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Short Communication

Determination of the neuroprotective agent 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione in plasma using high-performance liquid chromatography

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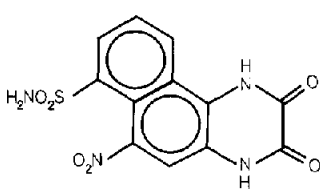
ABSTRACT

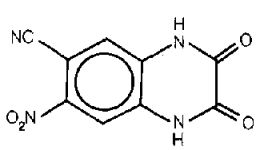
A rapid, sensitive and selective high-performance liquid chromatographic method for the determination of the neuroprotectant 6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione in rat plasma has been established and validated. Samples of 0.5 ml of plasma are extracted by elution from a Bond-Elut® column with methanol and analysed on a reversed-phase column. The wavelength of UV detection is 254 nm. The method is linear at least up to 30 μg/ml, with a lowest reliable determination level of 4 mg/ml. The assay has a coefficient of variation of 13% at 10 ng/ml and 4% at 1000 ng/ml. Small variations in the extraction procedure and the liquid chromatographic conditions have minimal or no influence on the assay.

INTRODUCTION

6-Nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione (I, Fig. 1) is a potent glutamate antagonist with a high affinity for the α-amino-3-hydroxy-5-methyl-4-



I



II

Fig. 1. Structures of I and the internal standard II.

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isoxazolepropionic acid (AMPA) site [1]. The compound is effective as a neuroprotectant for cerebral ischemia [2,3].

Very few compounds of related structure have been described in the analytical chemical literature. Page *et al.* [4] studied the metabolism of a sedative quinoxaline-2,3-dione. Isolation and identification were performed by thin-layer chromatography and combined gas chromatography–mass spectrometry. The enol form of quinoxaline-dione is the product in a derivatization procedure for the analysis of oxalic acid [5]. No other liquid chromatographic methods have been reported.

This paper describes a high-performance liquid chromatographic (HPLC) method for determination of I in rat plasma.

EXPERIMENTAL

Chemicals

Compound I (sodium salt) was from batch No. Rob 1.121 (Novo Nordisk, Soeborg, Denmark. The internal standard, 6-cyano-7-nitroquinoxaline-2,3-dione (II), was from batch No. PJA VI 16 (Novo Nordisk). Water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France). All organic solvents were HPLC-grade purity.

Animals

Blood samples from female Wistar rats (200 g, from Moellegaard, Ll. Skensved, Denmark) were collected in Vacutainers® (Grenoble, France)), and plasma was obtained by centrifugation at 1200 *g* for 10 min.

Instrumentation

A Vac-Elut® SPS24 solid-phase extraction apparatus (Analytichem International, Harbor City, CA, USA) was used for extraction. A Merck system consisting of an L6200 pump, a column oven, a 655A-40 autosampler and an L4200 UV detector (294 nm) was used. Data were collected on a Merck HPLC software (D-6000) system. The column was a 25 cm × 4 mm I.D. LiChrospher RP-18 end-capped, 5 μm particle size (Merck, Virum, Denmark). The mobile phase was tetrahydrofuran–13 m*M* phosphoric acid (pH 2.35) (15:85, v/v). The flow-rate was 1.0 ml/min at 40°C. The specificity was investigated using a Hewlett Packard 1040A diode-array detector.

Extraction procedure

A Bond-Elut C_8 extraction column (500 mg, 3 ml) placed in the Vac-Elut unit ("waste" position) was washed twice with 1 ml of methanol and twice with 1 ml of water, followed by 200 μl of 13 m*M* phosphoric acid (pH 2.35). A 500-μl sample of plasma, 50 μl of internal standard solution (normally 10 μg/ml) and 500 μl of 8 *M* urea in 13 m*M* phosphoric acid (for denaturation of proteins) were added, and the column was submitted to gentle suction. A 1-ml volume of 13 m*M* phosphoric

acid was then added, and gentle suction again applied. The Vac-Elut unit was switched to the "collect" position, and 2 ml of methanol were added under gentle suction. The collected eluate was transferred to a 10-ml tube and evaporated at 45°C under nitrogen. The residue was redissolved in 75 μl of the mobile phase, centrifuged (200 *g*, 5 min), transferred to HPLC autosampler vials and centrifuged (3500 *g*, 5 min) again, and injected into the HPLC system. Normally, 25 μl were injected but for small concentrations of I the injection volume was 50 μl.

Plasma samples were spiked by addition of aqueous solutions of I. This addition resulted in less than 5% water added to plasma.

RESULTS AND DISCUSSION

Linearity

Although the method was found to be linear in the range 0–30 μg/ml, for practical purposes it is necessary to use two sets of standard curves in order to get accurate results at the low concentration level.

Typical standard curves gave correlation coefficients $r = 1.000$ for the range 0–30 μg/ml and $r = 0.999$ for the range 0–100 ng/ml. The standard curves were obtained with different concentrations of internal standard.

Lowest reliable determination level and detection limit

The lowest reliable determination level, calculated as three times the standard deviation of the lowest assayed standard, was 4 ng/ml. The detection limit was 2 ng/ml, at a signal-to-noise ratio of 3.

Recovery

The absolute recovery of I varied because of the procedure using the internal standard. It was determined by measuring the amount of I recovered after extraction and analysis of spiked samples. More than 70% of the compound was recovered after extraction.

Accuracy and precision

Within-assay accuracy and precision were determined by analysing spiked plasma samples at five concentrations with up to six determinations at each level. Standard curves were constructed for (i) concentrations below 100 ng/ml and (ii) the whole concentration range. The concentration of I in each sample was determined using the appropriate standard curve. The procedure was carried out four or five times on different occasions, and the same standards were used each time. The results are shown in Table I. Minor deviations from nominal concentration were observed, but these were not considered to be important. The overall means and standard deviations were calculated.

Between-assay accuracy and precision were determined by analysing four plasma pools, spiked with different concentrations of I, on five or six occasions during

TABLE I

WITHIN-ASSAY ACCURACY AND PRECISION (REPEATABILITY)

Means ± S.D. were determined on different occasions (n = number of determinations). Pooled mean and standard deviations are calculated using AOVONEWAY (Minitab statistical software version 7.2, Minitab, State College, PA, USA).

Concentration added (ng/ml)	n	Concentration found		Coefficient of variation (%)
		ng/ml	%	
11.49	21	11.1 ± 1.4	97	13
114.9	26	115 ± 5.8	100	5.0
1149	26	1125 ± 51	98	4.5
11 490	28	11 412 ± 484	99	4.2
28 570	22	28 570 ± 1098	100	3.8

a period of two weeks. The determinations were carried out using fresh standards each time. The results are shown in Table II. The data show a 3% deviation from the nominal concentration at the 10.6 μg/ml level, which is not considered important.

Selectivity

The selectivity of the method was judged by injecting authentic plasma samples (30 and 120 min after administration of 30 mg/kg intraperitoneally) in an HPLC system with a diode-array detector. Comparison of UV spectra on different sites of the peak of I indicated no impurities. Typical chromatograms are shown in Fig. 2.

TABLE II

BETWEEN-ASSAY ACCURACY AND PRECISION (REPRODUCIBILITY)

Concentration added (ng/ml)	n	Concentration found		Coefficient of variation (%)
		ng/ml	%	
10.60	5	10.1 ± 1.3	95	13
106.0	5	106.6 ± 2.6	101	2.4
1060	6	1096 ± 42	103	3.8
10 600	6	10 898 ± 217	103[a]	2.0

[a] = Significantly different from nominal concentration ($p < 0.05$).

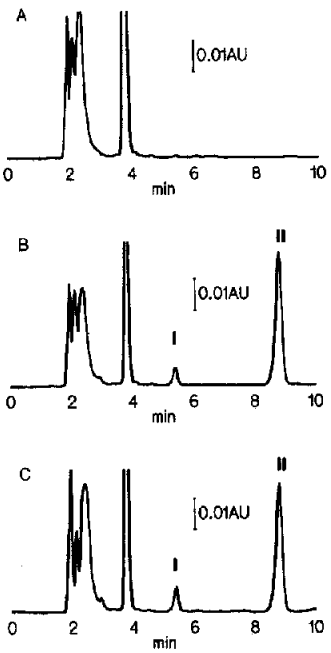

Fig. 2. Chromatograms of (A) blank rat plasma, (B) rat plasma spiked with I (208 ng/ml) and (C) rat plasma 1.5 h after intravenous administration of I at a dose of 2 mg/kg. The concentration of I is 280 ng/ml. The concentration of the internal standard (II) in B and C is 962 ng/ml.

Ruggedness

A number of parameters were investigated in order to see possible effects on the assay.

The influence of the evaporation temperature was studied by analysing three plasma pools. A standard curve was constructed using the standard procedure at

TABLE III

INFLUENCE OF THE AMOUNT OF PLASMA USED IN THE ASSAY OF I

The data are mean of two determinations.

Nominal concentration (ng/ml)	Concentration found					
	In 100 μl plasma		In 250 μl plasma		In 500 μl plasma	
	ng/ml	%	ng/ml	%	ng/ml	%
36	38	106	36	100	35	97
615	592	96	590	96	590	96
12 195	12 127	99	12 327	101	11 714	96

45°C. The concentrations of the plasma pools analysed by evaporation at 60°C were determined from the standard curve. The difference between nominal and measured concentrations was found to be less than 2%. Elevation of the temperature resulted in a 30% decrease of the evaporation time.

The influence of the amount of plasma used in the assay was studied by analysing 100, 250 and 500 μl of plasma from the same plasma pools. Standard curves were constructed as described above, and a linear relationship between the concentration and the peak-height ratio was found in all cases. Comparison of the results is made in Table III.

The effect of the column temperature (30–55°C) on the retention time was studied using plasma samples. No problems of selectivity were observed in this temperature range.

The consequences of small variations of pH of the mobile phase were investigated. Variations of pH in the interval 1.85–2.85 gave only minor variations in retention times.

The tetrahydrofuran concentration had a large influence on the retention time. It is possible to increase the amount of tetrahydrofuran in the mobile phase in order to minimize the analysis time, but this will lead to higher back-pressure and will, furthermore, make higher demands on the column quality.

The number of theoretical plates calculated for I (5–10 μg/ml of an aqueous standard in 25 μl) was, however, apparently not a critical parameter. Three columns were used during the validation procedure, and the number of theoretical plates was determined regularly. The initial plate count was *ca.* 9000. When the plate count fell below *ca.* 2000, the column was either turned around or replaced.

CONCLUSION

The method described in the present report has a sufficient detection limit, a good accuracy and precision, and a selectvity that does not change significantly with small variations in the analytical procedure. The method is well suited for plasma analysis in connection with pharmacokinetic studies of I in rats.

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