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Short communication

Simple determination of riluzole in rat brain by high-performance liquid chromatography and spectrophotometric detection

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Abstract

A simple method was developed for separation and quantification of riluzole in rat brain. The analyses were performed by high-performance liquid chromatography using a C_{18} reversed-phase column (Hypersil ODS) with UV detection at 264 nm. The mobile phase consisted of methanol–water containing 1% triethylamine adjusted with orthophosphoric acid to pH 3.2. The retention time was 8.6 min. A simple liquid–liquid extraction with ethyl acetate was used to obtain riluzole from brain samples. The limit of quantification was 10 ng/g. The recovery was about 80%. The relationship between peak areas and concentrations was linear over the range between 0.01 and 0.8 μg/g, with r^2 value over 0.99. The assay provided good reproducibility and accuracy and proved to be suitable for pharmacokinetic studies of riluzole.

Keywords: Riluzole; Spectrophotometric detection; Brain; Rat

1. Introduction

Riluzole (Fig. 1), 2-amino-6-trifluoromethoxybenzothiazole, is an anti-glutamatergic agent, which was found to be protective in several models of neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) [1], multiple sclerosis (MS) [2], Parkinson's disease (PD) [3], and ischemia [4]. Riluzole was demonstrated to modulate the anti-glutamatergic activity through glutamate and sodium receptors. Several studies showed that riluzole inhibits the release of glutamate and L-aspartate from nerve terminals, modulates the *N*-methyl-D-aspartate (NMDA) ionotropic receptors and stabilizes the voltage-dependent sodium channels in myelinated fibers [5]. Due to its interesting pharmacology properties and potential therapeutic applications a new and easy HPLC method for the determination of riluzole in rat brain could be useful to support pharmacokinetic studies. Previous studies described methods for determination of riluzole in human plasma and urine by high-performance liquid chromatography with ultraviolet detection [6] or coupled with tandem mass spectrometry [7], which were unsuitable to determine riluzole in rat brain. Pharmacokinetics studies of riluzole done in rat and monkeys used a radiolabeled assay [8]. However, this method of quantification is not very convenient for routine analysis. A recent method [9] based on high-performance liquid chromatography with ultraviolet detection has been described for riluzole. The method used a sample preparation procedure based on solid-phase extraction and a relatively long analysis time by HPLC. Therefore, the aim of this study was to develop and validate a rapid and sensitive method for the determination of riluzole in rat brain, with a simple liquid–liquid extraction, which could be easily applicable routinely.

2. Experimental

2.1. Chemicals

Riluzole was obtained from Sigma (Milan, Italy). All solvents and chemicals were of HPLC or analytical grade. Methanol and ethyl acetate were obtained from Merck

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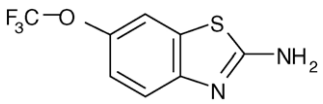

Fig. 1. Structure of riluzole.

(Milan, Italy). Triethylamine, 85% orthophosphoric acid, and perchloric acid were purchased from Aldrich (Milan, Italy).

2.2. Instrumentation

The pH was determined using a Metrohm 691 pH meter (Metrohm Ltd., Herisau, Switzerland). The sample preparation procedure utilized a Heidolph REAX 2000 vortex mixer, a Nüve NF 800 R centrifuge with RA 200 Swing Out rotor (AR-EL Group, Greece) and an ALC microcentrifugette 4214 centrifuge (ALC International, Milan, Italy). The HPLC apparatus (Agilent Technologies, Milan, Italy) was a Hewlett Packard HP 1100 chromatographic system interfaced with the HP ChemStation software and equipped with a binary pump G1312A, a diode array detector (DAD) G1315A and a thermostated column compartment G1316A. A Hypersil ODS C_{18} reverse-phase column (150 mm × 4.6 mm i.d., 5 μm) and a Hypersil ODS C_{18} column (7.5 mm × 4.6 mm i.d., 5 μm), utilized as a guard column, were purchased from Alltech (Milan, Italy).

2.3. Brain collection

Adult male Sprague–Dawley rats, weighing 250–300 g (Charles River, Calco, Italy), were used. The animals were treated in accordance with European guidelines for the care and use of laboratory animals (86/609/EEC).

Brain was excised from rats and stored at −20 °C until analyses.

2.4. Sample preparation

Rat brain was thawed and 1 g brain, accurately weighed, was added with 2 ml of borate buffer (0.055 M; pH 8.5). The sample was vortex-mixed vigorously with a pestle until complete homogenization and then extracted with 2 ml of ethyl acetate. The mixture was shaken for 4 min and then centrifuged at 5000 × *g* for 15 min. The organic layer was removed and the extraction repeated with another 2 ml of ethyl acetate. The organic layers were combined and evaporated to dryness under nitrogen. The residue was reconstituted in 200 μl of mobile phase, vortex-mixed and centrifuged at 10,000 × *g* for 5 min. The supernatant was aspirated with a tuberculine syringe, filtered through a 0.45 μm nylon membrane filter and injected onto the HPLC system.

2.5. Standards

A stock solution containing riluzole (220 μg/ml) was prepared by directly dissolving in a mixture of water/methanol (50/50). Aliquots of the stock solution were added to blank brain homogenate to give appropriate ranges of concentrations, from 0.01 to 0.8 μg/g riluzole, for the calibrations.

2.6. Chromatographic conditions

The mobile phase consisted of 68% CH_3OH and 32% of an aqueous solution containing 1% TEA adjusted to pH 3.2 with H_3PO_4. Prior to use, the mobile phase was filtered through a 0.2 μm nylon membrane filter. The UV detector was set at 264 nm with a flow rate of 0.5 ml/min. Chromatography was performed at 25 °C. The injected volume was 50 μl.

2.7. Calibration curves

One gram of drug-free rat brain, accurately weighed, was homogenate with 2 ml of borate buffer (0.055 M; pH 8.5) and spiked with standard solution containing riluzole in order to obtain a concentration in the range of 0.01–0.8 μg/g. Spiked brain samples were taken through the assay procedure and calibration graph was constructed by plotting riluzole peak-area versus the concentration of the analyte. Linear regression analysis was used to calculate the slope, intercept, and correlation coefficient of the calibration curve.

2.8. Recovery, intra- and inter-day precision

The recovery of riluzole from the brain was determined by comparing the peak areas obtained from the direct injection of standard solutions of compounds with those found by extraction ($n = 5$ for each concentration of riluzole) from spiked brain taken through the assay procedure already described. The measurements of intra- and inter-day variability were utilized to determine the accuracy and precision of the developed assay. Three concentrations of riluzole were chosen to test both intra- and inter-day variations. Relative standard deviation (R.S.D.) was taken as a measure of precision, and the percentage difference between determined and spiked amounts was considered a measure of accuracy. Samples at each given concentration were analyzed five times for intra-day variation. While, the inter-day reproducibility was examined three times on five separate days (Table 1).

2.9. Stability test

The stability of riluzole in rat brain was investigated. Spiked samples were prepared with drug-free brain at two concentration levels (0.08 and 0.8 μg/g). Spiked brain ho-

Table 1
Intra- and inter-day variation for the assay of riluzole

Analyte spiked (μg/g)	Mean calculated concentration (μg/g)	R.S.D. (%)[a]	Accuracy (%)[b]
Intra-day ($n = 5$)			
0.06	0.062 ± 0.005	8.6	104
0.30	0.306 ± 0.013	4.1	101.8
0.60	0.636 ± 0.031	4.8	106
Inter-day ($n = 15$)			
0.06	0.063 ± 0.006	9.5	105
0.30	0.308 ± 0.012	3.9	102.6
0.60	0.632 ± 0.030	4.7	105.3

[a] R.S.D. (%) = (S.D./mean) × 100.
[b] Accuracy (%) = [1 − (concentration spiked − mean concentration measured)/concentration spiked] × 100.

mogenate samples were divided in two portions. One portion was stored at −20 °C, thawed and analyzed on weeks 0, 1, 2, and 4. The other portion was treated as described in the sample preparation, divided in two portions and stored at 4 °C and room temperature, respectively. At 6, 12, and 24 h after extraction each sample in both portions was directly analyzed by HPLC.

3. In vivo rat study

In order to demonstrated the suitability of our newly developed riluzole HPLC assay in brain for pharmacokinetic studies, we performed a pilot study in the rat. A solution of riluzole (5 mg/kg) was injected intraperitoneally in the rat. Brain samples were collected 8, 16, and 30 h post-dose and stored at −20 °C until analysis by HPLC.

4. Results and discussion

This study was performed to determine the optimal conditions for extraction of riluzole from rat brain and for chromatographic separation.

Under the chromatographic conditions described in our study, retention time of 8.6 min for riluzole was observed. No interfering peaks were observed in the blank brain chromatogram, even at LOQ value of riluzole as shown in Fig. 2, indicating the efficient clean up method used. Representative chromatograms for riluzole in rat brain are shown in Fig. 3. Good recovery levels were achieved at all the concentrations that were studied (0.06, 0.3, and 0.6 μg/g) with a range of 75–80%. The detector response was linear over the concentration range from 0.01 to 0.8 μg/g. Linear regression analysis performed for calibration curves yielded correlation coefficients of 0.9988 and good linearity with a negligible intercept ($y = 59.1768x - 1.4232$). The limit of quantification, i.e. the lowest concentration of riluzole that could be determined in brain, was 10 ng/g

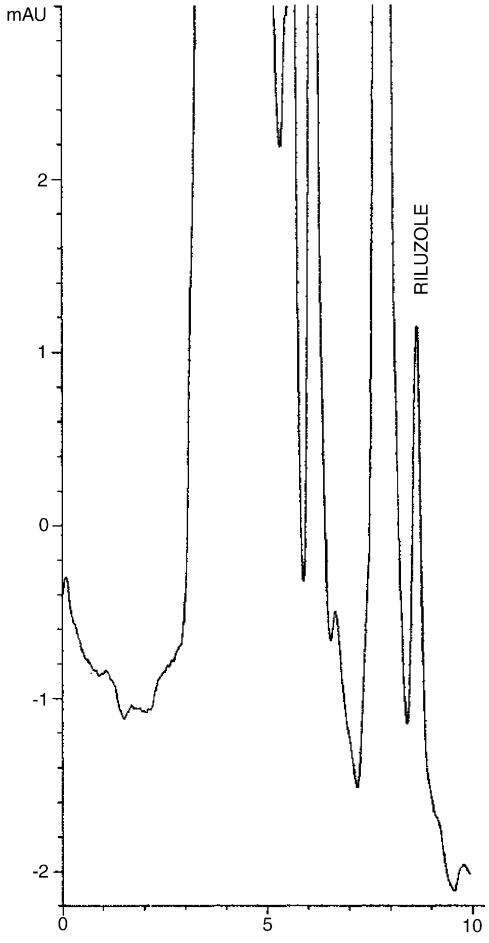

Fig. 2. Chromatogram of riluzole in brain extract corresponding to the limit of quantitation (LOQ = 10 ng/g).

with an R.S.D. of 11.4% and accuracy of 107.2% ($n = 8$) Validation of our assay method consisted of intra- and inter-day reproducibility studies at three concentration levels of riluzole: 0.06, 0.3, and 0.6 μg/g. Table 1 shows the intra-day precision, with R.S.D. range from 4.1 to 8.6%, and the inter-day precision, with R.S.D. range from 3.9 to 9.5%, indicating the very good reproducibility of this method.

A stability study was conducted to determine the best storage temperature for brain samples. The results demonstrated that riluzole was stable for longer than 12 h at 4 °C and for longer than 24 h at room temperature. Furthermore, riluzole was stable up to 4 weeks when stored at −20 °C. Therefore, all extracted samples were stored refrigerated at 4 °C for the same day analysis, whereas brain samples were frozen at −20 °C until analysis by HPLC.

To show the applicability of the method to pharmacokinetic studies, an in vivo pilot pharmacokinetic study was carried out in rat. A representative chromatogram of rat brain, removed 16 h after i.p. somministration of riluzole, is showed in Fig. 3.

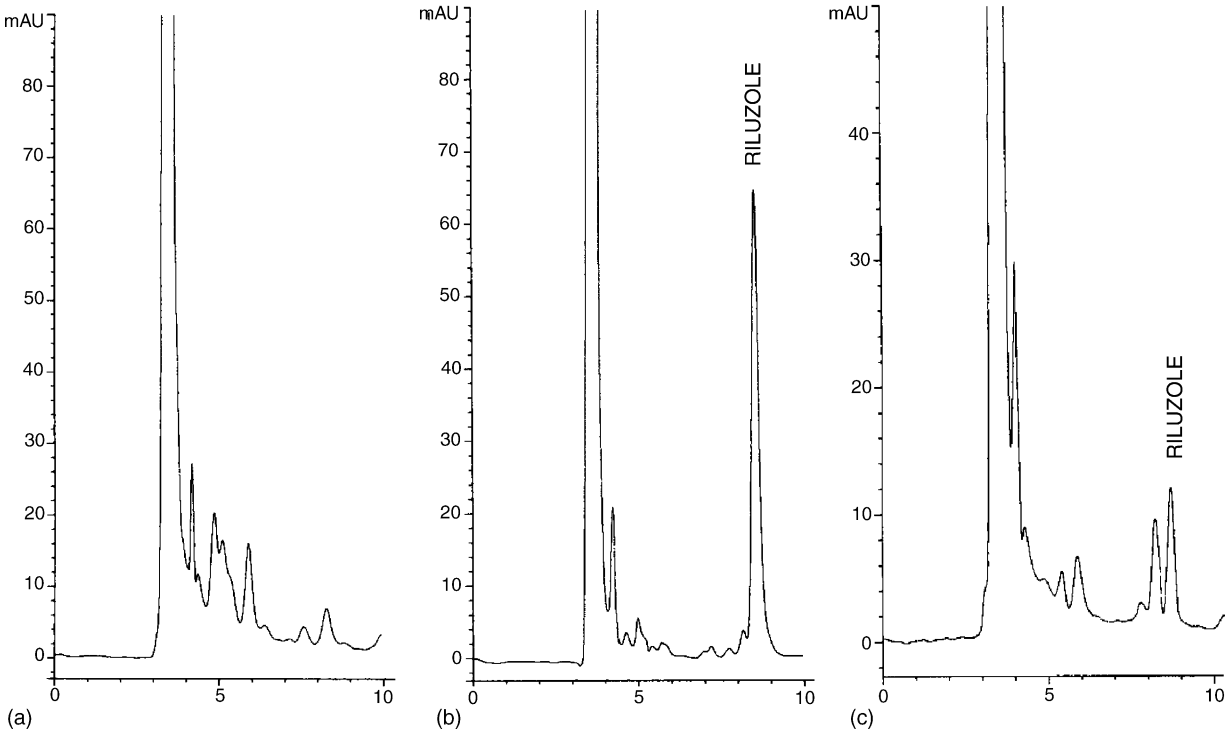

Fig. 3. Representative chromatograms of (a) blank brain, (b) brain sample spiked with riluzole (0.6 μg/g), (c) brain removed 16 h after i.p. somministration of riluzole (0.130 μg/g).

5. Conclusions

A specific HPLC method using spectrophotometric detection was developed and fully validated for the determination of riluzole in rat brain. The assay is rapid, selective and accurate. Furthermore, the extraction procedure is simple, allowing sufficient sample throughput to be applied to pharmacokinetic studies.

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