore *et al.*, 1989). A single venous blood sample was taken before the benorylate dose. Plasma was separated and stored at −80° C until analysis.

The studies had received approval by the Joint University/Health Authority Ethics Committee.

Benorylate hydrolysis

Plasma incubations Plasma (40 μl) was incubated with benorylate (1 mM) added in 30 μl acetone, and 300 μl Ca^{++} tris buffer pH 7.4 at 37° C in a shaking waterbath. At 0, 10, 20 and 40 min for all plasmas plus 5 min for four plasmas and to 120 min for three plasmas, hydrolysis was stopped by the addition of 300 μl cold perchloric acid (6% v/v) containing *p*-toluic acid (1 μg ml^{-1}). Following centrifugation to precipitate protein the incubations were placed on ice until analysis by h.p.l.c. Spontaneous hydrolysis of benorylate was less than 10% of plasma hydrolysis under these conditions.

Plasma aspirin esterase activity was measured as previously described (Williams *et al.*, 1989). Acetylsalicylate 1 mM was incubated with 0.2 ml plasma in 3 ml buffer at 37° C.

Preparation of liver microsomal and cytosolic fractions

Human liver tissue (approximately 200 mg) was thawed at +4° C and a weighed portion homogenised with 10 ml ice cold buffer pH 7.4 (0.25 M potassium phosphate, 0.15 M KCl) using a glass to glass homogeniser. Homogenates were centrifuged for 5 min at 1000 *g* followed by 10 min at 18,000 *g*. Microsomes were prepared by centrifugation of the 18,000 *g* supernatant for 60 min at 120,000 *g* and the cytosolic fraction was retained. The microsomal pellet was resuspended in 0.25 M phosphate buffer (pH 7.4 containing 0.15 M KCl) and recentrifuged for 60 min at 120,000 *g*. The pellet was finally resuspended in phosphate buffer pH 7.4.

Liver enzyme assays

Liver microsomes or cytosol (equivalent to 0.8 mg liver) were incubated with benorylate (300 μM added in acetone (10 μl)) in 500 μl 0.25 M phosphate buffer pH 7.4 at 37° C in a shaking water bath for varying times between 0 and 180 min. Hydrolysis was stopped by the addition of 500 μl cold perchloric acid (6% v/v) (no internal standard was used). Following centrifugation the incubations were stored on ice until analysis by h.pl.c. Spontaneous hydrolysis of benorylate in buffer was measured in parallel and subtracted to determine enzyme activity.

In vivo *studies*

Four fasted volunteers received single oral suspensions (10 ml made up to 50 ml in water) of benorylate (4 g) on an empty stomach. No volunteer had taken aspirin or paracetamol for at least 2 weeks prior to the study. Blood samples were taken from an indwelling venous catheter. For the early blood samples (blank plus 15, 30, 45 and 60 min), 2 ml heparinised blood was cooled on ice and centrifuged immediately in a cold centrifuge to separate plasma which was then added to an equal volume of ice cold perchloric acid (6% v/v containing *p*-toluic acid 1 μg ml^{-1}), centrifuged at +4° C and the supernatant immediately injected onto the h.p.l.c. The time for processing a blood sample from subject to h.p.l.c. was less than 15 min. A blank plasma sample was spiked with benorylate and processed in parallel to confirm that there was no significant loss of benorylate during preparation. Later blood samples taken at 90 min, 2, 3, 4, 6, 8, 12 and 24 h were processed in a similar manner and analysed the same day but with less emphasis on speed.

H.p.l.c. conditions

Plasma assays H.p.l.c. was performed using a Waters Z module containing an ODS C18 column with C18 precolumn with a mobile phase of 50:50 methanol and (0.072% w/v) orthophosphoric acid at a flow rate of 4 ml min^{-1} (u.v. detection 234 nm). Retention times were as follows: paracetamol 1.4 min, acetylsalicylic acid 2.0 min, salicylic acid 2.8 min, *p*-toluic acid 3.6 min, benorylate 4.8 min, and phenetsal 9.6 min. Extracted calibration curves were measured in parallel with the samples. Inter-assay variation for standards was salicylate (400 nmol) 6%, paracetamol (400 nmol) 6%, phenetsal (600 nmol) 13% and benorylate (600 nmol) 14%. Enzyme activity was expressed as nmol benorylate hydrolysed min^{-1} ml^{-1} plasma.

Liver assays H.p.l.c. was performed using a continuous gradient from 20% to 60% methanol with orthophosphoric acid (0.072% v/v). The column was Spherisorb ODS (5 μ) flow rate

1 ml min^{-1} u.v. detection at 234 nm. Retention times were as follows: paracetamol 4.6 min, acetylsalicylic acid 9.4 min, salicylic acid 9.9 min, *p*-toluic acid 10.7 min, benorylate 11.5 min, phenetsal 14.4 min.

Spiked standards in phosphate buffer were mixed with equal volumes 6% perchloric acid containing 1 μg ml^{-1} *p*-toluic acid before analysis. Activity was expressed as μmol benorylate hydrolysed or product formed min^{-1} g^{-1} liver.

Results

In vitro *studies*

Plasma When benorylate was incubated with plasma, benorylate disappeared rapidly in parallel with the formation of phenetsal so that no benorylate was detectable by 60 min (Figure 1). In later samples (after 20 min) low levels of salicylate and paracetamol were detected, but no acetylsalicylate was identified. The initial rate of benorylate hydrolysis up to 15 min, was assumed to be linear and was calculated for all plasmas from the concentrations measured at 0, 5 and 10 min after the start of incubation. The hydrolysis rate was 1050 ± 80 (± s.e. mean) nmol benorylate ml^{-1} plasma min^{-1} compared with 95.3 ± 8.0 nmol acetylsalicylate ml^{-1} min^{-1}. Table 1 contains individual results. For six of the subjects (1, 8 and 9 excluded) there appeared to be a good relation between plasma aspirin esterase and plasma benorylate esterase activity, however including all nine subjects the correlation was not significant $r = 0.38$ (Pearsons).

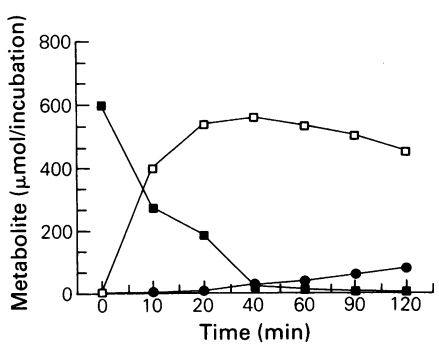

Figure 1 Hydrolysis of benorylate (1 mM) by human plasma *in vivo*. Benorylate ■, phenetsal □, salicylate ○ and paracetamol ●. Salicylate and paracetamol levels were similar and lines superimpose in the figure. Results shown are for one plasma, duplicate assays at each point.

Liver

Microsomal fraction Because of the very rapid hydrolysis of benorylate by liver samples, hydrolysis conditions of 300 μM benorylate plus microsomes (4 μg protein equivalent to 0.8 mg liver per incubation) were selected such that benorylate was detectable for 30 min and only 50% disappeared over the first 15 min. Benorylate disappearance at this concentration was proportional to microsomal protein between 0 and 20 μg per incubation. Four livers were studied and results are shown in Table 2 and Figure 2a. Benorylate disappeared from the incubations at 4.53 ± 0.45 μmol g^{-1} liver min^{-1} over the first 10 min. Phenetsal peaked by 15 min and then declined. Paracetamol and salicylate were rapidly formed and by 120 min hydrolysis was complete. The maximum concentration of acetylsalicylate formed was only 18 μmol g^{-1} liver.

Cytosol The profile of benorylate hydrolysis by cytosol was similar to that by microsomes

Table 1 Hydrolysis of benorylate and acetylsalicylate by human plasma *in vitro*

Subject	*Benorylate hydrolysis (nmol ml^{-1} min^{-1})*	*Acetylsalicylate hydrolysis (nmol ml^{-1} min^{-1})*
1	783	124
2	1374	104
3	1485	129
4	1174	85
5	960	71
6	1048	79
7	803	58
8	908	108
9	945	100

Benorylate (1 mM) was incubated with 40 μl plasma in 300 μl buffer and acetylsalicylate (1 mM) with 200 μl plasma in 3 ml buffer.

Table 2 Benorylate hydrolysis by human liver

Subject	Liver cytosol			Liver microsomes		
	Benorylate hydrolysis ($\mu mol\ g^{-1}\ liver\ min^{-1}$)	*Phenetsal hydrolysis* ($\mu mol\ g^{-1}\ liver\ min^{-1}$)	*Time to peak phenetsal* (*min*)	*Benorylate hydrolysis* ($\mu mol\ g^{-1}\ liver\ min^{-1}$)	*Phenetsal hydrolysis* ($\mu mol\ g^{-1}\ liver\ min^{-1}$)	*Time to peak phenetsal* (*min*)
1	4.8	7.6	10	3.6	2.5	10
2	3.9	4.1	20	3.3	3.5	20
3	4.7	8.1	10	5.5	3.0	20
4	4.8	4.2	20	4.7	1.8	30
Mean ± s.d.	4.53 ± 0.45	6.0 ± 2.1	15	4.2 ± 1.0	2.72 ± 0.71	20

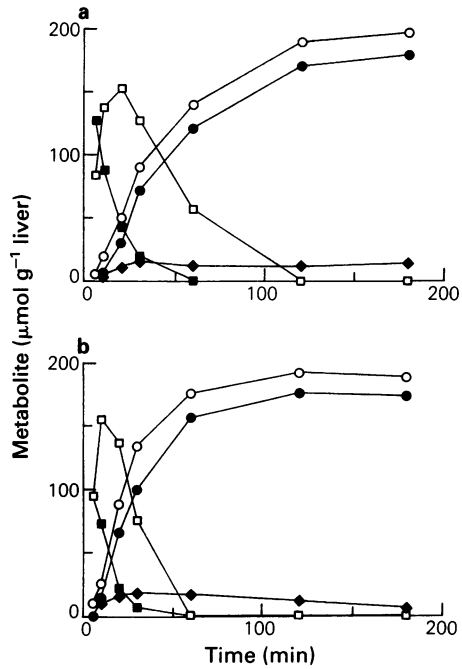

Figure 2 Hydrolysis of benorylate by (a) human liver microsomes or (b) human liver cytosol. Results shown are mean of four livers, duplicate assays at each point. Benorylate (300 μM) was incubated with microsomes or cytosol equivalent to 0.8 mg liver for 180 min. Benorylate ■, phenetsal □, salicylate ○, paracetamol ● and acetylsalicylate ◆.

(Table 2 and Figure 2b). Under equivalent conditions in terms of wet weight of liver, the rate of hydrolysis of benorylate to phenetsal was similar but time to peak phenetsal was faster and disappearance of phenetsal more rapid. Appearance of paracetamol and salicylate was more rapid than with microsomes. Again h.p.l.c. indicated a small amount of acetylsalicylate (17 $\mu mol\ g^{-1}$) which decreased with time from 15 min.

In vivo *studies* Following a single oral dose of 4 g benorylate, no unchanged benorylate, phenetsal or acetylsalicylic acid were detected in plasma at any time. Plasma paracetamol levels reached a maximum between 90 min and 4 h. Salicylate levels increased to peak between 3 h and 6 h (Figures 3a and b).

Discussion

Benorylate is hydrolysed to paracetamol and salicylate by plasma, liver microsomes and liver cytosol by a route involving initial hydrolysis of

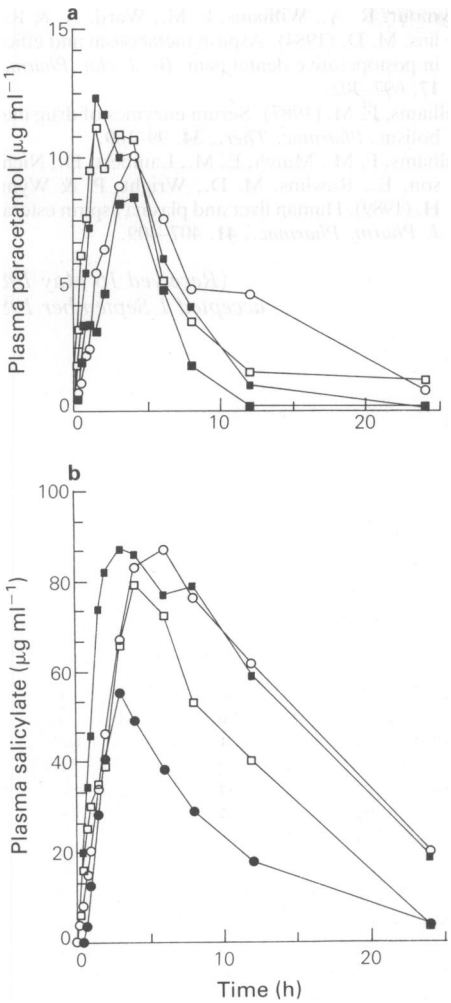

Figure 3 Plasma profile of (a) paracetamol and (b) salicylate in four volunteers following a single oral dose of benorylate (4 g).

the acetyl group to yield phenetsal followed by hydrolysis to paracetamol and salicylate. Only traces of acetylsalicylate were detected with liver indicating that hydrolysis by this route is minor.

Under the substrate conditions employed (300 μM with liver compared with 1 mM with plasma), human liver (microsomes and cytosol) was 10 times more active at hydrolysing benorylate than plasma. *In vivo* following an oral dose all benorylate will be hydrolysed via phenetsal to paracetamol and salicylate in the first pass through the liver. This would explain why following an oral dose of benorylate no benorylate or phenetsal was detected in the plasma even 15 min after the dose. In contrast about 60% of an oral dose of acetylsalicylate reaches the circulation and is detected within the plasma for 1 h after the dose (Rowland *et al.*, 1972; Seymour *et al.*, 1984). These results and previous studies have shown that the rate of removal of the acetyl group from benorylate by human liver and plasma is about 10 times that for aspirin (Williams *et al.*, 1989).

Benorylate was more actively hydrolysed by liver cytosol than microsomes and this parallels acetylsalicylate hydrolysis (Williams *et al.*, 1989). Benorylate and acetylsalicylate appear to be substrates for the same esterase enzymes but with differing affinities. Acetylsalicylate has been shown to be hydrolysed by cholinesterase and to a small extent albumin in the plasma, (Rainsford *et al.*, 1980) and by microsomal and cytosolic carboxylesterases in the liver (Williams *et al.*, 1989). The close relation between plasma benorylate and acetylsalicylate hydrolysis for six of the subjects studied, suggests that the same enzymes are involved. There is no explanation for the different relation in three subjects and this requires further investigation.

Previous studies have indicated that plasma aspirin esterase activity influences the analgesic effect of aspirin by influencing the circulating acetylsalicylate level even following significant liver hydrolysis (Seymour *et al.*, 1984). As benorylate (or phenetsal) does not reach the circulation unhydrolysed, the plasma enzymes may not contribute significantly to its hydrolysis *in vivo*. It therefore seems likely that the pharmacological and therapeutic effects of benorylate are mediated by salicylate and paracetamol.

References

Beales, D. L., Burry, H. C. & Grahame, R. (1972). Comparison of aspirin and benorylate in the treatment of rheumatoid arthritis. *Br. med. J.*, **2**, 483–485.

Hamalainen, M., Laine, V. A., Penn, R. G. & Vainio, K. (1973). The passage of benorylate into the synovial fluid and tissue of rheumatoid patients. *Rheum. Rehab., suppl.*, 85–91.

Mayhew, S. R. (1978). A comparison of benorylate and naproxen in degenerative arthritis. *Rheum. Rehab.*, **17**, 29–33.

Moore, U., Seymour, R. A., Williams, F. M., Nicholson, E. & Rawlins, M. D. (1989). The efficacy of benorylate in postoperative dental pain. *Eur. J. clin. Pharmac.*, **36**, 35–38.

Rainsford, K. D., Ford, N. L. V., Brooks, P. M. &

Watson, H. M. (1980). Plasma aspirin esterases in normal individuals, patients with alcoholic liver disease and rheumatoid arthritis. Characterisation and the importance of enzymatic components. *Eur. J. clin. Invest.*, **10**, 413–420.

Robertson, A., Glynn, J. P. & Watson, A. K. (1972). The absorption and metabolism in man of 4-acetamidophenyl-2-acetoxybenzoate (benorylate). *Xenobiotica*, **2**, 339–347.

Rowland, M., Riegelman, S., Harris, P. A. & Sholhoff, S. D. (1972). Absorption kinetics of aspirin in man following oral administration of an aqueous solution. *J. pharm. Sci.*, **61**, 379–385.

Seymour, R. A., Williams, F. M., Ward, A. & Rawlins, M. D. (1984). Aspirin metabolism and efficacy in postoperative dental pain. *Br. J. clin. Pharmac.*, **17**, 697–702.

Williams, F. M. (1987). Serum enzymes of drug metabolism. *Pharmac. Ther.*, **34**, 99–109.

Williams, F. M., Mutch, E. M., Lambert, D., Nicholson, E., Rawlins, M. D., Wright, P. & Wynne, H. (1989). Human liver and plasma aspirin esterase. *J. Pharm. Pharmac.*, **41**, 407–409.

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