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In vitro metabolism studies on oxamniquine and related compounds by chiral liquid chromatography*

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Abstract: A previously developed method based on α_1-acid glycoprotein for the resolution of the enantiomers of the Pfizer antischistosomal drug oxamniquine was used to examine possible enantioselectivity in the *in vitro* microsomal hydroxylation of a metabolic precursor, UK-3883, but was found to be limited by the poor operational stability of the analytical column ("EnantioPac") employed. As an alternative approach, a "Pirkle" covalently-bonded dinitrobenzoyl leucine column was used, with simple pre-column solute derivatization to the carbamate to improve chromatographic performance. The method allowed preliminary examination of the stereochemistry of the *in vitro* biotransformation, hydroxylation of UK-3883 to oxamniquine, which yielded evidence for substrate enantioselectivity in favour of the dextrorotatory enantiomer of UK-3883.

Keywords: *Oxamniquine; enantioselective metabolism; chiral chromatography; Pirkle phases; α_1-acid glycoprotein phase.*

Introduction

The numerous possible consequences of chirality in the biotransformation of a xenobiotic entity have been well recognized for several decades, although their study has been restricted by the poor applicability of conventional methods for enantiomeric assay [1]. Since the early 1980s, the commercial availability of a number of chiral stationary phases (CSPs) and chiral mobile-phase additives for use in high-performance liquid chromatography (HPLC) has facilitated the study of enantioselective phenomena in xenobiotic metabolism. The utility of representative examples of some commercially available CSPs in a model *in vitro* transformation reaction was examined.

The enantiomeric resolution of the Pfizer antischistosomal drug, oxamniquine (**I**, Fig. 1) on a first-generation α_1-acid glycoprotein (αGP) column ("EnantioPac") has been recently reported [2]. An attempt was made to use this method for studies on possible enantioselectivity in the *in vitro* microsomal hydroxylation of a compound related to oxamniquine (UK-3883; **II**, in Fig. 1), which in such systems is hydroxylated to yield **I**.

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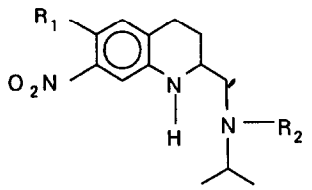

Compound	Name	R_1	R_2
I	Oxamniquine	CH_2OH	H
Ia	Ethyl carbamate	CH_2OH	$COOC_2H_5$
II	UK-3883	CH_3	H
IIa	Ethyl carbamate	CH_3	$COOC_2H_5$

Figure 1
Structures of oxamniquine, UK-3883 and the respective carbamate derivatives.

However, the EnantioPac column displayed marked fragility, severely limiting its application.

Thus an alternative approach was developed based on a "Pirkle" covalently-bonded leucine column, involving extraction of analytes from the incubation medium and simple solute derivatization to the carbamate. This allowed the preliminary investigation of the stereochemistry of the *in vitro* hydroxylation reaction, described in the present report. As part of this work, a second-generation αGP column ("Chiral-AGP"), which has recently become commercially available, was studied. This new phase has been found to be more stable than the original αGP column for metabolism studies on oxamniquine and related compounds.

Experimental

Chromatography

The modular chromatograph employed consisted of a Kratos SP400 pump (Kratos Inc., Ramsey, NJ, USA), a Rheodyne 7125 injection valve (Rheodyne, Berkeley, CA, USA), and either an LKB 2151 (LKB, Bromma, Sweden) variable wavelength detector, or a Kratos SP1000 multichannel detector at 246 nm. Peak integration, where required, was performed on a Hewlett–Packard HP 3396A (Hewlett–Packard Ltd, Avondale, PA, USA) integrator.

The columns and mobile phases used were:

(**a**) αGP: 10 cm × 4.6 mm (i.d.) (EnantioPac, LKB AB, Bromma, Sweden). Mobile phase: 10 mM sodium dihydrogen phosphate + 100 mM NaCl (pH 5.85)–propan-2-ol (99.5:0.5, v/v) at 0.3 ml min^{-1}.

(**b**) (3,5-Dinitrobenzoyl)-L-leucine, covalently bonded to silica: 25 cm × 4.9 mm (i.d.) (HiChrom Ltd, Reading, Berks). Mobile phase: hexane–propan-2-ol (90:10, v/v) at 1.0 ml min^{-1}.

(**c**) αGP immobilized on 5 μm silica particles: 10 cm × 4.0 mm (i.d.) (Chiral-AGP, ChromTech AB, Norsborg, Sweden). Mobile phase: 10 mM sodium dihydrogen phosphate (pH 6.0)–CH_3CN (99.4:0.6, v/v) at 0.9 ml min^{-1}.

Materials

Oxamniquine and UK-3883 were kindly supplied by Pfizer Central Research (Sandwich, UK). Solvents and buffer salts used were of analytical grade; eluents were passed through a 0.45-μm pore filter and degassed by ultrasound at reduced pressure before use. Ethyl chloroformate was obtained as a pure liquid, from Fluka AG Chemicals (CH-9470 Buchs, Switzerland).

Methods

The composition of the 16.0-ml incubation medium was (expressed in terms of final concentration): substrate (UK-3883), 0.02 mM; isocitrate dehydrogenase (Sigma Type IV, St. Louis, MO, USA), 640 μl/16 ml; magnesium chloride (hexahydrate, Analar grade), 5 mM; manganese chloride (tetrahydrate, Analar grade), 5 μM; isocitric acid (trisodium salt, Sigma), 5 mM; β-NADP (sodium salt, Sigma) 1.0 mM, and sufficient microsomal preparation to yield a final protein content of approximately 1 mg ml^{-1}.

Incubations were made at 37°C with dog or rat liver microsomes, prepared according to accepted methods (e.g. ref. 3). The final incubation volume was 16.0 ml; samples of 1.0 ml were taken at prescribed intervals.

Derivatization of **I** and **II** with ethyl chloroformate gave quantitative yields of the carbamate derivatives **Ia** and **Ib** within 15 min as follows: 1.0 ml incubate was basified (5 M NaOH) and extracted into ether (1.0 ml). To the supernatant at −10°C was added 200 μl ethyl chloroformate and 400 μl triethylamine (TEA; to trap HCl generated in the highly exothermic reaction). Precipitated TEA–HCl was dissolved in 1.0 ml added water, the ether supernatant aspirated, evaporated to dryness and the residue taken up in 200 μl eluent prior to injection.

The quantitative yield of the reaction was confirmed by HPLC (no residual **I** or **II**) and the absence of racemization during reaction was confirmed by performing the reaction procedure on the individual enantiomers; no trace of the product of the opposite enantiomer was detectable.

Optimization of the EnantioPac separation was achieved using the modified sequential simplex procedure as previously described [2]. For optimization on the Chiral-AGP column, a factorial design was employed followed by a modified simplex.

Results and Discussion

In most species studied, UK-3883 (**II**) is hydroxylated by hepatic microsomal (cytochrome P-450 dependent) enzymes to yield oxamniquine (**I**) [4]. Initial studies on the stereochemistry of this reaction, utilizing the first generation αGP (EnantioPac) column, provided evidence of substrate enantioselectivity, with preferential generation of the dextrorotatory (second eluting) enantiomer (Fig. 2). After incubation of **II** with rat liver microsomes for 2 h, the area of the peak corresponding to (+)-oxamniquine was approximately 1.5 times greater than that for the peak due to the laevo enantiomer. However, the fragility of the EnantioPac column made it impracticable to continue this approach in the study.

Although only poorly resolvable on the Pirkle leucine phase *per se*, the enantiomers of **I** and **II** were well resolved when chromatographed as their ethyl carbamate derivatives. Figure 3 presents a representative chromatogram depicting the resolution of the enantiomers of **IIa** on the covalent leucine phase, after extraction of **II** from a dog liver microsomal incubate and subsequent derivatization.

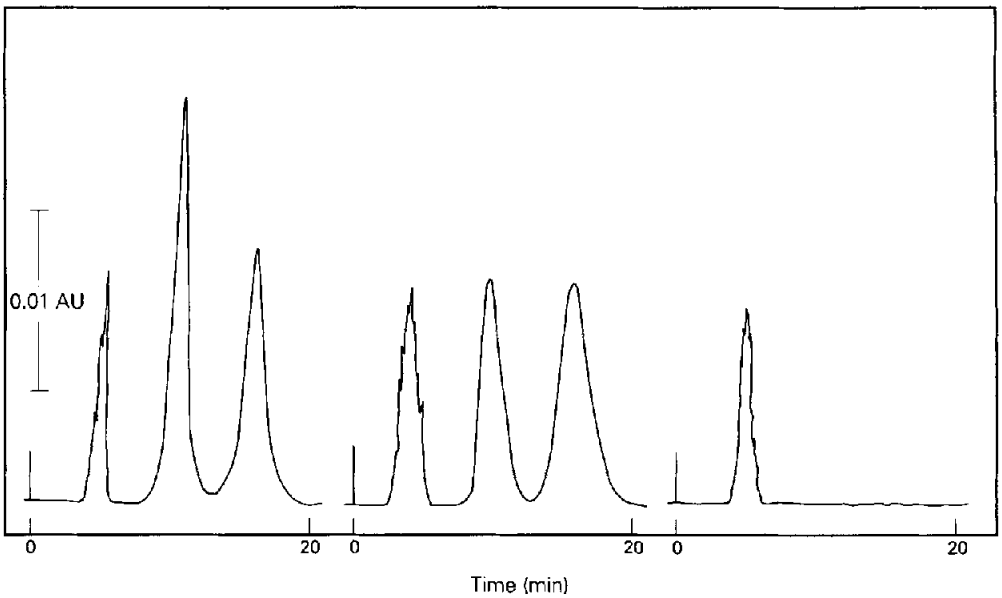

Figure 2
Chromatograms obtained from extracts of incubation medium of **II** with rat liver microsomes, showing differential generation of oxamniquine enantiomers. (a) Blank incubate, spiked with 50 ng ml^{-1} racemic oxamniquine. (b) After 2 h incubation of **II**. (c) Control. For chromatographic conditions, see text.

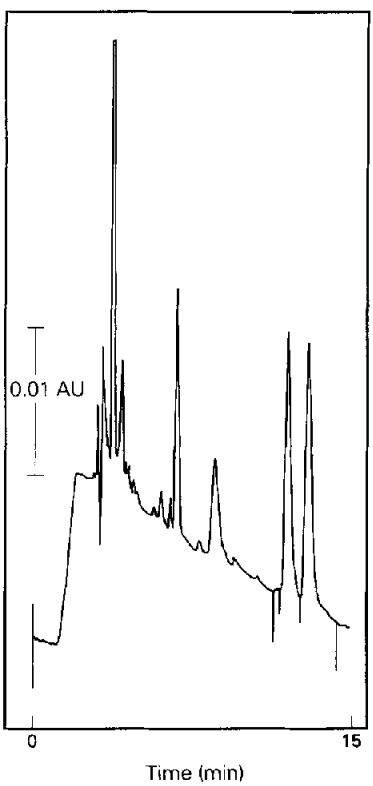

Figure 3
Chromatogram of the separation of the enantiomers of **IIa** on the Pirkle leucine phase, in a typical dog liver microsomal incubation extract. For chromatographic conditions, see text.

The detector response to **IIa** in standard solutions was linear in the examined range of 10 ng to 10 μg injected on-column (r = 0.9986, n = 7). The recovery of **II** from the incubation medium was 93%, and the relative standard deviation in the recovery method was 6.3% (n = 3). Thus the depletion of the enantiomers of **II**, during incubation with dog liver microsomes, could be readily followed after suitable extraction and derivatization (Fig. 4). The results of these incubations confirm that the mixed-function oxidase enzymes responsible for the hydroxylation of **II** exhibit a substrate enantioselectivity for the dextrorotatory antipode. This leads to a clearance rate approximately 1.5 times greater for the preferred enantiomer than for the other isomer.

Studies on further compounds related to **I**, being variously substituted at R_2 (Fig. 1) and the ring-nitrogen atom, yielded evidence that the chromatographic enantioselectivity observed on the leucine phase could be related to the attractive interaction between the analyte ring-nitrogen atom and a suitable acceptor group on the immobilized chiral ligand. Evidence for this is provided in the observation that loss of this interaction, as occurs when the two nitrogen atoms in **I** or **II** are linked to form a third ring, leads to concomitant loss of chiral recognition. The use of mobile phase modifiers other than aliphatic alcohols (which may behave as both hydrogen bond donors and acceptors), viz. chloromethane (which does not undergo hydrogen bonding, but has a strong dipole moment), diethyl ether (purely a hydrogen bond acceptor) and chloroform (a hydrogen bond donor), confirmed that an interaction in which the substrate was behaving as a hydrogen bond donor was crucial for chiral discrimination in this series of compounds.

The recently developed second-generation αGP column promises much for studies on chiral drug metabolism. Early experience has confirmed that this column has good long-term stability, high selectivity and high separation speed (T. A. G. Noctor, A. F. Fell and B. Kaye, paper in preparation). In the present studies, the Authors have found that oxamniquine enantiomers are well resolved, very quickly, with no requirement for prior derivatization. Work is in progress to determine the utility of this phase in continuing metabolism studies on oxamniquine, of the type described here.

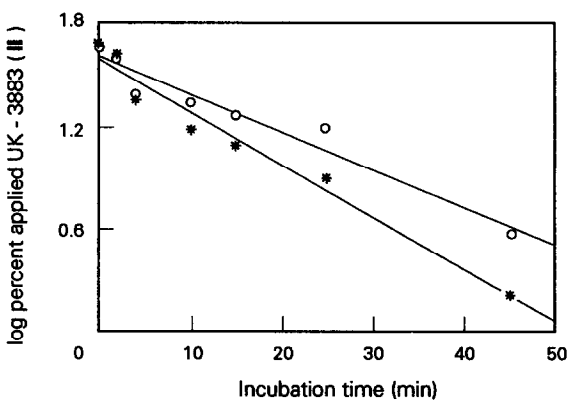

Figure 4
Relationship between log percent applied UK-3883 (**II**) and incubation time with dog liver microsomes. The slope of the plots is a measure of the intrinsic clearance of the enantiomeric substrates. ○, Laevorotatory enantiomer: regression equation $y = -2.21.10^{-2}x + 1.62$, r = 0.9672. *, Dextrorotatory enantiomer: regression equation $y = -3.23.10^{-2}x + 1.60$, r = 0.9850.

Conclusions

The first-generation αGP column proved to give good chiral resolution of **I** and of **II**, without requiring solute derivatization, but its poor stability performance greatly diminished its practical usefulness for *in vitro* metabolism studies.

Despite the requirement for pre-column solute derivatization, the use of a Pirkle covalent leucine column was found to be more appropriate, due to its greater long-term stability. The employment of this phase allowed the demonstration of substrate enantioselectivity for the dextrorotatory isomer of **II**, in its hepatic-microsomal hydroxylation to oxamniquine (**I**). Early experience with the second-generation αGP column has confirmed that when the phase is used routinely for filtered solvent extracts from samples of biological origin, with or without a Chiral-AGP pre-column, chiral drug metabolism studies can be greatly facilitated. The development of coupled-column systems combining chiral and achiral columns in HPLC, should prove to be of further benefit in bioanalytical studies of this kind [5], in rendering sample pretreatment less necessary, and improving method sensitivity by peak compression prior to detection.

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