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Liquid chromatographic assay for riluzole in mouse plasma and central nervous system tissues

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Abstract

An isocratic, reversed-phase high-performance liquid chromatographic procedure (HPLC) was developed for determination of the neuroprotective agent riluzole in mice plasma, brain and spinal cord. The procedure is based on isolation of the compound and the internal standard from plasma and central nervous system tissues using a Bakerbond spe™ C8 cartridge, with satisfactory recovery and specificity. Separation was on a C18 column, coupled with an UV detector at 263 nm. The assay was linear over a wide range, with a lower limit of quantification of 100 ng ml^{-1} or g^{-1} using 0.1 ml of plasma and about 100 mg of brain tissue. The precision and accuracy were within the acceptable limits for an HPLC assay. The method is currently used to support pharmacological studies of the activity of riluzole when given in combination with other potential neuroprotective agents in an animal model of familiar amyotrophic lateral sclerosis (SOD1-G93A transgenic mice).

Keywords: Riluzole

1. Introduction

Riluzole (2-amino-6-trifluoromethoxybenzothiazole) [1] is the current treatment for amyotrophic lateral sclerosis (ALS) [2–4], a chronic neurodegenerative disease causing progressive motor weakness resulting from selective motor neuron cell death. It is the only drug approved by the Food and Drug Administration (FDA) for this devastating disease where mortality is seen on the average four years after onset. Its mechanism of action in ALS is not completely understood. However, in a transgenic model of familial ALS [5] riluzole prolonged survival without any effect on the onset of clinical disease. Similar action was observed in patients [6,7]. This suggests there are multifactorial downstream pathogenic pathways implicated in ALS, so improving therapeutic efficacy will depend on implementing a strategy addressing these multiple components [2–4,8].

Recent preclinical studies in transgenic mouse models of ALS have aimed at assessing the possibility of boosting the pharmacolological activity of riluzole by combining it with neuroprotective drugs having different mechanisms of action [9]. Riluzole meaurements in plasma, and particularly in the central nervous system (CNS), of these animal models could help in adjusting the experimental design and interpreting the results. This, however, calls for an accurate analytical method to measure the drug at the site of action.

Analysis based on high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [10] or coupled with tandem mass spectrometry (LC/MS/MS) [11] have been described for riluzole. However, they do not appear to be able to quantitate the drug in CNS tissues of animals, since they are aimed only human plasma and urine. On the other hand, the methods used to study the CNS uptake and distribution of riluzole in rats and monkeys are based on whole body autoradiography and tissue dissection counting after oral ^{14}C-riluzole [12]. We therefore set up a relatively simple, yet highly selective HPLC procedure for the rapid determination of riluzole in plasma and CNS tissues of rodents.

The method relies on a solid-liquid extraction procedure and resolution by an isocratic reversed-phase system. This method was used to support pharmacological studies of the neuroprotective activity of riluzole when given alone and in combination with agents acting by different mechanisms in the complex pathway to neuronal death. Transgenic mice over-expressing a mutated form of the human enzyme SOD-1 (SOD1-G93A), which is among the most successful

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models for preclinical drug studies in ALS [13,14], were used in these studies.

2. Experimental

2.1. Materials and reagents

Riluzole was obtained from Rhône-Poulenc Rorer (Antony, France), L-acetyl-carnitine from Sigma–Tau (Pomezia, Italy) and minocycline from Sigma–Aldrich (Milan, Italy). The internal standard (IS) 1[2-methoxyphenyl]-4-[(1-benzo[b]furan-2-carboxamide)-butyl]-piperazine was synthetised by G. Campiani at the University of Siena (Italy). Stock solutions were prepared by dissolving riluzole and the IS in methanol at the concentration of 1 mg ml^{-1}. The solutions were stable for at least 1 week when stored at −20 °C. Working standard solutions were prepared from the stock solutions by dilution with methanol and kept at +4 °C in dark bottles.

Other chemicals and solvents, CH_3COOH, CH_3CN and CH_3OH (C. Erba, Milano, Italy), KH_2PO_4 (E. Merck, Darmstadt, Germany) were of analytical-reagent grade and were used without further purification. Water was deionised before use.

Drug-free plasma and brain tissues were obtained from female C57 mice and stored at −20 °C after collection and processing.

2.2. Chromatographic apparatus and conditions

HPLC analysis was done on a Waters system (Milford, MA, USA) equipped with a Wisp-717 sample processor, a model 510 solvent delivery system and a model 2487 UV detector set at 263 nm, coupled with a model C-R6A Chromatopac Shimadzu integrator (Shimadzu, Kyoto, Japan). Separation was on a μBondpack C18 column (3.9 mm × 300 mm) Waters (Milford, MA, USA), at room temperature. The mobile phase (CH_3COOH:CH_3CN:CH_3OH:0.01 M KH_2PO_4; 1:5:43:51, v/v/v/v) was a slight modification of that used for riluzole analysis in human plasma and urine [10]. The eluent was filtered through a 0.45-μm filter, degassed before use, and delivered isocratically at a flow-rate of 1 ml min^{-1}.

2.3. Extraction

Plasma samples (0.1 ml) were adjusted to a volume of 0.5 ml with drug-free plasma and mixed with 25 μl of a methanolic solution of the IS (10 μg/ml), then diluted to 1.0 ml with 0.01 M phosphate buffer, pH 7.4. The final solution was Vortex-mixed, centrifuged in a Sorval centrifuge for 10 min at 4 °C at 2000 rpm, and the supernatant was applied on 3 ml Bakerbond spe^{TM} Octyl (C8) disposable extraction columns (J.T. Baker, Deventer, Holland), previously activated with 2 ml CH_3OH and 2 ml H_2O.

Brain tissue was homogenised (10 ml g^{-1}) in deionised water. To 1 ml of this homogenate the IS (25 μl) was added, then Vortex-mixed and centrifuged at 2000 rpm for 10 min at 4 °C. The precipitate was re-dissolved in 0.5 ml of distilled water and centrifuged. Like plasma samples, the combined supernatants were processed as below.

After loading the biological material, and equilibration with K_2HPO_4 0.01 M, the extraction cartridges were washed with 0.5 ml H_2O and 0.5 ml 10% CH_3OH in H_2O. Riluzole and the IS were eluted with 2 ml with 1 M NH_4OH in CH_3OH and the eluent was dried under a stream of nitrogen. The residue was dissolved in 150 μl of the mobile phase and 100 μl were injected in to the HPLC system.

2.4. Data analysis

The precision and accuracy of the method were determined by replicate analyses of quality control samples (QC) containing small, medium and large known amounts of riluzole, stored at −20 °C. On different days these QC were assayed with standard samples and the calculated concentrations were compared (inter-assay variance). Intra-assay variance was checked by replicate analysis of QC samples on the same day.

Daily standard curves with six concentrations over the working range were plotted in duplicate with QC samples injected between the two sets of standards. The relative response factor was computed as the analyte to the IS peak-height ratio. Calibration lines were constructed by least squares linear regression on the relative response factors against the nominal concentration of the compound. Concentrations of QC, and unknown samples, were obtained by back-solving the usual equation ($y = a + bx$) of the calibration line.

2.5. Application

Female SOD1-G93A mice and their nontransgenic littermates (Consorzio Mario Negri Sud, S. Maria Imbaro, Chieti, Italy) were used in a study to evaluate the neuroprotective activity of riluzole when combined with other neuroprotective or neurotrophic agents [13–18] which are currently being tested as potential combination therapy for ALS [2–4]. The pharmacological evaluation of this study is still in progress and a more detailed account of the effect of the various combinations on the onset and progression of the disease in this model will be presented separately.

The pharmacological treatment started at the age of 7 weeks. Mice were dosed orally with riluzole dissolved in water (200 μg ml^{-1}), minocycline (100 mg/kg, every other day) or L-acetyl-carnitine dissolved in water (500 μg ml^{-1}), or their combination.

SOD1-G93A mice in the terminal stage of disease and their C57 littermates were killed by decapitation under deep anesthesia, respectively, 1 and 2 h after the last dose of acetyl-carnitine or minocycline. Blood samples were

collected in heparinized tubes centrifuged and the plasma was stored at −20 °C. Brains and spinal cords were rapidly removed, blotted with paper to remove excess surface blood and stored at −20 °C.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 12 December 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

3. Results and discussion

3.1. Chromatography

Riluzole was recovered selectively and efficiently from plasma and brain homogenate using 3 ml Bakerbond spe™ Octyl (C8) disposable extraction columns and an elution system consisting of 1 M NH_4OH in CH_3OH which extracted only a few impurities and no interfering substances from all tissues. Fig. 1 gives examples of chromatograms of extracts from brain spiked with riluzole (2500 ng g^{-1} (B)) and brain of a SOD1-G93A mouse given 200 μg ml^{-1} of riluzole in drinking water (C). The retention times were 21 min for riluzole and 11 min for the IS and no interference was observed from drug-free tissue (A).

There was also no interference when drug-free brain and plasma, spiked with riluzole, the IS and some potential neuroprotective and neurotrophic agents (the anti-excitotoxics gabapentin and verapamil, the anti-apoptotic minocycline, the antioxidants acetyl-carnitine and α-tocopherol, and buspirone) [2–4] were processed according to this procedure at the arbitrary concentration of 10 μg ml^{-1} or g^{-1} (see Table 1). Chromatography was on the μBondpack C18 reversed-phase column using a modification of the isocratic system described for the analysis of human plasma and urine [10].

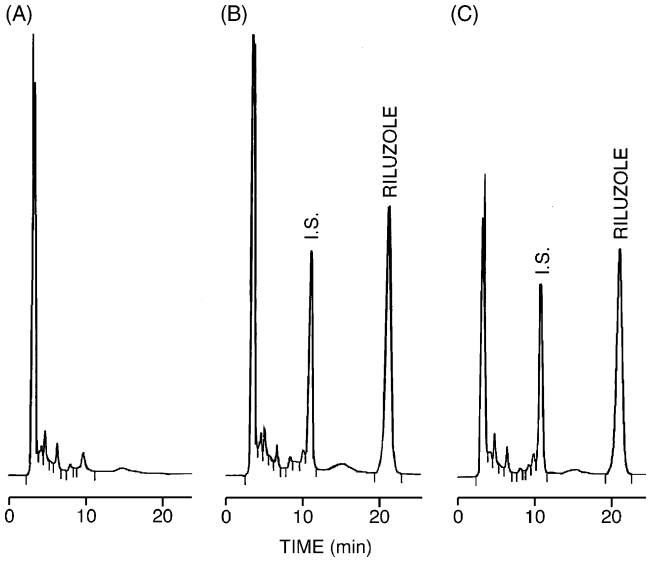

Fig. 1. Chromatograms of extracts from drug free rat brain (A); brain spiked with riluzole (2500 ng g^{-1} (B)); and brain of a SOD1-G93A mouse given 200 μg ml^{-1} of riluzole in drinking water (C). Column: μBondpack C18 (3.9 mm × 300 mm). Mobile phase, CH_3COOH:CH_3CN:CH_3OH:0.01 M KH_2PO_4 (1:5:43.3:50.7, v/v/v/v).

Table 1
Interference studies with potential neuroprotective and neurotrophic agents

Compound	Retention time (min)
Riluzole	21
Internal standard[a]	11
Acetyl-carnitine	–
Buspirone	6.1
Gabapentin	–
Minocycline	–
α-Tocopherol	–
Verapamil	14

No peaks (–) (10 μg ml^{-1}); chromatography was on the μBondpack C18 column using CH_3COOH:CH_3CN:CH_3OH:0.01 M KH_2PO_4 (1:5:43:51, v/v/v/v) as eluent system.

[a] 1[2-methoxyphenil]-4-[(1-benzo[b]furan-2-carboxamide)-butyl]-piperazine.

The overall mean recovery, determined by comparing the peak heights of riluzole from spiked plasma and brain plasma with those from direct injection of riluzole dissolved in the mobile phase, averaged 92 ± 5% in plasma, 91 ± 7% in brain and 85 ± 7% in spinal cord, with no significant dependence on concentration. Results were similar using higher plasma (0.5 ml, rat plasma) and brain homogenate (2 ml + 1 ml, rat brain) volumes, the recovery (91 ± 7 and 89 ± 7%, respectively) and selectivity (no interfering peaks, data not shown) both being relatively unaffected (see Table 2).

The relationships between the peak-height ratios of riluzole and the IS and the amount of the compound added to plasma and brain homogenate were always linear, with the r^2 invariably exceeding 0.995. The slopes of these curves ranged from 0.0040 to 0.0042 for plasma (average

Table 2
Extraction recoveries of riluzole from plasma and brain homogenate

Tissue/species	Volume (ml)	Extraction recovery (%)
Plasma		
Mouse	0.1	92 ± 5
Rat	0.5	94 ± 8
Brain		
Mouse	1.0[a]	91 ± 7
Rat	2.0[b]	89 ± 7

The recoveries were determined by comparing the peak heights of riluzole from spiked plasma and brain homogenates (0.25–1.0–5.0 μg ml^{-1} or g^{-1}) with those from direct injection of the drug dissolved in the mobile phase ($n = 16$).

[a] Approximately 100 mg of brain tissue.

[b] Approximately 200 mg of brain tissue.

Table 3
Summary of intra-day quality control samples results for riluzole analysis in rat plasma and brain

Added[a] ($\mu g\,ml^{-1}$ or g^{-1})	Found ($\mu g\,ml^{-1}$ or g^{-1})	CV (%)	RE (%)
Plasma			
0.25	0.25 ± 0.00	0.00	−0.40
1.00	1.01 ± 0.04	4.10	1.03
5.00	5.02 ± 0.23	4.52	0.37
Brain			
0.25	0.25 ± 0.00	0.00	1.20
1.00	1.03 ± 0.03	3.23	2.83
5.00	4.86 ± 0.16	3.37	−2.90

[a] Using 0.1 ml of plasma or approximately 100 mg of brain tissue.

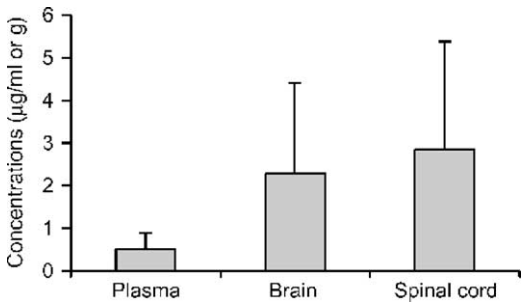

Fig. 2. Mean plasma, brain and spinal cord concentrations of riluzole in female C57 mice. Riluzole was given with the drinking water (200 $\mu g\,ml^{-1}$ or approximately 40 mg/kg). Each point represents the mean ± S.D. of six animals.

regression equation $y = 0.0041x + 0.0077$) and from 0.0043 to 0.0048 for brain ($y = 0.0045x + 0.0080$). The lowest calibration standard corresponded to the LOQ (100 $ng\,ml^{-1}$ or g^{-1} using 0.1 ml of plasma or approximately 100 mg of brain tissue), i.e. the lowest concentration that could be measured with acceptable accuracy and precision as calculated in separate studies (≤20%). This LOQ was sufficient for pharmacokinetic studies in the mouse although even lower limits could be reached by increasing the volume of plasma or brain homogenate.

Inter-day assay coefficients of variation (CV) ranged from 2.8 to 4.0% in plasma and from 2.9 to 3.9% in brain, with overall mean accuracy (RE), calculated from the deviation of the mean concentration from the nominal value, from 0.3 to 1.5% for plasma and from −0.2 to 2.7% for brain. The intra-day validation results are summarised in Table 3.

Riluzole was stable in plasma and brain homogenate for at least 2 h at room temperature, i.e. approximately the time needed for handling the daily series of biological samples. The drug was assumed to be stable when stored for longer times at −20 °C [10].

3.2. Drug measurements

The method was successfully applied for riluzole measurements in transgenic SOD1-G93A mice given this drug alone and in combination with neuroprotective agents having different mechanism of action. These included the tetracycline derivative minocycline and the naturally occurring compound L-acetyl-carnitine. Minocycline is reported to delay onset and extend survival in transgenic mouse models of ALS [16–18], and is currently undergoing clinical trials in ALS patients [19]. L-Acetyl-carnitine behaves as a neurotrophic and neuroprotective agent in cultured motoneurons and its potential in various neurodegenerative diseases are under investigation [20]. Both minocycline and L-acetly-carnitine enter the CNS of man and animals when taken orally [21,22] the tetracycline by passive diffusion [21] and carnitine by a sodium-dependent transport system [23].

Fig. 2 shows the plasma and CNS concentrations of riluzole in mice, at the end of the pharmacology study. Fig. 3 compares the whole-brain concentrations of riluzole in C57 and SOD1-G93A mice given the drug alone or with minocycline and L-acetyl-carnitine. Like in rats and monkeys [12], riluzole rapidly crossed the blood-brain barrier and concentrated in the CNS of mice, regardless of the genetic status. In C57 mice the mean whole brain-to-plasma concentration ratio averaged 4.3 (Fig. 2), slightly lower than the value calculated with ^{14}C-radiolabeled riluzole in rats [12]. In SOD1-G93A mice the brain-to-plasma distribution ratio could not be accurately determined because the drug plasma concentrations were near to or below the limit of quantitation in several animals. Riluzole spinal cord concentrations were slightly higher than whole brain concentrations, suggesting an uneven distribution within the central nervous system. No comparison can be made with other species as no attempt has yet been made to characterise this aspect of riluzole distribution, although ^{14}C-radiolabeled riluzole concentrate more in the white matter than in the grey matter of the rat brain [12].

Riluzole brain and spinal cord concentrations in C57 mice showed wide variability, with CV above 50% in both tissues. This is because the drug was dissolved and given with the drinking water and therefore the amount of drug taken varied from animal to animal, continually changing within the study period.

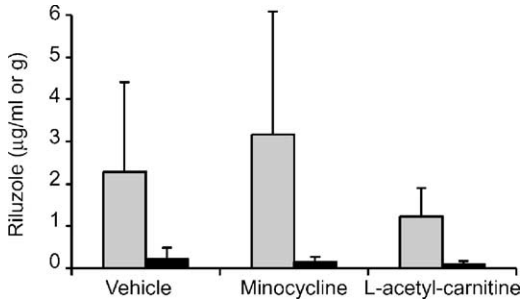

Fig. 3. Mean brain concentrations of riluzole in non transgenic and transgenic animals at the end of the treatment. Schedule with riluzole (200 $\mu g\,ml^{-1}$ or approximately 40 mg/kg), minocycline (100 mg/kg, orally) and L-acetyl-carnitine (500 $\mu g\,ml^{-1}$ or approximately 100 mg kg^{-1}). Each point is the mean ± S.D. of three to six animals for C57 (open bars) and SOD1-G93A mice (closed bars).

Variability was even wider in SOD1-G93A animals, which also had rather low riluzole brain concentrations (0.21±0.28 μg/g), compared to C57 mice (2.29±2.12 μg/g). This is possibly because in the terminal stadium of the disease the transgenic animals suffered severe paralysis and therefore could not move easily to take the daily riluzole dose like the normal mice. These findings should be taken into account when planning pharmacological studies of riluzole in animal models of ALS.

Minocycline and L-acetyl-carnitine did not affect riluzole brain-to-plasma partition in C57 mice (4.6 ± 1.6 and 3.8 ± 0.8, respectively) or the riluzole brain concentrations in SOD1-G93A transgenic animals (see also Fig. 3). Minocycline undergoes little metabolism in animals [24] (not in man) and L-acetyl-carnitine is rapidly eliminated with the urine after equilibration with L-carnitine via carnitine acetyl-transferase [22], whereas riluzole is extensively metabolised by oxidation and conjugation in all species so far investigated [12].

4. Conclusions

Current quests for therapy of ALS include studies of combination of agents that act by different neurochemical mechanisms [2–4]. Because riluzole provides only symptomatic relief its combination with other neuroprotective agents is also being evaluated [2–4]. These studies are usually in transgenic mouse models, which allow a relatively cheap and rapid evaluation of new potential therapies; pharmacokinetic studies may help clarify the mechanism(s) of the interaction and any relationships with the pharmacological response in these models, facilitating the extrapolation of results to the clinical situation. A fast and simple analytical method like the one proposed here is a pre-requisite for such studies.

The method requires only a small volume of plasma or tissue homogenate and the LOQ is sufficient for pharmacokinetic studies in small animals such as the mouse, though even lower limits could be reached by increasing the volume of plasma or brain homogenate. Moreover, riluzole is measurable over a wide range, with mean CV and RE generally below 10%. It appears, therefore that this is a workable means of quantitating riluzole for pharmacokinetic and concentration-response studies.

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