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Chromatographic determination of 7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones in rat plasma: relationship to their anticonvulsant activity

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Abstract

The present investigation was designed to develop an assay suitable for pharmacokinetic studies of new compounds, i.e. the novel 7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one derivatives (**2a** and **2b**), acting as non-competitive AMPA-receptor antagonists. A reversed-phase high-performance liquid chromatographic method has been developed to determine the time-course of plasma concentrations of derivatives **2a** and **2b** administered intraperitoneally to Sprague–Dawley rats. The separation of compounds studied and a *N*-methyl-2,3-benzodiazepin-4-one derivative as internal standard (I.S.) from plasma, were carried out by liquid–liquid extraction using diethyl ether. The samples were injected onto the analytical column (Partisil 10 ODS) eluted with acetonitrile/0.01 *M* acetate buffer (pH 5.3) at a flow-rate of 2 ml/min and detected at 240 nm. Compounds **2a**, **2b** and I.S. gave retention times of 8.5, 5.25 and 11.1 min, respectively. The selectivity of the method was satisfactory. The mean recovery from spiked rat plasma ranged from 86.7 to 91.6% for **2a**, and from 85.1 to 87.0% for **2b**. The procedures were validated with a good reproducibility and linear response from 0.0625 to 2 μg/ml, with a regression coefficient of 0.9932 for **2a** and 0.9854 for **2b**. The lower limit of quantification (LOQ) was taken as 15 ng/ml for the two compounds. **2a** and **2b** showed no signs of significant degradation in rat plasma during storage at −20 °C and following freeze/thaw cycles. Moreover, plasma levels of the tested compounds have been correlated with their anticonvulsant activity, determined in vivo in genetically epilepsy-prone rats. Due to its sensitivity, the method was suitable for application to pharmacokinetic study. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: 7,8-Methylenedioxy-4*H*-2,3-benzodiazepin-4-ones; Anticonvulsant; Pharmacokinetic

1. Introduction

GYKI 52466, 1-(4′-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H*-2,3-benzodiazepine (Fig. 1), is the prototype of selective non-competitive 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionic acid

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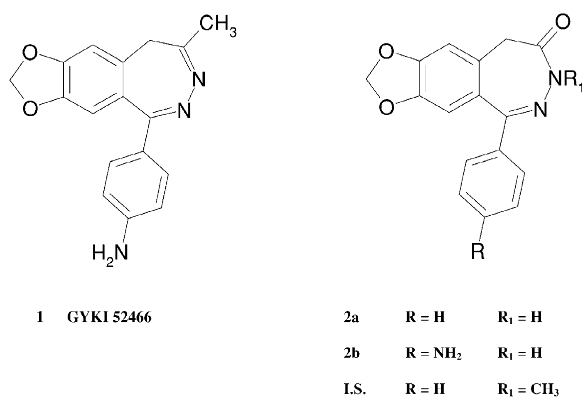

Fig. 1. Chemical structure of 2,3 benzodiazepines.

(AMPA)-receptor antagonists acting via an allosteric site on the receptor complex [1,2]. GYKI 52466 has remarkable anticonvulsant properties [3,4] but, contrary to the classical 1,4-benzodiazepines, it is devoid of any sedative and anxiolytic activity and does not bind to the benzodiazepine site of the $GABA_A$ receptor complex. Furthermore, GYKI 52466 behaves as a neuroprotective agent in focal and global ischemia [5,6].

We have recently investigated [7,8] a new series of 1-aryl-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones (e.g. **2a** and **2b**), structurally related to GYKI 52466, which proved to possess remarkable anticonvulsant activity in various seizure models such as DBA/2 mice [9–12], and acting as non-competitive antagonists at the AMPA receptor complex [13].

The **2a** and **2b** 2,3-benzodiazepin-4-one derivatives differ from GYKI 52466 in a modification in position 4 of the heptatomic nucleus, i.e. the introduction of a carbonyl moiety; moreover, the derivative **2a** lacks the amino group on the phenyl ring present in **2b** and **1**.

In the present study, in order to test the role of the amino substituent on the pharmacokinetic profile in rat, we have determined the time course of plasma concentrations of **2a** and **2b** after intraperitoneal administration in Sprague–Dawley rats using a high-performance liquid chromatographic method and a liquid–liquid extraction [14]. Moreover, the anticonvulsant activity of **2a** and **2b** has been evaluated in vivo in the genetically epilepsy-prone rats (GEPR-9s), an animal model of generalised clonic-tonic seizures, widely used to evaluate the anticonvulsant properties of novel and conventional compounds [15,16].

2. Experimental

2.1. Analytical study

2.1.1. Drugs

Compounds **2a** [3,5-dihydro-7,8-methylenedioxy-1-phenyl-4*H*-2,3-benzodiazepin-4-one], **2b** [1-(4′-aminophenyl)-3,5-dihydro-7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one] and the internal standard 3,5-dihydro-3-methyl-7,8-methylenedioxy-1-phenyl-4*H*-2,3-benzodiazepin-4-one (Fig. 1) were synthesized in our laboratories as previously described [7]. GYKI 52466 was obtained from Research Biomedicals (Natick, MA, USA).

2.1.2. Drug administration and collection of sample

Compounds **2a** and **2b** were dissolved in a freshly prepared solution in 50% dimethylsulfoxide and 50% sterile saline (0.9% NaCl); GYKI 52466 was dissolved in sterile saline. For systemic administration, all compounds were administered intraperitoneally (i.p.) (0.4 ml/100 g of body weight of the rat), as a freshly ultrasonicated solution. At least six animals were used once for each dose level studied. Blood samples were withdrawn from the animals 15, 30, 45, 60, 75, 90, 105 and 120 min after i.p. administration of the drugs. Blood cells were removed by centrifugation and separated plasma was stored at −20 °C until assay.

2.1.3. Chemicals and standards

Diethyl ether, acetonitrile, methanol and HPLC grade water (Carlo Erba, Milan, Italy) were used and deionized water was used to prepare buffer for the aqueous standards. All other reagents were of analytical reagent-grade (Carlo Erba, Milan, Italy). Stock solutions (1 mg/ml) of the 7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-one derivatives (**2a** and **2b**) were prepared in methanol. Working solutions were made by dilution with methanol and used to prepare aqueous standards and spiked plasma sam-

ples. The internal standard (I.S.) was used at a concentration of 10 μg/ml.

2.1.4. Chromatography

The HPLC system consisted of a Jasco PU 980 pump and LG 980-02 ternary unit (Tokyo, Japan) with a 100-μl loop injection valve and a variable-wavelength ultraviolet Jasco 975 detector (Tokyo, Japan) set at 240 nm. A Partisil 10 ODS Watman (250×4.6 mm, 10 μm) reversed-phase column (Carlo Erba, Milan, Italy), was used at 30 °C with a ODS guard (45×4.6 mm). The column was eluted with acetonitrile/0.01 *M* acetate buffer adjusted to pH 5.3 with pure acetic acid (35:65, v/v), at a flow-rate of 2 ml/min.

2.1.5. Extraction

A 0.5-ml aliquot of plasma was mixed with 100 μl of 2 *M* NaOH and 100 μl of I.S. solution (10 μg/ml). After vortexing for 5 s, the sample was applied to an Extrelut NT (E. Merck, Darmstadt, Germany) pre-packed glass column. After 10 min, diethyl ether (10 ml) was added to the column. The eluate was collected and evaporated to dryness at 45 °C under a stream of nitrogen. The residue was dissolved in 300 μl of methanol and a 100-μl aliquot was injected into the chromatographic system. All standard and spiked samples were similarly treated.

2.1.6. Calibration curve and method validation

Compounds **2a**, **2b** and I.S. (1 μg) were injected into the column and identified by their relative retention times. Calibration curves were obtained by plotting the peak area ratio of the drugs to the I.S., versus the known concentration of each reference substance added to drug-free rat plasma. The curves were constructed from six replicate measurements of six concentrations of each compound over a range of 0.0625–2 μg/ml and analysed one curve a week. Plasma samples containing a known amount of all compounds studied and I.S. were prepared and stored frozen at −20 °C until use. These samples were used as quality control specimens. The within-day and between-day precision and accuracy were calculated by six replicate analysis at each chosen concentration (0.05, 0.2, 0.5, 1 and 2 μg/ml) and analysed over a period of 10 weeks.

2.1.7. Extraction efficiency and stability

The recovery from plasma and aqueous standard of all compounds and I.S. was evaluated to test the efficiency and reproducibility of the extraction procedure. The determination of the extraction efficiency in rat plasma was made by adding amounts of 0.5–1 and 2 μg/ml in replicate ($n=6$). The extraction was conducted as described above and 100 μl of internal standard working solution was added prior to the extraction. The responses of these standards taken by means of the extraction procedures have been compared with those of standard solution at the same concentration injected directly into the liquid chromatographic apparatus. The peak-area ratios were compared to the ratio of the standard aqueous samples without extraction. Six replicate samples were determined at each point and performed once a day for 6 days.

The stability of **2a** and **2b** was assessed in heparinised rat plasma by analysing 200 μl stability samples at concentrations of 0.2 and 2 μg/ml. Samples were analysed immediately after preparation and after storage at room temperature (ca. +22 °C) for ca. 24 h, frozen (ca. −20 °C) after 2, 4 and 10 weeks of storage. The effect of freeze-thawing on the samples was evaluated at low and high concentrations (equivalent to low and high quality control sample concentrations). Three replicate determinations were made in each case and at each time for each substance. The concentrations of **2a** and **2b** found in the stability samples were compared to the theoretical spiked concentrations.

2.2. Pharmacological study

2.2.1. Animals

Genetically epilepsy-prone rats (GEPR-9s), a strain derived from Sprague–Dawley rats, were housed three per cage in stable conditions of humidity (60±5%) and temperature (22±2 °C), and allowed free access to food and water until the time of the experiments. The animals were maintained on a 12-h light, 12-h dark cycle (lights on 7.00 a.m.–7.00 p.m., off 7.00 p.m.–7.00 a.m.). GEPR-9s were tested three times at weekly intervals between 6 and 8 weeks of their life, and only animals that showed audiogenic seizures in all three exposures to sound stimulation were used for these experiments.

2.2.2. Anticonvulsant activity

Anticonvulsant activity was evaluated in genetically epilepsy-prone rats (180–260 g, 12–18 weeks old, male, $n=80$) by exposing them to a mixed frequency sound of 12–16 kHz, 109-dB intensity. Individual animals were placed under a hemispheric Plexiglas dome (Ø 58 cm) and 60 s were allowed for habituation and assessment of locomotor activity. Auditory stimulation was applied for 60 s or until tonic extension occurred, and induced a sequential seizure response in treated and control GEPR-9s rats. In particular, the audiogenic seizure response was assessed on a scale which describes the locomotor activity recorded for each animal and goes from 0 (no response) to 9 (maximum response), as previously reported [17]. A full-seizure response (S.R.) consisted of an early wild running phase, followed by generalised myoclonus and tonic flexion and extension to give a score of 9. The control (vehicle-treated) and drug-treated rats were scored for latency to and incidence of the different phases of the seizures. The time course of the anticonvulsant action of **1**, **2a** and **2b** was determined following the intraperitoneal (i.p.) administration of 2,3-benzodiazepines (50 μmol/kg) to groups of six rats for each time; the animals were tested for sound-induced seizure responses from 5 to 240 min after drugs administration.

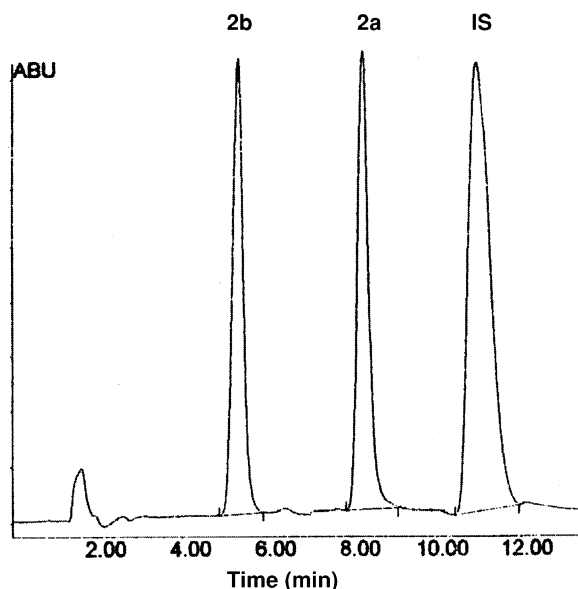

Fig. 2. Retention time of **2a**, **2b** and I.S.

3. Results

3.1. Detection and sensitivity

Fig. 2 shows the retention time of the tested compounds. The drugs and the internal standard were detected at 240 nm and the retention times were 5.25 min (±0.018) for **2b**; 8.15 min (±0.017) for **2a**; 11.1 min (±0.025) for I.S. The lower limit of detection (LOD), with a signal-to-noise ratio of 3, was 10 ng/ml for **2a** and 8.5 ng/ml for **2b**. The sensitivity of the method allowed a quantification of 15 ng/ml (LOQ) of these drugs in plasma samples.

3.2. Linearity

A linear response was observed over the examined concentration range (0.0625–2 μg/ml). The regression coefficient was 0.9932 for **2a** ($y=0.8423x+0.0869$; slope SE=0.0111, intercept SE=0.0073; $n=6$); and 0.9854 for **2b** ($y=0.9088x-0.0425$; slope SE=0.0326, intercept SE=0.0621; $n=6$).

3.3. Precision, accuracy and stability

Precision and accuracy for within-day and between-day assay of both **2a** and **2b** are shown in Tables 1 and 2. In the within-day assay, the average relative error (RE) value was 1.34% for **2a** and 2.24% for **2b**. Acceptable precision was achieved for all concentrations investigated. In the between-day assay, the accuracy ranged from 94.4 to 104.5% for **2a**, from 93 to 108% for **2b**. The extraction efficiency, expressed as coefficient of variation, ranged from 1.19 to 2.94% for **2a** and from 1.59 to 3.40% for **2b**; it was independent of the concentration, as reported in Table 3.

The two compounds studied showed no signs of significant degradation in rat plasma during 10 weeks of storage at −20 °C. The percentage of spiked concentrations ranged from 91.4 to 108.5%. Both 2,3-benzodiazepines were stable in rat plasma at ambient temperature (ca. 22 °C) for up to 2 h, stored frozen at ca. −20 °C for 70 days and following three freeze/thaw cycles (data not shown).

Table 1
Within-day precision of the HPLC assay ($n=6$)

Concentration spiked (μg/ml)	Concentration found[a] (μg/ml)	Accuracy (%)	C.V. (%)	R.E. (%)
2a				
0.05	0.048±0.004	96.0	8.61	−3.3
0.2	0.207±0.009	103.5	4.43	3.5
0.5	0.507±0.014	101.4	2.78	1.4
1	1.037±0.052	103.7	4.98	3.7
2	2.028±0.066	101.4	3.23	1.4
2b				
0.05	0.052±0.005	104.0	9.61	4.6
0.2	0.196±0.003	98.0	1.44	−2.0
0.5	0.516±0.008	103.2	1.58	3.2
1	1.036±0.046	103.6	4.43	3.6
2	2.036±0.025	101.8	1.25	1.8

C.V., coefficient of variation; R.E., mean relative error.
[a] Mean±SD.

3.4. Chromatography

Fig. 3 shows a representative chromatogram of drug-free rat plasma sample. There are no interfering peaks at the retention time of the studied 2,3-benzodiazepines.

Fig. 4 shows two chromatograms: one refers to rat plasma sample obtained 30 min after i.p. administration of **2b** giving a concentration of 1.48 μg/ml (Fig. 4A); the other chromatogram refers to rat plasma obtained 30 min after i.p. administration of **2a** giving a concentration of 1.07 μg/ml (Fig. 4B).

Table 2
Between-day precision of the HPLC assay ($n=6$)

Concentration spiked (μg/ml)	Concentration[a] found (μg/ml)	Accuracy (%)	C.V. (%)	R.E. (%)
2a				
0.05	0.047±0.004	94.4	7.67	−6.0
0.2	0.209±0.006	104.5	2.89	4.5
0.5	0.521±0.052	104.2	9.95	4.2
1	1.037±0.052	103.7	4.98	3.7
2	2.045±0.077	102.2	3.78	2.2
2b				
0.05	0.054±0.003	108.0	4.68	7.3
0.2	0.186±0.007	93.0	3.78	−7.0
0.5	0.518±0.013	103.2	2.45	3.6
1	1.066±0.069	106.6	6.49	6.6
2	2.045±0.077	102.2	3.78	2.2

C.V., coefficient of variation; R.E., mean relative error.
[a] Mean±SD.

Table 3
Determination of extraction recovery of 2,3-benzodiazepines from plasma ($n=6$)

Concentration spiked (μg/ml)	Mean recovery (%)	SD	C.V. (%)
2a			
0.5	91.6	0.014	2.94
1	87.8	0.017	1.98
2	86.7	0.021	1.19
2b			
0.5	87.0	0.015	3.40
1	85.1	0.018	2.06
2	86.8	0.028	1.59

SD, standard deviation; CV, coefficient of variation.

3.5. Time-course of plasma concentrations of 2,3-benzodiazepines

Time profiles of plasma concentrations of rats treated i.p. with **2a** and **2b** are reported in Fig. 5. The i.p. administration of **2a** determined a peak plasma concentration of 1.42 μg/ml (C_{max}) that was achieved after 45 min (T_{max}).

The i.p. administration of **2b** determined a peak plasma concentration of 1.48 (C_{max}) that was achieved after 30 min (T_{max}).

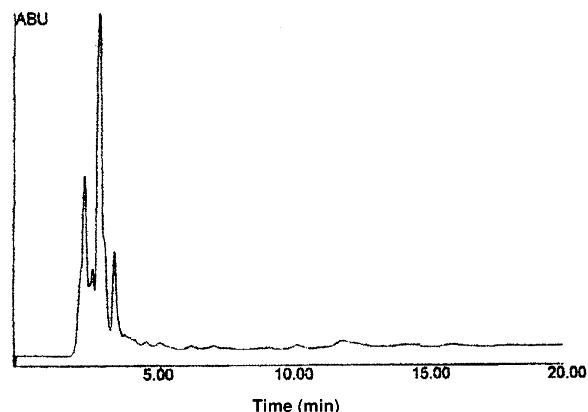

Fig. 3. Representative chromatogram of drug-free rat plasma sample.

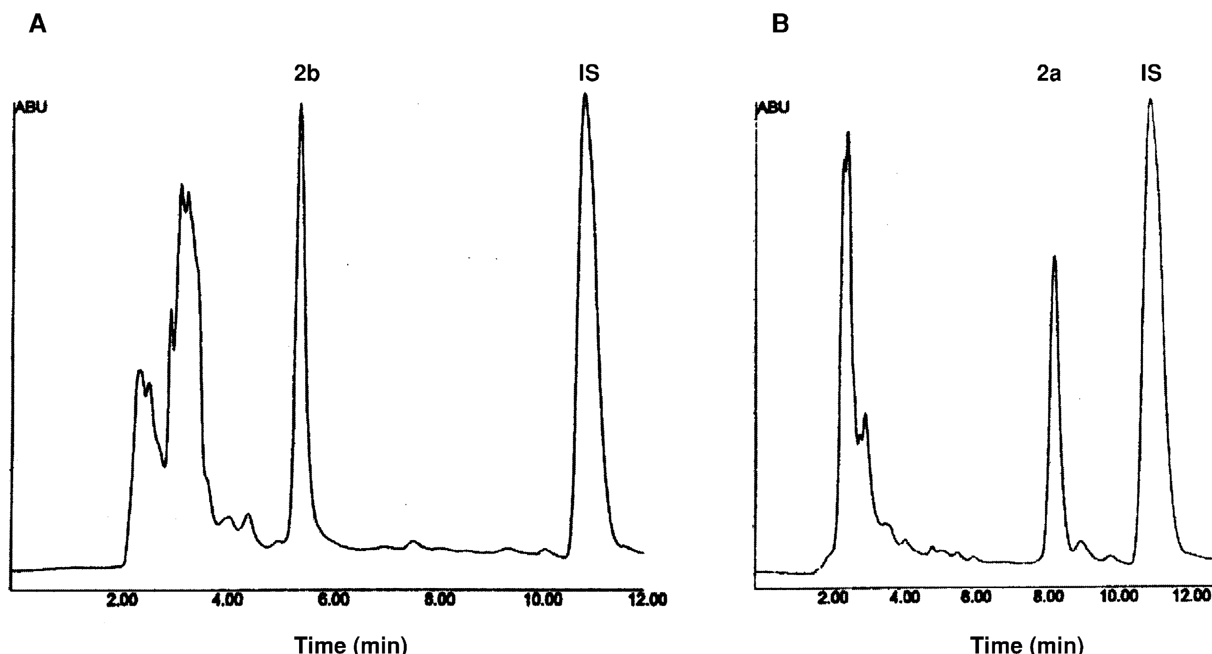

Fig. 4. Representative chromatograms of rat plasma sample: (A) Representative chromatogram of rat plasma sample obtained 30 min after i.p. administration of **2b** giving a concentration of 1.48 μg/ml; (B) Representative chromatogram of rat plasma sample obtained 30 min after i.p. administration of **2a** giving a concentration of 1.07 μg/ml.

3.6. Anticonvulsant properties of 2,3-benzodiazepines

The novel 7,8-methylenedioxy-4*H*-2,3-benzodiazepines were tested for anticonvulsant activity against audiogenic seizures in GEPR-9s and the results were compared with that of GYKI 52466 (**1**), chosen as the reference compound. The time course of the anticonvulsant action of **1**, **2a** and **2b** was determined after their i.p. administration and results are reported in Fig. 6. The time-course studies revealed that GYKI 52466 showed the maximum

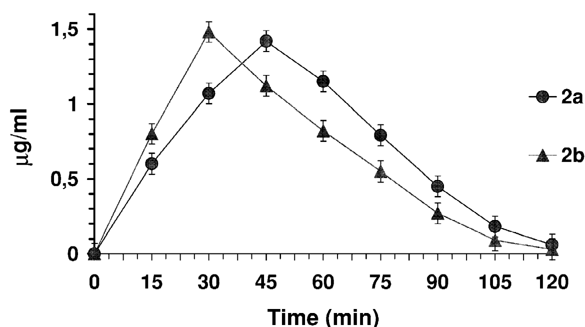

Fig. 5. Time profiles of plasma concentrations of rats treated i.p. with **2a** and **2b** (0.4 ml/100 g of body weight of the rat; $n=6$ for each dose). The abscissa shows the analysed times and the ordinate the plasma levels of the determined drugs. The **2a** peak plasma concentration (1.42 μg/ml) was achieved after 45 min i.p. administration. The **2b** peak plasma concentration (1.48 μg/ml) was achieved after 30 min i.p. administration.

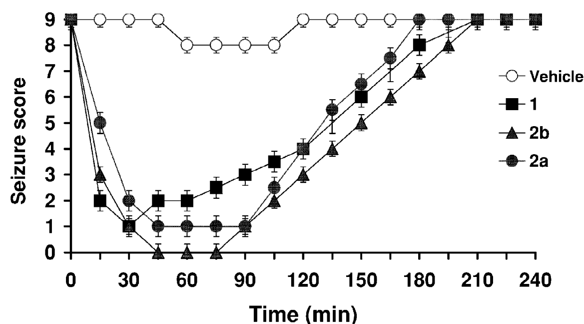

Fig. 6. Anticonvulsant effects of **2a**, **2b** and GYKI 52466 (**1**) (50 μmol/kg) against audiogenic seizures in genetically epilepsy-prone rats. The abscissa shows the time and the ordinate the seizure score: 0, total protection; 3, clonic phase; 9, tonic extension ($n=6$ rats for each time).

activity from 15 to 45 min, whereas that of derivatives **2a** and **2b** was in the range of 30–90 min. Indeed, the curve of the compound **2b** shows a more complete protection against seizures (seizure score= 0) from 45 to 75 min.; interestingly, derivative **2b** is also provided with the longer-lasting anticonvulsant activity.

4. Discussion and conclusions

This report describes the methodology and validation of a HPLC assay for the determination of 2,3-benzodiazepine derivatives in rat plasma. The method has been applied to study the pharmacokinetic profile of compounds **2a** and **2b** in Sprague–Dawley rats.

The present study demonstrated that the 7,8-methylenedioxy-4*H*-2,3-benzodiazepin-4-ones showed anticonvulsant activity in the audiogenic seizure test in genetically-epilepsy prone rats. In particular, **2a** and **2b** exhibit a potency higher than GYKI 52466 and longer-lasting anticonvulsant action. Moreover, we determined earlier a lower toxicity of **2a** and, in more remarkable way, of **2b** than that of the parent compound, as expressed by the therapeutic index values in DBA/2 mice [8].

In a previous study, we reported a peak plasma concentration of GYKI 52466: the plasma C_{max} was reached 15 min after i.p. administration [18]. In the present study, we observed a difference between the two compounds studied in reaching the T_{max}, that could be related to their different lipophilicity. Indeed, the presence of the amino group at 4′-position of the phenyl ring at C-1, inducing reduction of lipophilicity, is responsible for a faster systemic distribution of **2b** than **2a**.

A comparison between time-course of anticonvulsant activity and plasma levels indicates that the compounds under study showed the maximum protection against seizures 15 min after the peak plasma concentrations were reached, as for that previously observed for GYKI 52466 [18]. It is noteworthy that, at variance with GYKI 52466, the maximum anticonvulsant effects of compounds **2a** and **2b** were maintained until 90 min after administration. One possible explanation for the presence of anticonvulsant activity of **2a** and **2b** even if the plasma levels were particularly low is the possibility of accumulation of these derivatives in the brain or a slow clearance of them from the brain. This hypothesis is supported by the evidence that other 2,3-benzodiazepine derivatives (GYKI 52322, GYKI 51189), have been detected in rat brain [19–21].

In conclusion, the data reported showed that the pharmacological profile of 2,3-BZs is highly dependent on the substitution pattern at the phenyl ring at C-1. The previous observation of a more favourable therapeutic index of **2b**, and the longer lasting anticonvulsant activity demonstrated in the present paper, suggest that the pharmacological properties of the **2b** derivative merit further investigation.

Acknowledgements

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