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Short communication

Stereospecific high-performance liquid chromatographic assay of zopiclone in human plasma

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Abstract

A high-performance liquid chromatographic (HPLC) assay for the analysis of the enantiomers of zopiclone (ZPC), a cyclopyrrolone hypnotic, in plasma was developed. Following the addition of chlordiazepoxide as internal standard (I.S.), plasma containing the ZPC enantiomers and I.S. was extracted by liquid–liquid extraction at an alkaline pH. After evaporation of the organic layer, the drug and I.S. were reconstituted in ethanol–hexane (80:20, v/v) and injected onto the HPLC column. The enantiomers were separated at ambient temperature on a 25-cm Chiralcel OD-H column with ethanol–hexane (60:40, v/v) as the mobile phase pumped at a flow-rate of 0.6 ml/min. The enantiomers of ZPC were quantified by fluorescence detection with excitation and emission wavelengths of 300 and 470 nm, respectively. The assay described allows for the direct quantitation of ZPC without pre-column derivatization, and is suitable for clinical studies of ZPC in humans after administration of therapeutic doses.

1. Introduction

Zopiclone [ZPC, (±)-6-(5-chloro-2-pyridyl)-7-(4-methyl-1-piperazinyl)-carbonyloxy-6,7-dihydro[5H]pyrrole[3,4-b]pyrazin-5-on, Fig. 1] is a hypnotic which is a member of the cyclopyrrolone class and is marketed as the racemate. Although the chemical and pharmacologic properties of ZPC enantiomers are not well known, receptor binding studies have suggested that (+)-ZPC has a 50-fold greater affinity to benzodiazepine receptors than (−)-ZPC [1]. The enantiomeric differences in benzodiazepine receptor affinity are likely to be important, as the mechanism of ZPC activity is related to this receptor [1]. As the receptor affinity of the enantiomers of ZPC differs, analytical proce-

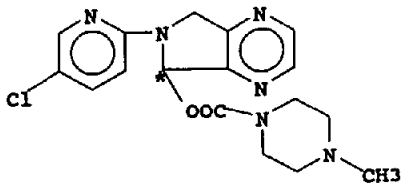

Fig. 1. Structure of zopiclone (ZPC). The asterisk denotes the chiral center.

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dures measuring concentrations in biological samples should be stereospecific.

To date, several analytical methods for the quantitation of ZPC have been reported in the literature. These techniques have utilized gas chromatography [2,3] and high-performance liquid chromatography [4–8]. When assaying for ZPC, it has been suggested [7] that it is necessary to measure the N-oxide and the desmethyl ZPC metabolites, which are active and inactive, respectively. However, these metabolites were not detectable in plasma after administration of clinical doses of ZPC [4,5]. The other known metabolites are formed via carbamate hydrolysis and are inactive [9]. As the known metabolites in plasma do not contribute to the hypnotic activity of the drug, it is more important to develop analytical methods which are free from interferences and which will delineate the time-course of ZPC after administration of the racemate when conducting clinical studies rather than measure drug and metabolite plasma concentrations non-stereospecifically. To date, however, there have only been two reports on the stereospecific analysis of ZPC [1,10]. Although the method reported by Fernandez *et al.* [10] utilized a cellulose carbamate column, the column was not commercially available. Furthermore, it was necessary to first separate endogenous constituents from the plasma samples containing ZPC on an achiral silica column and collect the eluent containing the ZPC enantiomers. This eluent was then chromatographed on a chiral column. Recently, Blaschke *et al.* [1] reported the use of either a polymethacryloyl-1*R*,2*S*-norephedrine or an Ultron ES ovomucoid stationary phase. However, data supporting the analytical validity of these two stationary phases was not presented, as the assay was not applied to clinical studies.

It is well recognized that for the determination of the individual enantiomers of drugs administered as racemates stereospecific methods must be used [11]. In our laboratory, we have generally utilized pre-column derivatization with a homochiral reagent to separate chiral xenobiotics [12–16]. This approach was initially attempted for ZPC but was not successful, as ZPC does not possess a suitable functional group for derivatization. In a preliminary attempt to create a reactive functional group for pre-column derivatization, the carbamate moiety was hydrolyzed followed by derivatization with optically pure reagents including, for example, various alkyl chloroformates. Interestingly, although hydrolysis of the carbamate has been reported for ZPC [17], under the experimental conditions used by us the carbamate did not appear to be successfully hydrolyzed. More importantly, however, was that chemical hydrolysis of the carbamate prior to derivatization yields the same product as one of the major metabolites [9]. Hence, overestimation of the formation of these metabolic products via this approach is likely.

In this paper, we report the separation of ZPC enantiomers using a commercially available chiral column. The method reported here does not require any pretreatment or achiral chromatography prior to chiral column chromatography.

2. Experimental

2.1. Chemicals

Zopiclone (ZPC) and chlordiazepoxide HCl (internal standard) were obtained as gifts from Rhône Poulenc Canada (Montreal, Canada) and Hoffmann-La Roche (Montreal, Que., Canada), respectively. Monopotassium phosphate and disodium phosphate, as well as all HPLC grade solvents including hexane, water, methyl-*tert.*-butyl-ether (MTBE), and iso-octane (2,2,4-trimethylpentane) were obtained from BDH chemicals (Toronto, Ont., Canada). HPLC grade ethanol and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA).

2.2. Chromatography

The HPLC apparatus consisted of a multisolvent delivery system Model 600E system controller attached to a Model 717 autosampler, a Model 470 scanning fluorescence detector, and a NEC Powermate 486/33i computer with Millenium 2010 chromatography manager software (Waters Scientific, Mississauga, Ont., Canada). Separation of the enantiomers was accomplished

at ambient temperature using a 25 × 0.46 cm I.D. Chiralcel OD-H chiral column 5 μm particle size, supplied by Daicel Chemical Industries (Exton, PA, USA). Detection of the enantiomers was performed at wavelengths of 300 and 470 nm for excitation and emission, respectively, with detector gain set at 1000 times. The mobile phase consisted of ethanol–hexane (60:40, v/v), pumped at a flow-rate of 0.6 ml/min.

2.3. Standard solutions

A stock solution made from 10 mg racemic ZPC (1 mg/10 ml) was prepared in acetonitrile. The I.S. solution (chlordiazepoxide-HCl, 1 mg/10 ml) was dissolved in water. Both solutions were stored at 4°C.

2.4. Sample preparation

Racemic ZPC was added to 1.0 ml drug-free plasma in glass tubes to give final concentrations of 0, 2.5, 5.0, 10.0, 25.0, 50.0, and 75.0 ng/ml of each enantiomer. To each tube 50 μl of I.S. solution and 100 μl of 0.07 *M* phosphate buffer (pH 8) were added, followed by the addition of 5 ml of MTBE–iso-octane (75:25, v/v). The resultant mixture was vortex-mixed for 30 s using a Vortex Genie 2 mixer (Fisher Scientific, Edmonton, Alb., Canada) and centrifuged for 5 min at 1800 *g* using a Dynac II centrifuge (Becton Dickinson, Parsippany, NJ, USA). The organic layer was transferred to a clean tube and evaporated to dryness using a Savant Speed Vac concentrator-evaporator (Fisher Scientific, Edmonton, Alb., Canada). The residue was reconstituted in 75 μl of ethanol–hexane (80:20, v/v). Aliquots of 50 μl were injected onto the HPLC system. Both sample preparation and analysis were conducted at ambient temperature (25°C).

2.5. Quantitation

Calibration curves were obtained by plotting the peak-area ratios (ZPC/I.S.) after extraction and analysis *versus* their corresponding added plasma concentrations. Results are reported as mean ± standard deviation (S.D.).

2.6. Accuracy and precision

Drug-free plasma was spiked with racemic ZPC at seven different concentrations corresponding to enantiomer concentrations ranging from 2.5 to 75.0 ng/ml ($n = 9$ for each concentration). Accuracy, expressed as the percent analytical recovery (% A.R.) was measured by determining the concentration of drug measured in each sample relative to the known amount of each enantiomer added. Precision, expressed as percent coefficient of variation (%C.V.) was calculated from the back-calculated concentrations generated using the regression curves from each calibration curve ($n = 9$).

2.7. Extraction efficiency

Extraction efficiency was calculated by adding known concentrations of ZPC to drug-free plasma and comparing them to unextracted samples by using equivalent volumes of standard solutions. The extraction was performed as described above without the addition of I.S. All samples were evaporated and reconstituted in 75 μl of ethanol–hexane (80:20, v/v). For this study the average of 5 extracted plasma samples was compared to the average of 5 unextracted standard samples at 2.5 and 25 ng/ml.

3. Results and discussion

Separation of the enantiomers of ZPC was accomplished using a commercially available Chiracel OD column. Interestingly, initial attempts to find a suitable internal standard were less than satisfactory, as the peak corresponding to the I.S. was not sufficiently separated from the solvent front. These initial results were obtained using a Chiracel OD (10 μm particle size) column. None of a number of different I.S. tested, several of which were benzodiazepines, proved satisfactory. However, by substituting a Chiracel OD-H column (high efficiency) for the OD column, satisfactory results were obtained (*i.e.* improved separation of the analytes with similar run-times). Both columns only differ in

their particle size (5 *versus* 10 μm for the OD-H and OD columns, respectively). Fig. 2 shows representative chromatograms obtained from blank plasma, spiked plasma (5 ng/ml of each enantiomer of ZPC, to illustrate clinical utility), and a 10-h plasma sample obtained from a healthy volunteer receiving a single 7.5-mg oral dose of ZPC. The peaks corresponding to ZPC-1 and ZPC-2 enantiomers eluted at 19 and 28 min, respectively. Although the exact order of elution of the enantiomers was not precisely determined using pure enantiomers, it may be suggested that the first- and second-eluting peaks of ZPC corresponded to the (−)- and (+)-enantiomers, respectively. The order of elution of the ZPC peaks was determined by comparing the data obtained with the Chiracel OD-H to that reported by Fernandez *et al.* [10] using a cellulose carbamate column and by comparing the enantiomer concentration–time curve data previously obtained with that obtained in the present study. The ZPC enantiomer concentration–time profile in plasma confirmed that the enantiomer with the higher plasma concentrations [(+)-ZPC] was the second enantiomer peak to elute on the chromatograms. The resolution (R_s) and selectivity (α) for the peaks corresponding to the ZPC enantiomers were 4.5 and 1.7, respectively. The I.S. (chlordiazepoxide) peak eluted at 9.5 min.

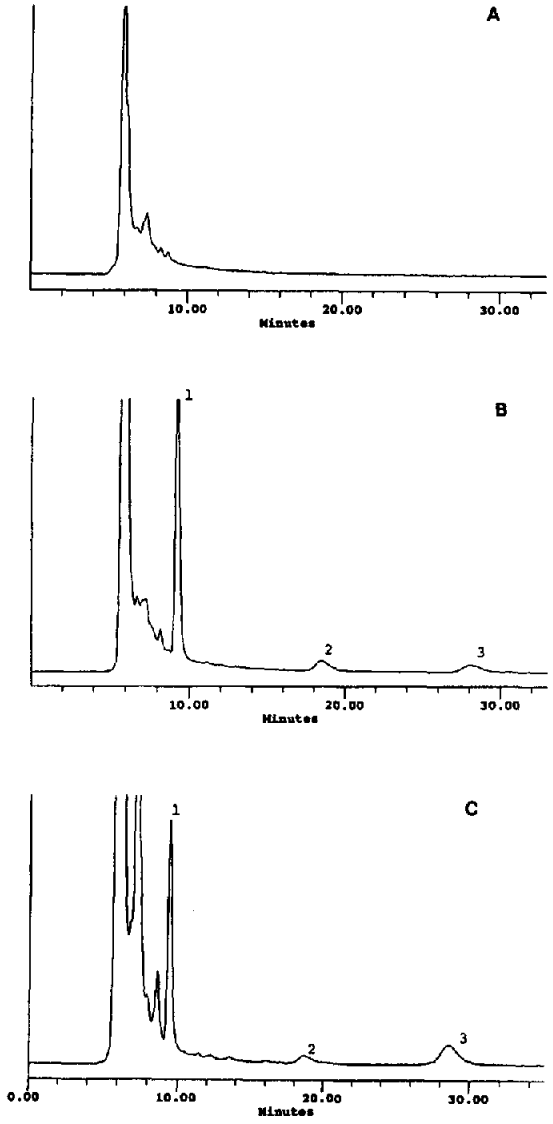

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked with 5 ng/ml of each ZPC enantiomer and 5 μg/ml of I.S., and (C) plasma sample taken 10.0 h after an oral 7.5 mg dose of racemic ZPC; ZPC-1 and ZPC-2 corresponded to 4.85 ng/ml and 11.61 ng/ml, respectively. Peaks: 1 = I.S.; 2 = ZPC-1; 3 = ZPC-2.

Table 1 summarizes the accuracy and precision of the method. Although the lowest calibration concentration examined was 2.5 ng/ml, the lowest concentration that could be detected was approximately 1 ng/ml. However, the day-to-day accuracy and precision at 1 ng/ml were not acceptable at this concentration. Nevertheless, the accuracy and precision were judged to be acceptable over the entire concentration range examined. The mean extraction yields were 91.1% and 95.1% at 2.5 and 25 ng/ml, respectively when extracted *versus* non-extracted samples were compared. Excessive alkalinization was not warranted as inadvertent hydrolysis of the carbamate could result in overestimation of ZPC clearance via metabolic routes. Although the total run-time per sample was 33 min., there was significant variation in run-times if column temperature was altered. In fact, if column temperature dropped below ambient (25°C), run-times were considerably longer. Thus temperature was kept at 25°C for all assays.

Fig. 3 shows the plasma enantiomer concentration–time profile obtained for a healthy vol-

Table 1
Accuracy and precision of method ($n = 9$)

Enantiomer concentration added (ng/ml)	Measured concentration (mean ± S.D.) (ng/ml)		Accuracy (A.R., %)		Precision (C.V., %)	
	ZOP-1	ZOP-2	ZOP-1	ZOP-2	ZOP-1	ZOP-2
2.5	2.70 ± 0.2	3.00 ± 0.5	107.8	119.8	8.03	16.2
5	5.24 ± 0.9	5.16 ± 0.6	104.8	103.2	16.8	12.0
10	9.47 ± 0.9	9.80 ± 0.9	94.7	98.0	10.0	8.81
25	25.4 ± 0.9	24.9 ± 1.0	101.5	99.6	3.55	3.96
50	49.4 ± 2.4	48.8 ± 2.7	98.8	97.7	4.81	5.45
75	75.3 ± 1.6	75.8 ± 1.7	100.5	101.1	2.13	2.26

unteer receiving 7.5 mg oral ZPC as a single dose. Although this is only a preliminary investigation of one subject, the concentration–time profile of ZPC enantiomers suggests that, as previously reported [10], the disposition of ZPC is stereoselective; ZPC-2 concentrations exceed those of ZPC-1.

In conclusion, a sensitive, convenient stereospecific assay for ZPC was developed using a commercially available chiral column. The need for pre-column derivatization with a homochiral reagent was obviated, as was the need to couple achiral chromatography to chiral chromatography. The assay is valid for the determination of the enantiomers of ZPC with minimal sample preparation, and thus the method can be used for clinical studies.

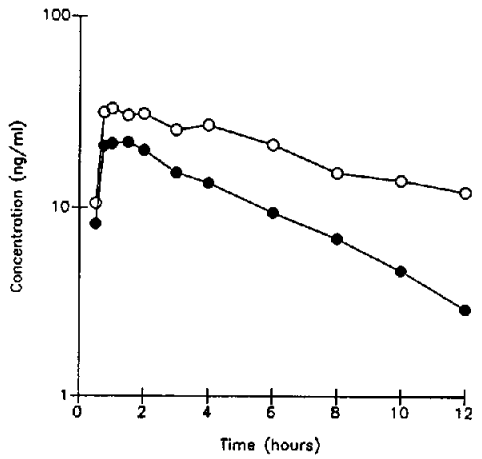

Fig. 3. Plasma concentration–time profile of ZPC-1 (●) and ZPC-2 (○) in a healthy volunteer following a single 7.5-mg oral racemic dose of ZPC.

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