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Journal of Chromatography B, 705 (1998) 149–153

JOURNAL OF CHROMATOGRAPHY B

Short communication

High-performance liquid chromatographic determination of new 2,3-benzodiazepines

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Received 2 June 1997; received in revised form 15 August 1997; accepted 8 September 1997

Abstract

A simple high-performance liquid chromatographic assay with ultraviolet detection at 254 nm for simultaneous determination of 2,3-benzodiazepine derivatives (2,3-BZ2 and 2,3-BZ2Me) and their metabolites in rat plasma is described. The procedure involves a fast extraction of the drugs from the buffered sample using methanol. The extract is evaporated to dryness at 45°C and the residue is redissolved in methanol (twice). A 20-μl aliquot is injected into the liquid chromatograph and eluted with methanol–water (65:35, v/v) on a C_{18} reversed-phase column. At a flow-rate of 1.5 ml/min the detection time was 3.1 min for 2,3-BZ2, 5.06 min for 2,3-BZ2Me and 10.9 min for prazepam, used as internal standard for the quantification of the studied compounds. The method has been used to investigate the steady-state concentrations of two 2,3-benzodiazepine derivatives in Sprague–Dawley rat plasma. © 1998 Elsevier Science B.V.

Keywords: 2,3-Benzodiazepines; AMPA/kainate antagonist

1. Introduction

GYKI 52466 is a 2,3-benzodiazepine derivative which differs pharmacologically from classical 1,4- and 1,5-benzodiazepines in that it possesses muscle relaxant and anticonvulsant properties acting as a highly selective non-competitive antagonist at excitatory amino acid (EAA) receptor α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and shows no affinity for the benzodiazepine receptors (BZRs) [1,2].

Recently, new series of 2,3-benzodiazepines (2,3-BZs), chemically similar to GYKI 52466, were synthesized in our laboratories and proved to possess anticonvulsant activity in various models of seizures [3,4]. It has been demonstrated that these 2,3-BZ derivatives are non-competitive antagonists at the AMPA/kainate receptor and do not affect NMDA and GABA receptor-mediated responses [4]. In particular, the 2,3-benzodiazepines 2,3-BZ2 and 2,3-BZ2Me (Fig. 1) have marked anticonvulsant activity comparable to that of GYKI 52466 and their effects are longer lasting than those of GYKI 52466. The time-course of anticonvulsant effects of 2,3-BZ2 and

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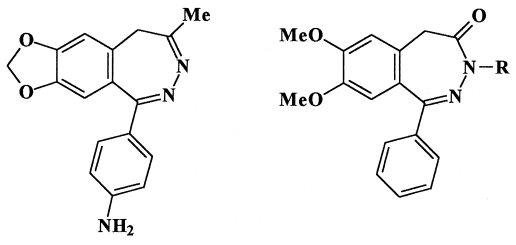
0378-4347/98/$19.00
PII S0378-4347(97)00463-5



GYKI 52466

2,3-BZ2 R = H
2,3-BZ2Me R = Me
2,3-BZ2Ac R = COMe

Fig. 1. Structures of GYKI 52466 and 2,3-BZs.

2,3-BZ2Me suggested that the N-substituted derivative underwent a metabolite activation in vivo and biotransformation in the parent compound 2,3-BZ2 by loss of the N-3 substituent.

In order to demonstrate this hypothesis, in the present study we have developed an assay suitable for pharmacokinetic studies of the 2,3-BZ derivatives and for their simultaneous determination in rat plasma. A high-performance liquid chromatographic (HPLC) method has been developed to detect the 2,3-BZ derivatives and their detectable metabolites and, to determine the time-profile of plasma concentrations of 2,3-BZ2 and 2,3-BZ2Me compounds administered intraperitoneally (i.p.) to Sprague–Dawley rats at a dose of 10 mg/kg. Our goal was to develop a sensitive method to detect drug concentrations of the order of nanograms in only 0.5 ml of plasma.

2. Experimental

2.1. Chemicals and standards

Methanol and water HPLC grade (Carlo Erba, Milan, Italy) were used. Compounds 2,3-BZ2, 2,3-BZ2Me and 2,3-BZ2Ac (Fig. 1) were synthesized in our laboratories. Prazepam used as internal standard (I.S.), was obtained from Parke Davis (Milan, Italy) and prepared at a concentration of 0.2 μg/ml. Stock solutions (1 mg/ml) of 2,3-BZs were prepared in methanol. Work solutions were made by dilution with methanol and used to prepare plasma and aqueous standards.

2.2. Chromatography

The HPLC system consisted of a Beckman, System Gold 125 solvent module with a 20 μl loop injection valve, a variable-wavelength ultraviolet 166 Detector set at 254 nm and a Epson Endeavoir 4DX2/50 L integrator. A Beckman Ultrasfere ODS (250×4.6 mm I.D.) reversed-phase column was used with an ODS guard (45×4.6 mm). The mobile phase was methanol–water (65:35, v/v) at a flow-rate of 1.5 ml/min. The separations were performed at room temperature. A Millex G.V. Millipore filter (0.22 μm) with a syringe kit were used to filter the methanolic solution of sample extracts.

2.3. Drug administration

The 2,3-BZ derivatives, 2,3-BZ2 and 2,3-BZ2Me, were dissolved in a solution containing 50% dimethylsulphoxide and 50% sterile saline, and administered intraperitoneally to Sprague–Dawley rats (10 mg/kg of body mass). Blood samples were withdrawn from the animals after 15, 30, 45, 60, 90 and 120 min after i.p. administration of drugs. Blood cells were removed by centrifugation and separated plasma was stored at −20°C until assay.

2.4. Extraction

An aliquot of 0.5 ml of plasma was mixed with 0.5 ml of the phosphate buffer (pH 7.0) in a 15 ml centrifuge tube. After vortexing for 5 s, 2 ml of I.S. solution was added and the contents vortexed for 1 min and centrifuged (4000 *g*) for 10 min. The supernatant was carefully decanted, transferred into a 7 ml centrifuge tube and evaporated to dryness at 45°C under a stream of nitrogen. The residue was dissolved in 1 ml of methanol and filtered. The filtrate was evaporated and again taken up with 500 μl of methanol, filtered and evaporated. The residue was dissolved in 100 μl of methanol and an aliquot

of 20 μl was injected into the chromatographic system.

2.5. Mass spectra

Mass spectra were recorded on a Hewlett-Packard, mod.A GC–MS system (Cernusco S/N, Milan, Italy).

3. Results and discussion

3.1. Quantitation and calibration curves

2,3-BZ2 and 2,3-BZ2Me (0.5 μg) and prazepam (0.4 μg) as I.S., were injected into column and were identified from their relative retention times. Calibration curves were obtained by plotting the peak area ratio of the drugs to the I.S. against the known concentration of 2,3-BZs added to drug-free rat plasma. The curves were constructed from four replicate measurements of four concentrations of each compound over the range, from 62.5 to 500 ng/ml. Plasma samples containing a known amount of 2,3-BZ2 and 2,3-BZ2Me and I.S. were prepared and stored frozen at −20°C until use. These samples were utilized as quality control specimens.

3.2. Linearity

A linear response was observed over the examined concentration range (62.5–500 ng/ml). The regression coefficient was 0.998 for 2,3-BZ2 ($y=8.073x+0.003$; slope standard error: 0.085; $n=16$) and 0.999 for 2,3-BZ2Me ($y=4.168x-0.026$; slope standard error: 0.047; $n=16$).

3.3. Detection and sensitivity

The drugs and I.S. were detected at 254 nm, the lower limit of detection (signal-to-noise ratio of 3) being 6.5 ng/ml for 2,3-BZ2 and 8.0 ng/ml for 2,3-BZ2Me. The sensitivity of the method allowed an easy quantitation of 20 ng/ml of these drugs in a 0.5 ml plasma sample.

3.4. Precision and accuracy

Within-run precision was established on two drug-free plasma pools. Compounds 2,3-BZ2 and 2,3-BZ2Me were added at two different concentrations (0.1 and 0.5 μg/ml). Similarly day-to-day precision was established at 0.1 μg/ml of concentration for these drugs, and analyzed every week for a period of two months. The results of both studies are shown in Table 1.

The relative recovery from plasma of these drugs and I.S. was evaluated to test the efficiency and reproducibility of the extraction procedure. The two compounds were added to drug-free plasma to achieve concentrations of 0.1, 0.2 and 0.5 μg/ml. The recovery ranged from 96.4 to 103.8% and was independent of the concentration, as reported in Table 2.

3.5. Application to pharmacokinetic study

The present method has been applied in the pharmacokinetic study of 2,3-BZ2 and 2,3-BZ2Me after i.p. administration to Sprague–Dawley rats (10 mg/kg of body mass).

Fig. 2 shows chromatograms from (A) a plasma standard spiked with a mixture of 2,3-BZ2 (0.5 μg/ml), 2,3-BZ2Me (0.5 μg/ml) and prazepam; (B) a rat plasma sample obtained 15 min after administration of 2,3-BZ2 and spiked with I.S.; (C) a rat

Table 1
Precision of the HPLC assay ($n=7$)

Compound	Concentration added (μg/ml)	Concentration found (μg/ml)[b]	C.V.[a] (%)
2,3-BZ2			
Within-run	0.1	0.094±0.002	2.1
	0.5	0.460±0.019	4.1
Day-to-day	0.1	0.092±0.004	5.4
2,3-BZ2Me			
Within-run	0.1	0.103±0.004	3.9
	0.5	0.519±0.019	3.6
Day-to-day	0.1	0.099±0.006	6.0

[a] C.V.=Coefficient of variation.
[b] Mean±S.D.

Table 2
Relative recoveries of the two 2,3-benzodiazepines from plasma (n=5)

Compound	Concentration added (μg/ml)	Recovery (%)	C.V.[a] (%)
2,3-BZ2	0.1	96.4	1.9
	0.2	101.5	3.1
	0.5	98.5	2.3
2,3-BZ2Me	0.1	102	3.9
	0.2	98.6	3.2
	0.5	103.8	2.1

[a] C.V.=Coefficient of variation.

plasma sample obtained 15 min after administration of 2,3-BZ2Me and spiked with I.S. In the chromatogram C it could be observed also the peak corresponding to 2,3-BZ2, thus suggesting that 2,3-BZ2Me was partially converted in vivo into 2,3-BZ2. In plasma samples of both compounds an identic metabolite is present, identified as N-acetyl derivate. Indeed, 2,3-BZ2Ac (Fig. 1) synthesized in our laboratories by comparison, gave a peak with the same retention time of 4.2 min; 2,3-BZAc was shown to be lacking in anticonvulsant activity [4], and we did not examine this compound further in the present study. In addition, fractions 1 and 2 (Fig. 2A) from HPLC analysis of methanolic extracts of rat plasma spiked with 2,3-BZ2 and 2,3- BZ2Me were collected and studied by mass spectrometry (MS). The mass spectra showed that molecular ions were present at a m/z value of 296 in the fraction 1 and at a m/z value of 310 in the fraction 2, due to 2,3-BZ2 and 2,3-BZ2Me, respectively. The fragmentation was consistent with the proposed structures and with a chromatographic data. The loss of a methyl radical (m/z 295) was also demonstrated by MS. We collected a fraction (1; Fig. 2C) after HPLC analysis of samples from plasma of animals treated with 2,3BZ2Me. Owing to its low concentration the evidence of 2,3-BZ2Ac metabolite was suggested taking into account its retention time.

Fig. 3 shows the time-profiles of plasma concentrations of Sprague–Dawley rats treated with 2,3-BZ2 (A) and 2,3-BZ2Me (B). Peak (or maximal) plasma concentration was achieved after 45 min from i.p. administration of 2,3-BZ2 (A). Plasma concentration–time profiles (B) of rats treated with 2,3-BZ2Me showed two curves, one is referred to inoculated drug and the other to its demethylated metabolite, that is 2,3-BZ2. The 2,3-BZ2Me peak (or maximal) plasma concentration was achieved 15 min after i.p. administration, whereas after 45 min its plasma concentration decreased significantly. On the contrary, for its metabolite (2,3-BZ2) maximal plasma concentration is achieved 45 and 60 min after drug administration. The 2,3-BZ2 and 2,3-BZ2Me

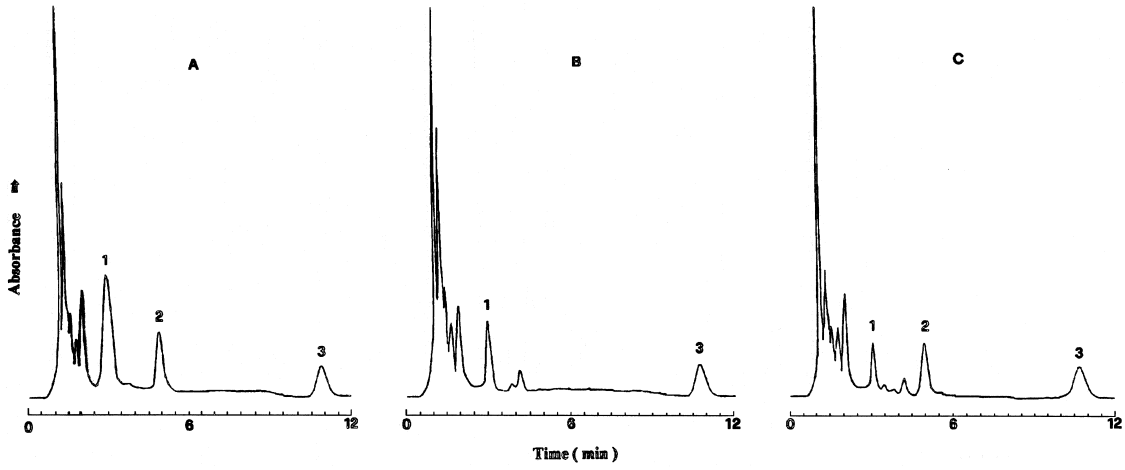


Fig. 2. Representative chromatograms of 2,3-BZs and the I.S. used in the analytical procedure, the relative retention times are: t'=3.1 for 2,3-BZ2 (1); t'=5.06 for 2,3-BZ2Me (2); t'=10.9 for prazepam (3). (A) Rat plasma spiked with 2,3-BZ2 (0.5 μg/ml), 2,3-BZ2Me (0.5 μg/ml) and the internal standard; (B) plasma sample obtained 15 min after i.p. administration of 2,3-BZ2 spiked with I.S.; (C) plasma sample obtained 15 min after i.p. administration of 2,3-BZ2Me spiked with I.S.

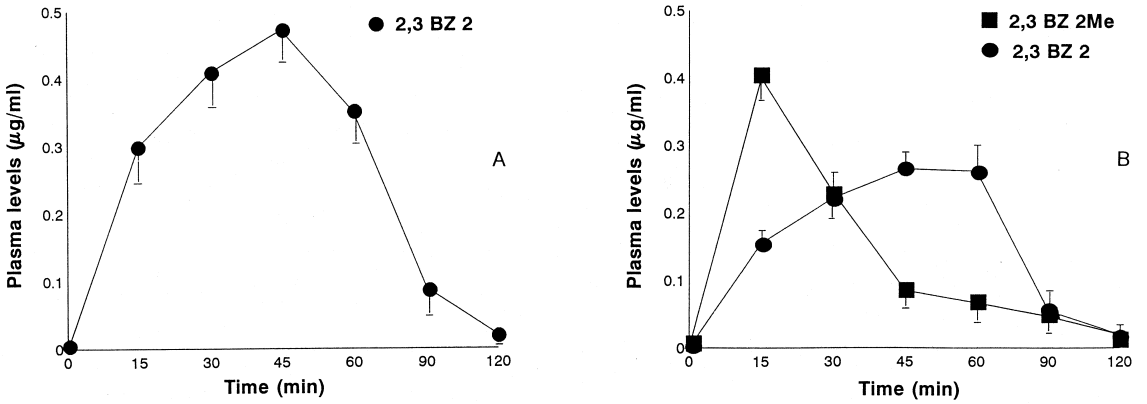


Fig. 3. Time profiles of plasma concentrations after i.p. administration to rats of (A) 2,3-BZ2; (B) 2,3-BZ2Me.

plasma levels gradually decreased and the drugs disappeared from plasma 120 min after i.p. administration. It should be noted that as 2,3-BZ2Me plasma concentration decreased, the 2,3-BZ2 plasma concentration increased, thus confirming that compound 2,3-BZ2Me was metabolically changed to 2,3-BZ2 by N-demethylation at 3-position of the heptatomic ring.

4. Conclusions

This report describes the methodology and validation of a high-performance liquid chromatographic assay for the simultaneous determination of 2,3-BZ2 and 2,3-BZ2Me and their metabolites in rat plasma. The method appeared rapid, simple and suitable also for therapeutic 2,3-benzodiazepine monitoring. In fact, following the administration of 10 mg/kg of compounds studied the detected plasma levels were in agreement with previously demonstrated anticonvulsant activity [3]. This method is the first approach to the plasma detection of these novel AMPA/kainate receptor antagonist. This analytical study showed also that the main metabolic pathway for compound 2,3-BZ2Me is demethylation, thus confirming, as previously suggested [3], that it is a prodrug which undergoes biotransformation to 2,3-BZ2.

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