Journal of Chromatography, 643 (1993) 419–425
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2746

Determination of paraquat in rat brain by high-performance liquid chromatography

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ABSTRACT

The applications of a method based on ion-pair solid-phase extraction and reversed-phase HPLC are reported. The method was used to measure paraquat concentrations in discrete brain areas at different times after its systemic administration in rats. In addition, the method was employed in the determination of paraquat levels in whole-brain samples from rats of various ages systemically treated with several doses of the herbicide.

INTRODUCTION

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a potent non-selective foliage-applied contact herbicide. It was discovered in 1955 and introduced in agriculture and horticulture for weed control in 1962. Paraquat is inactivated rapidly in sunlight and on contact with most soils so that no biologically active residues remain in the soil and almost immediate sowing or planting is allowed. The lack of biologically active residues, together with its speed of action, rainfastness and lack of selectivity, makes paraquat an essential agent in chemical weed control and, indeed, it is widely used in many countries of the world [1].

The herbicide, however, is toxic to man, and since its marketing in 1962 hundreds of cases of human death have been attributed to paraquat poisoning [2]. Almost all of the cases resulted from suicidal or accidental ingestion of the herbicide, although there are some reports of deaths caused by dermal exposure (see ref. 3).

The herbicide displays a peculiar toxicity for the lungs, where it accumulates, in an energy-dependent manner, by an uptake system shared by polyamines, leading to acute alveolitis, widespread fibrosis and fatal hypoxia [4–6]. The mechanism of paraquat toxicity involves the cyclic reduction/reoxidation of the herbicide with production of reactive oxygen species, consumption of NADPH and induction of lipid peroxidation, resulting in cell death [7–9]. Although paraquat poisoning produces mainly lung injury, toxic effects on heart [10], liver [11], kidney [12] and brain [13,14] have also been described.

Recently, the discovery that 1-methyl-4-phenylpiridinium ion (MPP^+), a compound exhibiting a striking chemical analogy with paraquat and marketed as herbicide under the name Cyperquat, induces a parkinsonian-like state in humans and primates [15,16] has renewed interest about the possibility that environmental chemicals, including paraquat, may be related to the development of Parkinson's disease [17–19].

Several epidemiological studies indicate a strong correlation between exposure at an early age to rural environment and drinking well water and development of idiopathic Parkinson's disease [20,21]. In addition, a higher prevalence of Parkinson's disease

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0021-9673/93/$06.00

has been found in those rural areas of Quebec specializing in market gardening and pulp milling and utilizing higher levels of herbicides and pesticides [22].

Bocchetta and Corsini [23] reported the case of early-onset Parkinson's disease in two men exposed to herbicides and pesticides. However, for several reasons, including the poor penetration of paraquat into the central nervous system (CNS) due to its chemical structure, doubt has been expressed about the ability of paraquat to cause Parkinson's disease (see ref. 24).

Rigidity and akinesia have been reported in frogs after intraperitoneal injection of paraquat [25]. In addition, following systemic administration of [^{14}C]paraquat in frogs it was demonstrated that, in spite of its poor penetration into the brain, a high concentration of radioactivity can be observed in the neuromelanin-containing nerve cells [26].

Until recently, no analytical techniques had been established for accurate detection of paraquat in the brain. We have developed a method based on the use of ion-pair reversed-phase high-performance liquid chromatography (HPLC) with UV detection that is reliable and easy to perform [27]. This technique allowed the detection of whole-brain paraquat concentrations as low as 0.030 μg/g wet brain mass in rats systemically treated with the herbicide (1 mg/kg subcutaneously). Owing to the sensitivity of the method, we have now studied the concentrations of paraquat in specific brain regions after its systemic injection in rats.

Evidence exists in the literature indicating that the permeability of the blood-brain barrier (BBB) can be affected by ageing processes (see ref. 28). Therefore, we studied brain paraquat concentrations in rats of different ages after systemic injection of the herbicide.

EXPERIMENTAL

Experimental animals, collection and preparation of samples

Two-week-old and adult (3, 12 and 24 months old) male Wistar rats, housed in stable conditions of humidity (65%) and temperature (22 ± 2°C), were used. They were fed with a standard diet and water *ad libitum*. Paraquat dichloride (1,1′-dimethyl-4,4′-bipyridinium dichloride; Sigma, St. Louis, MO, USA), dissolved in bidistilled pyrogen-free water, was administered by subcutaneous (s.c.) injection (1, 2.5, 5, 20 or 100 mg/kg) in a volume of 1 ml/kg body mass. After 1, 3 or 24 h, the animals were sacrificed and the brain rapidly removed, weighed and stored at −20°C until analysis. To measure paraquat levels in specific areas of the CNS, the brain was placed on an ice-chilled Petri dish and the hypothalamus, prefrontal cortex, pyriform cortex, mesencephalon, hippocampus, caudate, pons and medulla oblongata were rapidly dissected out, weighed and stored as for whole-brain samples.

Paraquat was determined in whole-brain samples using ion-pair solid-phase extraction and reversed-phase HPLC with UV detection as recently described [27]. The same extraction procedure and HPLC method were employed with only minor modifications in the measurement of paraquat concentrations in discrete brain areas. Briefly, tissue samples were homogenized with a Branson Model 250 ultrasonic cell disruptor in 2–4 ml of 0.10 *M* perchloric acid containing known amounts (0.375–7.5 μg/g wet tissue mass) of internal standard (1,1′-diethyl-4,4′-bipyridinium diodide). Following centrifugation (15 min at 15 000 *g*), the supernatants were transferred to polypropylene tubes containing 250–500 μl of ammonium hydroxide. After vortexing (30 s) and subsequent centrifugation (15 000 *g*; 15 min), the resulting supernatants were passed through disposable Sep-Pak C_{18} cartridges (Water), previously prepared for ion-pair extraction by eluting with 10 ml of alkaline sodium heptanesulphonate. The cartridges were washed with water (5 ml) and methanol (5 ml). Paraquat and internal standard were then eluted with 5 ml of acidic methanol. The eluates were evaporated to dryness at 40°C under a stream of air and reconstituted in the HPLC mobile phase (0.4–8 ml/g wet tissue mass). The injection volume was 20 μl.

Chromatography and mobile phase

The HPLC system consisted of a Beckman Model 110B pump with an Altex Model 210A injection valve connected to a 45 mm × 4.6 mm I.D. Beckman Ultrasphere ODS guard column (C_{18} reversed-phase, particle size 5 μm) followed by a 25 cm × 4.6 mm I.D. Altex Ultrasphere ODS analytical column (C_{18} reversed-phase, particle size 5 μm). A Beckman Model 163 variable-wavelength UV detector

monitored the column effluent at 258 nm. The mobile phase consisted of 7.5 m*M* sodium heptanesulphonate and 0.10 *M* orthophosphoric acid (pH adjusted to 3.00 with triethylamine) plus acetonitrile to yield a 10% (v/v) mixture. The flow-rate was 1.0 ml/min at room temperature.

Calibration and statistical analysis

To construct the calibration curves, aliquots of brain tissue from untreated animals were spiked with known amounts of paraquat and internal standard to give final paraquat concentrations ranging from 0.015 to 0.960 or from 0.300 to 9.600 μg/g wet tissue weight and taking the samples through the entire procedure. Calibration curves for whole-brain sample analysis ranged from 0.015 to 0.360 μg/g wet brain mass. Results were plotted as the paraquat/internal standard peak-height ratio *versus* concentration of paraquat, and the ratio for an unknown sample was converted into concentration by interpolation.

The results are expressed as mean ± S.E.M. Differences between means were assessed by unpaired Student's *t*-test and were considered significant when p was <0.05. In addition, the differences in the mean regional brain paraquat concentrations at 1-, 3- or 24-h intervals were evaluated by one-way analysis of variance (ANOVA). To verify whether herbicide levels in discrete brain areas were dose-related, analysis of the regression line was performed by using the mean concentration values obtained 24 h after 5, 20 and 100 mg/kg paraquat; the correlation was considered significant when the correlation coefficient (r) yielded a values of $p < 0.05$.

RESULTS AND DISCUSSION

The systemic administration of paraquat (5.0 mg/kg s.c.) yielded different brain regional distributions at 1, 3 and 24 h following treatment (Table I). In particular, 1 h after the acute administration, the lowest levels of herbicide were observed in the caudate nucleus (0.073 ± 0.016 μg/g wet tissue mass), highest concentrations being obtained in the prefrontal cortex (1.047 ± 0.151 μg/g); paraquat concentrations ranging from 0.177 ± 0.020 (hippocampus) to 0.721 ± 0.052 μg/g (hypothalamus) were obtained in the other regions studied (Table I).

The rate of paraquat elimination was time dependent in the prefrontal cortex and the hypothalamus, where the lowest levels were detected 24 h after treatment; by contrast, in the other brain areas the

TABLE I

PARAQUAT CONCENTRATIONS (μg/g WET TISSUE MASS) IN DISCRETE AREAS OF RAT BRAIN AS DETERMINED 1, 3 OR 24 h AFTER ADMINISTRATION OF 5.0 mg/kg s.c. HERBICIDE

Values are the mean ± S.E.M. of 3–9 determinations. Each sample of discrete area was the pool from six brains. Significant regional differences in paraquat concentration: $*p < 0.01$, $F = 17.87$; $**p < 0.01$, $F = 79.35$; $***p < 0.01$, $F = 8.85$ (one-way ANOVA).

Brain area	Time after administration		
	1 h*	3 h**	24 h***
Hypothalamus	0.721 ± 0.052	0.573 ± 0.030[a]	0.328 ± 0.023[a]
Prefrontal cortex	1.047 ± 0.151	0.280 ± 0.030[c]	0.166 ± 0.031[c]
Mesencephalon	0.241 ± 0.021	0.118 ± 0.010[c]	0.176 ± 0.030
Hippocampus	0.177 ± 0.020	0.108 ± 0.005[c]	0.137 ± 0.009[d]
Caudate	0.073 ± 0.016	0.046 ± 0.005	0.085 ± 0.009[e]
Pons	0.491 ± 0.110	0.115 ± 0.010[c]	0.283 ± 0.042[e]
Medulla oblongata	0.376 ± 0.080	0.116 ± 0.006[c]	0.159 ± 0.036

[a] $p < 0.05$ *vs.* 1-h concentrations.
[b] $p < 0.01$ *vs.* 1- and 3-h concentrations.
[c] $p < 0.01$ *vs.* 1-h concentrations.
[d] $d < 0.05$ *vs.* 3-h concentrations.
[e] $p < 0.01$ *vs.* 3-h concentrations (unpaired Student's *t*-test).

TABLE II

PARAQUAT CONCENTRATIONS (μg/g WET TISSUE MASS) IN DISCRETE AREAS OF RAT BRAIN AS DETERMINED 24 h AFTER ADMINISTRATION OF 5.0, 20 OR 100 mg/kg HERBICIDE

Values are the mean ± S.E.M. of 3–5 determinations. The data concerning 5.0 mg/kg dose were obtained in samples of discrete areas consisting of pools from six brains. Analysis of the regression line: * significant correlation at the $p < 0.01$ level; ** significant correlation at the $p < 0.05$ level. r = correlation coefficient.

Brain area	Treatment (mg/kg s.c.)			r
	5.0	20	100	
Hypothalamus	0.328 ± 0.023	1.610 ± 0.370	7.746 ± 0.959	0.9998*
Prefrontal cortex	0.166 ± 0.031	1.223 ± 0.594	7.398 ± 1.484	0.9999*
Pyriform cortex	0.208 ± 0.020	0.870 ± 0.389	4.006 ± 0.930	0.9998*
Mesencephalon	0.176 ± 0.030	1.208 ± 0.486	4.206 ± 0.548	0.9948
Hippocampus	0.137 ± 0.009	0.700 ± 0.184	2.740 ± 0.349	0.9982**
Caudate	0.085 ± 0.009	0.483 ± 0.181	1.944 ± 0.298	0.9983**
Pons	0.283 ± 0.042	1.033 ± 0.384	5.726 ± 1.232	0.9998*
Medulla oblongata	0.159 ± 0.036	0.792 ± 0.292	3.370 ± 0.486	0.9992**

initial drop observed at 3 h was followed by a second phase of significant accumulation, which in some regions yielded concentrations of paraquat similar to those seen 1 h after administration.

Compared with rats receiving 5.0 mg/kg, animals treated with doses of 20 and 100 mg/kg showed a similar pattern of paraquat distribution in the brain as observed 24 h following administration; in addi-

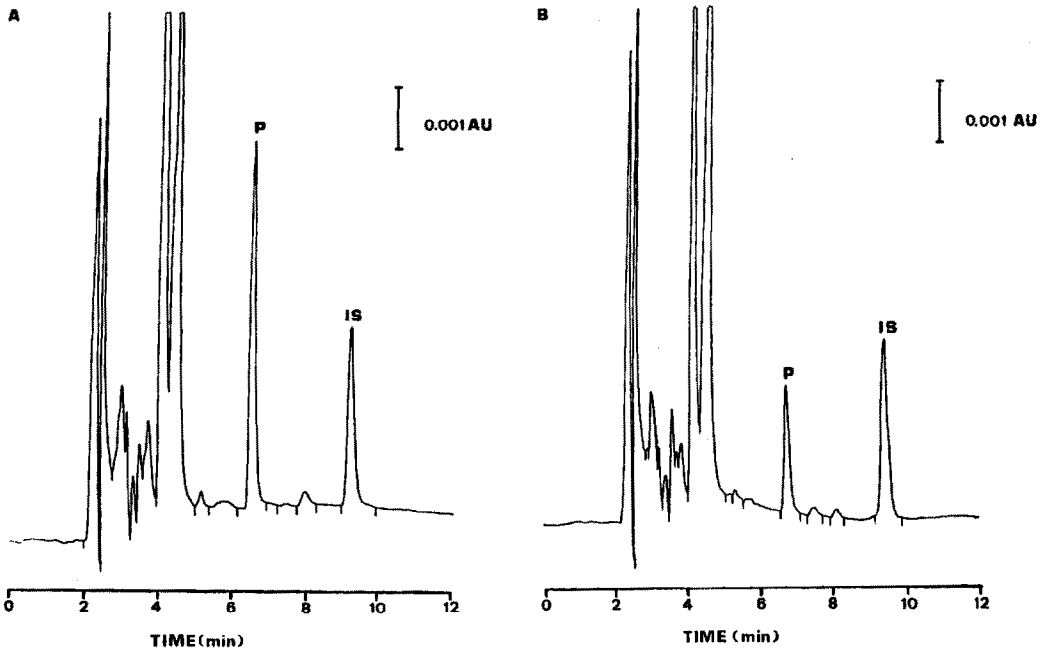

Fig. 1. HPLC elution profiles of discrete rat brain area extracts: (A) hypothalamus, (B) hippocampus. Each extract was the pool of six brains of rats treated 24 h earlier with paraquat. P = paraquat; IS = internal standard (0.375 μg/g wet tissue mass). Paraquat concentrations: (A) = 0.378 and (B) = 0.131 μg/g wet tissue mass.

tion, a dose-dependent increase in the regional levels of the herbicide was observed (Table II). Representative chromatograms of extracts from discrete brain areas are given in Fig. 1.

In the hypothalamus and medulla oblongata, two brain regions known to contain sites lacking BBB [29], the concentrations of paraquat were significantly different. This would indicate that the distribution of the herbicide in the brain does not depend solely on the ease with which it reaches a specific area via the bloodstream. The possibility that paraquat damages the capillary wall [14], facilitating its penetration into the CNS, cannot be excluded.

In rats, the microinfusion of paraquat into several brain areas produces locomotor disorders accompanied by electrocortical (ECoG) epileptic discharges and neuronal cell death, regardless of the site of injection [30,31]. The lack of selective neurotoxicity can be explained by its proposed mechanism of toxicity, *i.e.* the ability to generate oxygen free radicals and lipid peroxides with consequent cell damage and death. Paraquat can also be neurotoxic after systemic administration in rats [32,33]; in fact, it evokes limbic motor seizures, ECoG epileptic discharges and, of greater interest, selective neuronal cell death in the pyriform cortex, the last effect being observed 24 h after treatment (20 mg/kg s.c.); a lower dose (5 mg/kg s.c.) is ineffective.

Paraquat concentrations in the pyriform cortex, measured 24 h following administration of doses of 5 and 20 mg/kg, were 0.208 ± 0.020 and 0.870 ± 0.389 μg/g wet tissue mass, respectively.

These data indicate that in the pyriform cortex a paraquat concentration of about 0.200 μg/g is not neurotoxic, at least at 24 h after acute treatment with the herbicide, whereas paraquat levels of 0.870 ± 0.389 μg/g are associated with neuronal cell death.

Different areas of the CNS seem to exibit different sensitivities to the toxic effects elicited by paraquat. In contrast to the pyriform cortex, no neuronal damage was observed 24 h after treatment in other brain areas with higher or almost similar paraquat levels, *i.e.* the mesencephalon and hippocampus. The greater vulnerability of pyriform cortex to neuropathological insults has been well documented [34,35], and it could account for the selective neuronal cell death observed in this area 1 day after systemic administration of paraquat.

However, we cannot exclude the possibility that neuronal damage could also occur in brain areas other than pyriform cortex after longer exposure to paraquat.

The concentrations of paraquat detected in the brain of 3-, 12- and 24-month-old rats 1 h after the injection of the herbicide were dose-related and age-dependent, as shown in Table III.

In comparison with 3-month-old rats, higher paraquat levels were found in the brain of older animals (12 and 24 months old) following each dose of herbicide used (1.0, 2.5 and 5.0 mg/kg). In particular, the differences in brain paraquat concentrations were statistically significant over the range of doses administered when the data obtained in 3- and 24-month-old animals were compared (see Table III). In addition, in comparison to 3-month-old rats, statistically significant ($p < 0.05$) higher levels of paraquat were also obtained in the brain of 12-

TABLE III

PARAQUAT CONCENTRATIONS (μg/g WET BRAIN WEIGHT) AS MEASURED 1 h AFTER ITS SYSTEMIC (s.c.) ADMINISTRATION IN RATS OF DIFFERENT AGES

Values are the mean ± S.E.M. of 5–8 determinations. * $p < 0.05$ and ** $p < 0.01$ *vs.* the data obtained in 3-month-old rats (unpaired Student's *t*-test)

Dose (mg/kg)	Age (months)			
	0.5	3	12	24
1.0	0.064 ± 0.011	0.039 ± 0.006	0.047 ± 0.005	0.079 ± 0.016*
2.5	0.117 ± 0.005*	0.082 ± 0.012	0.132 ± 0.018*	0.170 ± 0.013**
5.0	0.334 ± 0.019*	0.185 ± 0.017	0.216 ± 0.022	0.243 ± 0.016*

month-old animals following the 2.5 mg/kg dose. The comparison of data obtained in 12- and 24-month-old rats revealed no statistically significant differences between these two groups of animals.

These data, whilst confirming the ability of paraquat to cross the BBB and to give rise to dose-related brain concentrations following systemic administration in rats [27], show that the diffusion of paraquat across the BBB is age-related. In fact, 1 h after the treatment, higher brain levels of paraquat are found in aged rats than in young animals, thus supporting the hypothesis of some degree of BBB leakage during ageing due to alterations in cerebral microvasculature (see ref. 28).

In addition, in comparison with 3-month-old rats, higher levels of paraquat were also detected in the brains of very young animals (2 weeks old) 1 h after acute administration of the herbicide (1.0, 2.5 and 5.0 mg/kg s.c.) (Table III). These results support data reported previously in the literature [36] indicating that full maturity of BBB is not completely reached in very young animals. Taken together, these data emphasize the possibility that the vulnerability of the CNS to environmental chemicals may be greater in the early stages of life and during ageing.

CONCLUSIONS

The availability of a specific and sensitive analytical technique for the determination of paraquat brain concentrations has allowed us to obtain more, and interesting, information on kinetic profile of paraquat after its systemic administration in rats.

The ion-pair solid-phase extraction and reversed-phase HPLC method previously described for the determination of paraquat concentrations in whole-brain samples [27] has been employed to detect herbicide levels in discrete rat brain areas. This has allowed us to demonstrate that a differential regional distribution of paraquat occurs in the brain 1, 3 and 24 h after systemic injection in rats, and that a different pattern of paraquat elimination exists among the brain regions studied. In addition, the concentrations of the herbicide in discrete brain areas were dose-related.

Age-dependent paraquat brain concentrations were detected in rats with the highest herbicide brain levels being obtained in very young and older animals.

In conclusion, the present results, whilst adding new data on paraquat kinetic profile, confirm the use of HPLC as a reliable analytical technique for the detection of environmental chemicals in biological samples.

ACKNOWLEDGEMENTS

Partial financial support from the Ministry for University and Scientific Research (Rome) and FIDIA SpA (Abano Terme, Italy) is gratefully acknowledged. We also thank Mrs. Tiziana Macrì and Mr. Giovanni Politi for their skillful technical assistance.

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