Chromatographic Resolution, Chiroptical Characterization and Preliminary Pharmacological Evaluation of the Enantiomers of Butibufen: a Comparison with Ibuprofen

J. R. S. HOULT, B. R. JACKSON, E. BENICKA*, B. K. PATEL* AND A. J. HUTT*

*Division of Pharmacology and Therapeutics, King's College London, London, SE1 9RT and
Department of Pharmacy, King's College London, London, SE1 8WA, UK

Abstract

Enantiomeric resolution of butibufen has been achieved on a cellulose tris(3,5-dimethyl-phenylcarbamate) chiral stationary phase with hexane−isopropanol−trifluoroacetic acid, $100:1\cdot2:0\cdot02$ (v/v/v) as mobile phase at a flow rate of $1\cdot0\,\text{mL}\,\text{min}^{-1}$.

Semi-preparative isolation of the enantiomers then chiroptical characterization indicated that the order of elution was $(-)$-R- before $(+)$-S-butibufen. When tested for their effects on the cyclooxygenase and 5-lipoxygenase pathways of eicosanoid metabolism in calcium ionophore-activated rat peritoneal leukocytes it was found that $(+)$-S-butibufen inhibited generation of thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2) (cyclooxygenase pathway), with an IC50 of $1\cdot5\,\mu\text{M}$ (approx.), whereas the $(-)$-R enantiomer was essentially inactive. Neither enantiomer inhibited the 5-lipoxygenase pathway. In this regard, $(+)$-S-butibufen was approximately five times less potent as a cyclooxygenase inhibitor than $(+)$-S-ibuprofen.

These results show the enantiomeric specificity and pathway selectivity of this novel non-steroidal anti-inflammatory drug.

R,S-Butibufen (2-(4-isobutylphenyl)butyric acid) is a non-steroidal anti-inflammatory drug (NSAID) structurally related to ibuprofen (Aparicio 1977; Carretero et al 1978). Substitution of the α-methyl group in ibuprofen for an α-ethyl group in butibufen has been reported to result in reduced potency in the UV−erythema and rat foot analgesia models (Nicholson 1982). However, others have claimed that butibufen is comparable in potency with ibuprofen in several tests (Aparicio 1977; Carretero et al 1978). Clinical studies have shown butibufen to be an effective and well tolerated drug with few gastrointestinal side effects (Houssay et al 1990; Bucheli et al 1994).

The major pharmacological activity of ibuprofen and related 2-arylpropionic acid NSAIDs, inhibition of cyclooxygenase, resides predominantly in the enantiomers with the S absolute configuration (Hutt and Caldwell 1984; Brune et al 1992; Evans 1992; Boneberg et al 1996; Neupert et al 1997). However, recent evidence has indicated that the R enantiomers of these agents do have anti-nociceptive activity unrelated to inhibition of cyclooxygenase (Brune et al 1991). Although stereochemical aspects of the action and disposition of 2-arylpropionic acids have been extensively investigated (Caldwell et al 1988; Williams 1990; Brune et al 1992; Evans 1992; Hayball 1996), relatively little is known about the 2-arylbutyric acid derivatives. The most extensively studied compound in this group is indobufen (Cerletti et al 1990; Perrone and Farina 1990; Strolin-Benedetti et al 1990; Grubb et al 1993), the S enantiomer of which is known to be responsible for the anti-inflammatory activity (Cerletti et al 1990).

The chromatographic resolution and some preliminary observations of the pharmacological effects of the enantiomers of butibufen on the enzymes of the arachidonate cascade are the subject of this report.

Materials and Methods

Chemicals

Racemic butibufen was kindly donated by Justesa Imagen (Madrid, Spain), R-, S- and racemic ibu-

Correspondence: A. J. Hutt, Department of Pharmacy, King's College London, Franklin-Wilkins Building, Stamford Street, London, SE1 8WA, UK.

profen were generous gifts from Boots (Nottingham, UK). Solvents for high-performance liquid chromatography were purchased from Rathburn (Walkerburn, UK). Other reagents were of analytical grade or better and were from BDH (Poole, UK).

High-performance liquid chromatography (HPLC)
HPLC was performed with either a Waters 6000A or an LDC Constametric 3000 pump, linked to an LDC Spectromonitor 3100 detector and a CI 4000 computing integrator. Samples were introduced on column by means of an LKB 2157 autosampler fitted with a 100-μL loop. Chiral separations were performed on a 250 mm × 4·6 mm i.d.; 10 μm cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phase (Chiralcel OD) with a matching guard column (50 mm × 4·6 mm i.d.; 10 μm) both supplied by HPLC Technology (Macclesfield, UK). The mobile phase was 100 : 1·2 : 0·02 (v/v/v) hexane−isopropanol−trifluoroacetic acid and the flow rate was 1·0 mL min^{-1} at room temperature. The UV detector was operated at 225 nm.

To obtain sufficient quantities of both butibufen enantiomers for chiroptical characterization and pharmacological evaluation, repeated injections (20 μL) of a concentrated (100 mg mL^{-1}) drug solution were made on to the chiral stationary phase. The column eluate containing the individual enantiomers was collected between 10·5 and 12·0 min and between 13·1 and 15·3 min. The mobile phase was evaporated under a stream of nitrogen and the samples were stored at −20°C until required.

Circular dichroism
Circular dichroism spectra were recorded by means of a Jasco J720 spectropolarimeter. The individual enantiomers of butibufen were dissolved in acetonitrile, at a concentration of 1·0 mg mL^{-1} (approx.), and spectra were recorded over the range 300−240 nm in a cell of pathlength 1·0 cm and between 260−200 nm in a cell of pathlength 0·02 cm. Optical rotation was determined by use of a Jasco J600 spectropolarimeter at an enantiomer concentration of 4 mg mL^{-1} (approx.) in acetonitrile, in a cell of pathlength 5 cm.

Eicosanoid generation by ionophore-stimulated rat peritoneal leukocytes
The methods used have been described elsewhere (Moroney et al 1988). In brief, mixed peritoneal leukocytes (85% polymorphonuclear leukocytes,

the remainder mononuclear cells; 95% viability) were suspended at 2·5 × 10^6 cells mL^{-1} in Hanks balanced salt solution (HBSS) containing Ca^{2+} (1·26 mM) and Mg^{2+} (0·9 mM). These cells were elicited from male Wistar rats by intraperitoneal injection of 6% glycogen 16 h previously. Cells in HBSS (0·5 mL) were pretreated for 10 min at 37°C with the potential inhibitor or its vehicle (2 μL dimethylsulphoxide), then stimulated for a further 15 min with the calcium ionophore A23187 (calcimycin, Sigma; 1 μM), and the cells pelleted by centrifugation. The supernatant was retained for analysis by radioimmunoassay for thromboxane B$_2$ (TXB$_2$), prostaglandin E$_2$ (PGE$_2$) and leukotriene B$_4$ (LTB$_4$); this analysis was performed by taking samples (2–50 μL), diluting to 100 μL with phosphate buffer (pH 7·5, 50 mM) containing 0·1% human γ-globulin and 0·9% saline, then adding polyclonal rabbit anti-eicosanoid serum diluted 1 : 1500 (200 μL), and tracer (100 μL) containing 10 nCi [^{3}H]$_8$TXB$_2$, 10 nCi [^{3}H]$_8$PGE$_2$ or 4 nCi [^{3}H]$_8$LTB$_4$ (NEN or Amersham), mixing, and incubating at 4°C for 18 h. After this, bound label was separated from free by use of 200 μL dextran-coated charcoal and the bound disintegrations min^{-1} counted in a Packard model 1900TR liquid scintillation analyser.

Results and Discussion

Under the chromatographic conditions employed the enantiomers of butibufen eluted from the Chiralcel OD chiral stationary phase with retention times of 11·0 and 14·4 min, yielding separation factors (α) and resolution values (R$_S$) of 1·44 and 2·17 respectively (Figure 1). Under identical conditions *R*- and *S*-ibuprofen eluted at 12·6 and 14·8 min respectively, with α and R$_S$ values of 1·24 and 1·14. Thus the less retained enantiomer of butibufen elutes more rapidly than *R*-ibuprofen, presumably because of increased, unfavourable interaction with the chiral stationary phase associated with the increased steric bulk of the α-ethyl group, compared with the α-methyl group. Semi-preparative-scale isolation of the individual enantiomers of butibufen was performed by multiple injection of a concentrated stock solution on column with collection of the column eluate between 10·5 and 12·0 min and between 13·1 and 15·3 min. Evaporation of the mobile phase under a stream of nitrogen yielded 40 mg (approx.) of each enantiomer as oils which did not solidify on storage at −20°C for one year. Re-injection of the individual enantiomers on to the chiral stationary phase during this period indicated that racemization did not
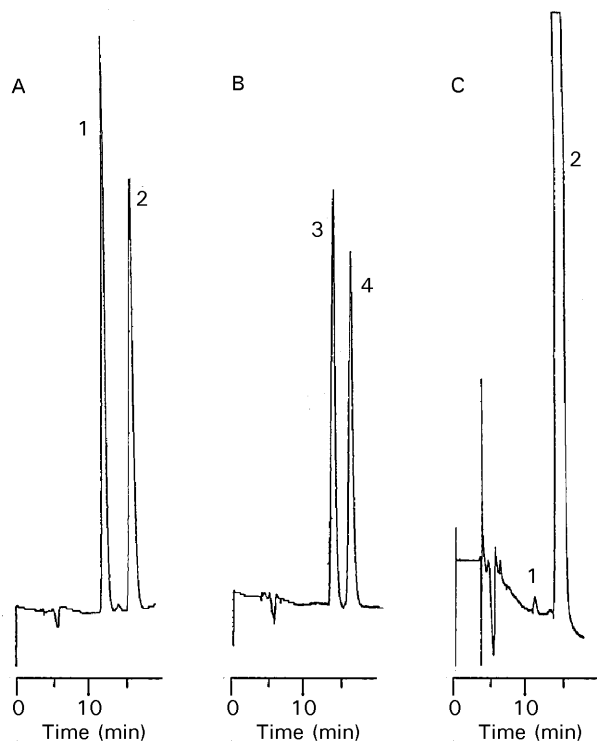
Figure 1. Chromatographic resolution of the enantiomers of A. butibufen (retention times: peak 1, 11·0 min and peak 2, 14·4 min) in comparison with B. ibuprofen (retention times: peak 3, R-, 12·6 min; peak 4, S-, 14·8 min) on Chiralcel OD. C. Determination of the stereochemical purity of the second eluting butibufen enantiomer after semi-preparative isolation (enantiomeric composition peak 1: peak 2, 0·27:99·73 by peak area).
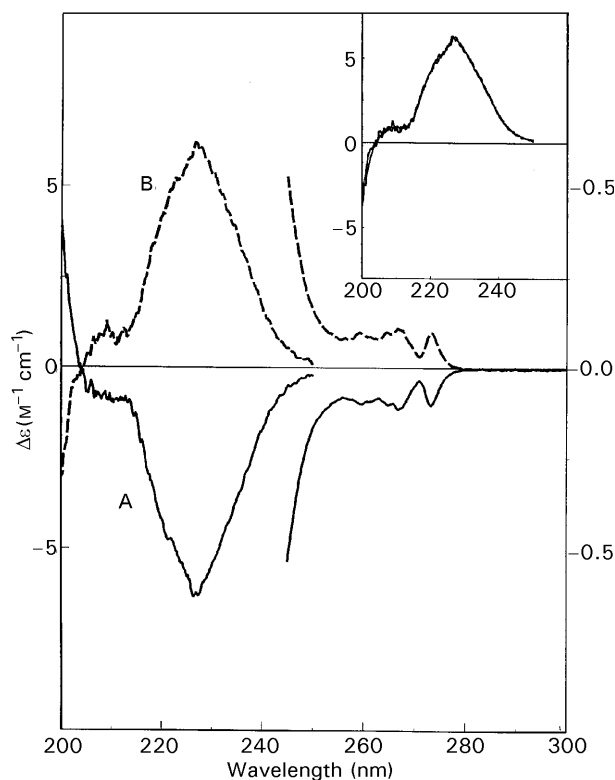

Figure 2. Circular dichroism spectra of A. the first- and B. the second-eluting enantiomers of butibufen. Inset: overlay of spectra with inversion of that of the first-eluting enantiomer. (The $\Delta\varepsilon$ scale between 300 and 245 nm is magnified tenfold compared with that between 245 and 200 nm).

occur; the enantiomeric purity was ≥99·7% for both isomers (Figure 1C).

Circular dichroism spectra of the isolated enantiomers, determined in acetonitrile, are presented in Figure 2. The mirror image relationship and the essentially identical spectra obtained on inversion and overlay of the circular dichroism spectra confirm the stereochemical purity of the enantiomers as determined by chromatography (see inset Figure 2). The spectra show intense absorption bands at 227 nm and a series of weaker absorption bands with maxima at 259, 266 and 273 nm similar to those of ibuprofen and related α-alkylphenylacetic acid derivatives (Barth et al 1970; Teulon et al 1978; Tan et al 1997). The band at 227 nm is assigned to n→π* transitions of the carbonyl group, whereas those in the 250–280 nm region are associated with ^1L_b transitions of the phenyl group (Barth et al 1970). The positive and negative circular dichroism at 227 nm correlate with S- and R-α-alkylphenylacetic acid stereochemistry respectively. The optical rotations of the individual enantiomers were found to be −82·8° and +80·1°, respectively, for the first and second eluting enantiomers. Thus, on the basis of this spectroscopic

data the order of elution of the enantiomers of butibufen on the Chiralcel OD chiral stationary phase is (−)-R before (+)-S.

The purified enantiomers and racemic butibufen were examined for their effects on two key enzymes of the arachidonate cascade, cyclooxygenase and 5-lipoxygenase. These enzymes are both expressed constitutively in elicited rat peritoneal leukocytes which can be stimulated with the calcium ionophore A23187 to yield thromboxane B_2 (TXB$_2$), prostaglandin E_2 (PGE$_2$) (products of the cyclooxygenase pathway) and leukotriene B_4 (LTB$_4$) (5-lipoxygenase pathway), as shown in Figure 3. The results show that the yield is LTB$_4$≫ TXB$_2$ > PGE$_2$. Pre-incubation with (+)-S-butibufen resulted in dose-dependent inhibition of the cyclooxygenase pathway, but was without effect on 5-lipoxygenase (Figure 3). The (−)-R enantiomer was essentially inactive (IC50 (the concentration causing 50% inhibition) > 100 μM), whereas the racemate was active, though correspondingly less so than the pure (+)-S enantiomer (Table 1). These results are in agreement with the expectation that the capacity to inhibit cyclooxygenase is exclusive to those compounds with the S absolute configuration. However, none of the three preparations of
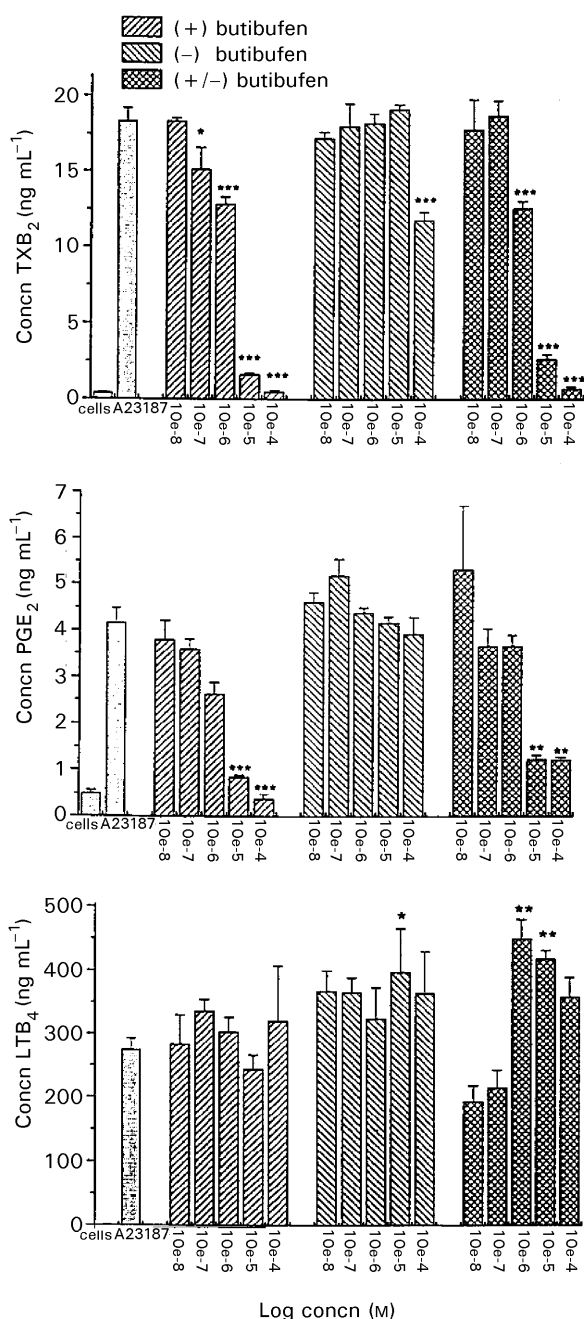
Figure 3.　Effects of the enantiomers of butibufen on eicosanoid generation by rat peritoneal leukocytes. Cells were preincubated with the indicated concentrations of butibufen for 10 min and then stimulated with 1 μM A23187 for a further 15 min. The supernatant was retained for radioimmunoassay of the three eicosanoid products TXB_2, PGE_2 and LTB_4. Results show means $\pm$ s.e.m. for three tests at each concentration, except for A23187 (n = 18). The significance of differences after drug treatment was evaluated by use of Student's unpaired t-test; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Table 1.　Profiles of inhibition by the enantiomers of butibufen and ibuprofen against the generation of prostaglandin E_2 and thromboxane B_2 (cyclooxygenase pathway) and leukotriene B_4 (5-lipoxygenase pathway).

Drug	Eicosanoid	Concentration causing 50% inhibition (μM)		
		$(+)$-S	$(-)$-R	R,S
Butibufen	Prostaglandin E_2	1·5	$\gg$100	4
Butibufen	Thromboxane B_2	1·5	$\gg$100	1·5
Butibufen	Leukotriene B_4	Inactive	Inactive	Inactive
Ibuprofen	Prostaglandin E_2	0·2	10	0·3
Ibuprofen	Thromboxane B_2	0·3	12	0·5
Ibuprofen	Leukotriene B_4	Inactive	Inactive	Inactive

Approximate micromolar IC50 values were determined on the basis of tests similar to those shown in Figure 3.

s.e.m. values), and that occasionally after butibufen treatment the yields were significantly increased above the values obtained on treatment with A23187 alone. Although in theory this might reflect "pathway diversion", i.e. as a result of substantial inhibition of the cyclooxygenase pathway, the substrate arachidonate might instead be converted into 5-lipoxygenase products, if this enzyme is unaffected by the drug. Further and more extensive studies are needed to verify this concept.

Comparative investigations, on the same tissue preparations, were performed using both the individual enantiomers and racemic ibuprofen. The data obtained indicate that S-ibuprofen is approximately five times more potent as a cyclooxygenase inhibitor than S-butibufen but is also inactive against 5-lipoxygenase (Table 1). Unexpectedly R-ibuprofen also inhibited the formation of PGE_2 and TXB_2 with a potency one fortieth that of the S enantiomer. Further chromatographic analysis of this sample on the chiral stationary phase (Chiralcel OD) indicated an enantiomeric purity of only 94·3%. Thus the presence of the S-ibuprofen impurity accounts for the observed activity.

In conclusion, this investigation has shown that, similarly to the related 2-arylpropionic acids, inhibition of cyclooxygenase by butibufen resides exclusively in the S enantiomer and confirmed the reduced potency of butibufen, compared with ibuprofen, previously observed in in-vivo models of analgesia (Nicholson 1982).

butibufen inhibited 5-lipoxygenase, showing the pathway-selectivity of these compounds. Inspection of Figure 3 indicates that the yields of LTB_4 were comparatively large and occasionally varied substantially between replicates (as shown by the

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