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Short Communication

High-performance liquid chromatographic method for the enantiomeric separation of the chiral metabolites of midazolam

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ABSTRACT

The enantiomeric resolution of the chiral metabolites of midazolam, 4-hydroxymidazolam and 1,4-dihydroxymidazolam, by high-performance liquid chromatography on a chiral stationary phase containing immobilized ovomucoid is described (no derivatization was necessary). Large separation factors (up to $\alpha = 2.9$) were obtained by varying the type and amount of organic modifier in the mobile phase. The system so far developed is recommended for monitoring possible differences in the stereoselective metabolism of midazolam.

INTRODUCTION

Different bioactive properties of enantiomers of pharmaceutical racemates have often been outlined [1]. As a consequence, pharmacological and pharmacokinetic studies of a new drug are nowadays carried out only on single enantiomers. Whereas much attention has been focused on the different properties of chiral drugs, less attention has been paid to the stereoselective properties of chiral metabolites, especially when the drug itself is achiral.

The achiral short-acting benzodiazepine midazolam is commonly used as preanaesthetic medication and in the induction and maintenance of anaesthesia [2,3]. The metabolic pathway of midazolam is shown in Fig. 1. The main metabolic route of the drug in humans is the hepatic formation of the achiral metabolite 1-hydroxymethylmidazolam (1-OH) [4], which is further conjugated to form the inactive 1-OH-glucuronide. From the stereochemical point of view, the metabolic pathways, which include the formation of 4-hydroxymidazolam (4-OH) and 1,4-dihydroxymidazolam (1,4-diOH), are of interest. These metabolites contain an asymmetric centre, and therefore some additional pharmacological routes are theoretically possible, especially in different disease states.

Analytical assays for midazolam and its metabo-

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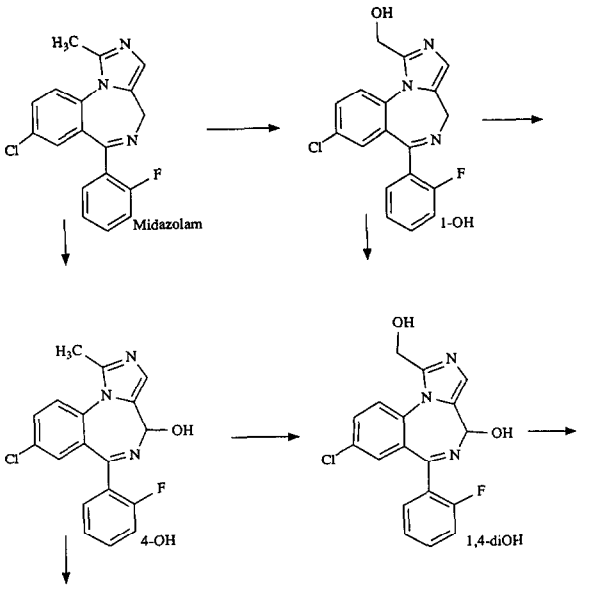

Fig. 1. Structures of midazolam and its main metabolites, 1-hydroxymidazolam, 4-hydroxymidazolam and 1,4-dihydroxy midazolam.

lites include gas chromatography [5–12] and high-performance liquid chromatography (HPLC) [7,11,13–17]. No cited approach considers stereochemical aspects.

For monitoring possible differences in the stereoselective metabolism it is necessary to develop analytical methods, that allow the enantiomeric separation of 4-OH and 1,4-diOH. The aim of this publication is to show the enantiomeric separation of 4-OH and 1,4-diOH using HPLC with a chiral stationary phase.

EXPERIMENTAL

Materials

4-OH and 1,4-diOH were kindly provided by Hoffmann-La Roche (Basle, Switzerland). Acetonitrile and ethanol were of analytical grade.

Apparatus

A Kontron HPLC pump (Model 420) was used in conjunction with a variable-wavelength Kontron UV detector (Model 430). The separation was performed on an ovomucoid-based HPLC column (Ultron ES-OVM, 5 μm particle size with 100-Å pores, 15 × 0.46 cm I.D.; Shinwa Chemical Industries, Kyoto, Japan). Mobile phase and chromatographic parameters are given in the legends of Figs. 2 and 3.

RESULTS AND DISCUSSION

The present investigations were made with a column containing immobilized ovomucoid. Its use for enantiomeric separation has been reported by Miwa *et al.* [18,19]. The advantages of this ovomucoid column for chiral HPLC separations include good

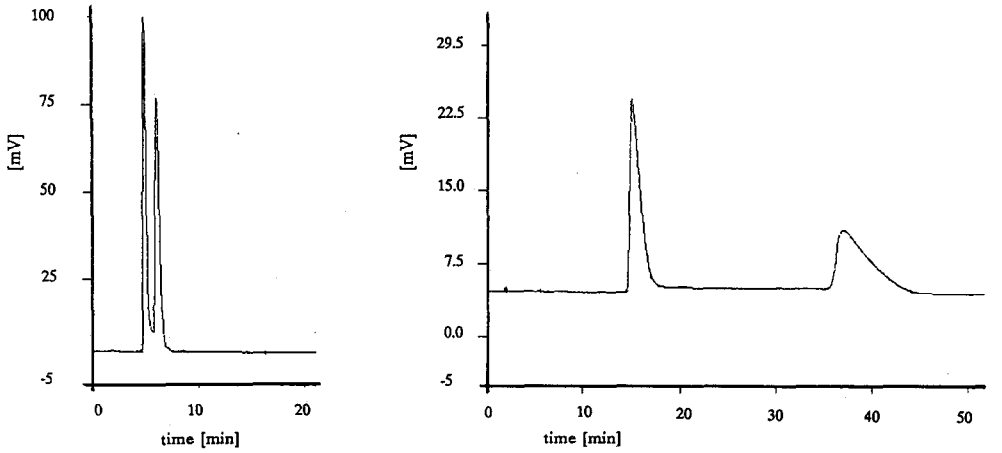

Fig. 2. Enantiomeric separation of 4-hydroxymidazolam. Mobile phase: 20 m*M* ammoniumphosphate buffer with 20% acetonitrile (left curve) and 10% acetonitrile (right curve), with phosphoric acid adjusted to pH 7. Flow-rate: 1.0 ml/min. UV detection: 220 nm. Injected volume: 20 μl (containing 2 μg of metabolites).

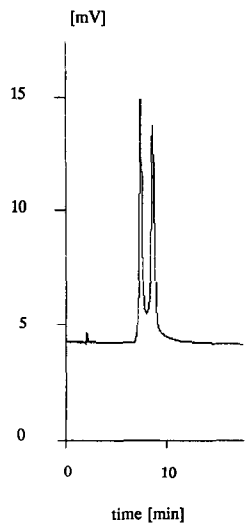

Fig. 3. Enantiomeric separation of 1,4-dihydroxymidazolam. Mobile phase: 10% acetonitrile with phosphoric acid adjusted to pH 7; other parameters as in Fig. 2.

stability to changes in temperature, pH, organic solvent composition and antitrypsin activity [20]. Systematic procedures have recently been developed by Kirkland *et al.* [21] and Wada *et al.* [22].

The aim of the present study was to achieve baseline separations of the enantiomers of both 4-OH and 1,4-diOH with capacity factors below 15 for all four components in a single run. A buffer concentration of 20 m*M* was used because lower ionic strengths are expected to give higher k'-values [22]. The pH value was maintained at 7 and the temperature at 25°C. Changes were made only with respect to the type and amount of uncharged modifier. The influence of organic modifier is shown in Table I for the enantiomeric separation of 4-OH. The chromatograms are displayed in Fig. 2 (for 4-OH) and Fig. 3 (for 1,4-diOH). As expected from an earlier work by Kirkland *et al.* [21], large decreases in α and k'-values were observed with increasing amounts of acetonitrile and ethanol. The retention times and the separation factor obtained with ethanol are significantly larger than those obtained with acetonitrile. Not having the pure enantiomers as a reference, the separations were confirmed only with diode-array detection. Owing to its less hydrophobic character, the retention of 1,4-diOH is lower than that of 4-OH.

For a simultaneous determination of the enantiomers of 4-OH and 1,4-diOH, 10% acetonitrile should be chosen as organic modifier. With this chromatographic system studies involving stereoselective pharmacokinetics and/or metabolism could be achieved.

TABLE I

INFLUENCE OF ORGANIC MODIFIER ON k' AND SEPARATION FACTOR (α)

Modifier	k_1	k_2	α
4-Hydroxymidazolam			
10% Ethanol	No separation within 2 h		
10% Acetonitrile	7.8	19.3	2.5
20% Ethanol	3.8	11.1	2.9
20% Acetonitrile	2.6	3.3	1.2
1,4-Dihydroxymidazolam			
10% Acetonitrile	3.6	4.2	1.2

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