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Note

Quantitative analysis of the cholinesterase inhibitor paraoxon in brain tissue using high-performance liquid chromatography

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In order to study pharmacokinetics and to obtain more information about the site of action of drugs, the availability of sensitive, precise and accurate analytical methods for the quantitative determination of drugs is essential. Various analytical methods have been developed for the analysis of small amounts of organophosphorus insecticides like parathion and its structurally related compound paraoxon (diethyl-4-nitrophenylphosphate, Fig. 1).

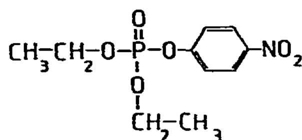

Fig. 1. Chemical structure of paraoxon.

Gas chromatography [1], using phosphorus-selective detection [2, 3], and high-performance liquid chromatography (HPLC) using UV detection [4], polarographic detection [5] or fluorimetric detection [6], have proved to be pre-eminently suitable for the analysis of residues in food.

Until now no HPLC method for the detection of the cholinesterase inhibitor paraoxon in animal tissues has been developed. In the present study a sensitive method for the determination of paraoxon in the presence of its hydrolysis product 4-nitrophenol in brain tissue of the cat using HPLC is described. In addition, concentrations of paraoxon in various brain tissues are presented after intravenous administration of various doses of paraoxon into the cat.

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EXPERIMENTAL PROCEDURES

Apparatus

The liquid chromatograph was custom-made and comprised a constant-flow pump (Varian 8500), a high-pressure injection valve (Valco) equipped with a 250-μl sampling loop, and a variable-wavelength UV detector (Pye Unicam LC3). The wavelength was set at 274 nm. All chromatograms were recorded on a linear potentiometric recorder (Goerz, Servogor). In all experiments stainless-steel 316 columns with dimensions 125 × 3 mm were used.

Materials

In all experiments double-distilled water was used. All solvents and chemicals were of analytical grade and used without further pretreatment. The column support was silica gel Si 60 (Merck, Darmstadt, G.F.R.) with a mean particle size of 5 μm. Paraoxon was obtained from Sigma, St. Louis, MO, U.S.A.

Chromatography

The HPLC columns were packed by a pressurized balanced slurry method. The slurry liquid consisted of a mixture of tetrabromoethane and chloroform of specific gravity of 2.09 for the bare silica (Si 60). After packing, the columns were washed with 200 ml of methanol and subsequently equilibrated with the eluent until constant retention of the solutes was obtained. Standard solutions of the drug and its metabolite (4-nitrophenol) were prepared from stock solutions of the compounds in methanol and were stored at 4°C. The mobile phase used for the analysis of paraoxon in extracts of brain tissue consisted of *n*-hexane containing 4% 2-propanol. The flow-rate was 60 ml/h.

Administration of drugs

Mongrel cats of either sex (weight 2—4 kg) were anaesthetized with α-glucochloralose (60 mg kg^{-1}). A femoral vein was cannulated for the intravenous administration of paraoxon. The trachea was cannulated for artificial respiration. After left-sided thoracotomy the aorta was ligated. Two or 10 min after dosing the animals were killed by occluding the aorta. The brain was removed and various brain regions were isolated on ice.

Sample preparation

The samples were homogenised with 0.1 *M* phosphate buffer (pH = 8.0) in appropriate glass tubes with Teflon pads at 0°C. For each 100 mg of tissue 1.0 ml buffer was added. After the addition of 0.2 ml of 2.5 *M* perchloric acid, the sample was diluted to 5.0 ml with 0.1 *M* perchloric acid. The mixture was homogenised and centrifuged at 2000 *g* for 15 min. Four millilitres of the clear supernatant were combined with another 4 ml of supernatant obtained after suspending and centrifuging the remaining pellet in 5.0 ml of 0.1 *M* perchloric acid. For the extraction of paraoxon the combined aqueous solutions (total volume 8.0 ml) were shaken vigorously with 2.0 ml of benzene in a glass stoppered centrifuge tube by means of a Vortex test-tube mixer. After centrifugation at 900 *g* for 5 min, the sample loop was filled with 250 μl of the organic layer.

RESULTS AND DISCUSSION

Chromatography

The retention behaviour of paraoxon and its metabolite 4-nitrophenol in liquid chromatography has been described for straight-phase [7] and reversed-phase systems [8, 9]. Of these two systems reversed-phase chromatography in combination with extraction and evaporation of the organic extraction fluid is not suitable for the analysis of low concentrations of these volatile solutes in aqueous solutions. Straight-phase liquid chromatography has also been described for a number of related organophosphorus insecticides [10, 11] in which hexane, containing 2-propanol or ethanol as the organic modifier, is used as the mobile phase. The main advantages of straight-phase chromatography are very stable separation systems and the compatibility of the system with organic extraction fluids. As can be seen from Fig. 2, a plot of $1/k_i'$ against percentage organic modifier yields a linear relationship which is commonly found in straight-phase systems. Excellent selectivity is obtained as is demonstrated by the test chromatogram of a solution containing paraoxon, 4-nitrophenol and benzene (Fig. 3).

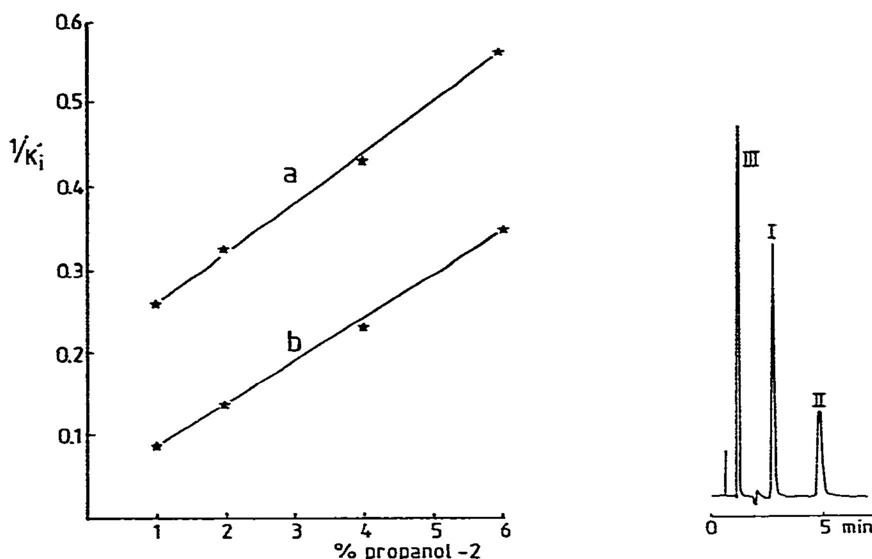

Fig. 2. Influence of the 2-propanol concentration in the mobile phase on the capacity ratio (k_i'). Stationary phase, Si 60. a = paraoxon; b = 4-nitrophenol.

Fig. 3. Separation of the solutes paraoxon (I), 4-nitrophenol (II) and benzene (III) in a test mixture.

Quantitative aspects

The precision and linearity of the method were determined by injecting 250 μl of solutions of paraoxon and 4-nitrophenol in different concentrations. The linear regression of peak height versus injected amount yields a correlation coefficient of 0.9993 for both solutes which indicates a high degree of linearity. The precision of the method was estimated by injecting solutions

of the solutes at high and at low concentration levels ($n = 6$). For both solutes the standard deviations for 250 ng and 25 ng were 1.45% and 1.82%, respectively. The peak-to-peak value of the baseline noise was determined as $5 \cdot 10^{-5}$ a.u. This leads to a calculated limit of detection for both solutes of 1.5 ng for a signal-to-noise ratio of 3. Consequently, for an injection volume of 250 μl the calculated limit of detection amounts to 12 ng of paraoxon per 100 mg of brain tissue.

Typical chromatograms were obtained from a blank brain tissue sample (Fig. 4a) and a sample obtained from a cat which received paraoxon (Fig. 4b). The possible degradation of paraoxon was studied by incubating the drug for 24 h in phosphate buffer (pH 7.4) at 37°C and in the presence of cat brain homogenates (at pH 7.4) at 37°C. The results are presented in Fig. 5. No chemical hydrolysis occurs, whereas a transient reduction of intact paraoxon can be observed under the influence of brain tissue. The degradation half-life in brain tissue homogenates was calculated to be 168 min.

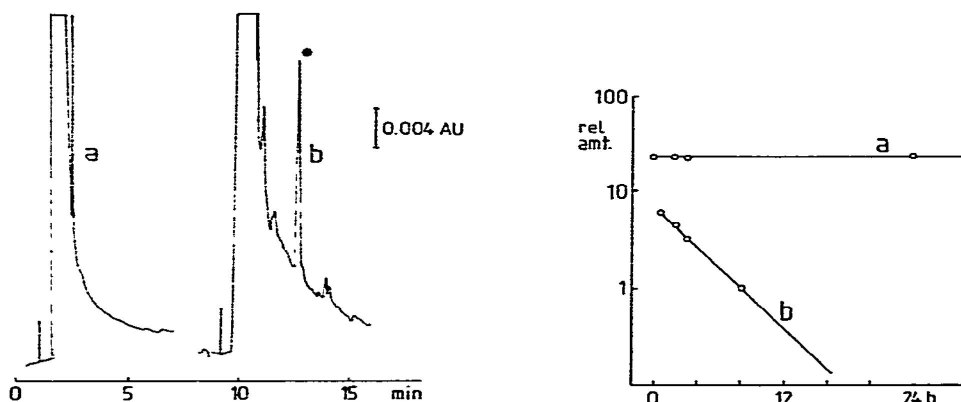

Fig. 4. Chromatograms of a blank brain tissue sample (a) and of a paraoxon(*)-containing tissue sample obtained after a pharmacological experiment (b). Mobile phase: 4% 2-propanol in n-hexane. Stationary phase: silica gel Si 60. Flow-rate: 60 ml/h.

Fig. 5. Semilogarithmic plot of the degradation of paraoxon in (a) phosphate buffer (pH 7.4, 37°C) and (b) in a brain tissue homogenate (pH 7.4, 37°C).

Brain concentrations in vivo

The developed method for the quantitative analysis of paraoxon was applied to brain tissue samples of cats which received paraoxon intravenously. Thus, the animals were given 550, 275 or 150 μg kg^{-1} via a femoral vein. Drug concentrations were measured in the medulla oblongata, pons and hypothalamus 2 and 10 min after dosing. The results are presented in Table I. With respect to the drug concentrations in the various brain parts, it should be emphasized that the relatively high standard errors of the mean are due to biological differences (blood pressure, blood flow) between the animals. Similar standard deviations were found after intravenous administration of radioactive compounds [12]. From Table I it is obvious that the amount of paraoxon decreases when lower doses are applied. Moreover, upon administration of 275 and 150 μg of paraoxon, equal concentrations are measured in

TABLE I

PARAOXON (Px) CONCENTRATIONS AND ACETYLCHOLINESTERASE (AChE) INHIBITION IN VARIOUS BRAIN REGIONS, 2 AND 10 min AFTER THE INTRAVENOUS ADMINISTRATION OF 550 μg, 275 μg and 150 μg OF PARAOXON PER kg
Values are presented as mean ± S.E.M. *n* represents the number of experiments.

Dose (μg/kg)	Brain part*	t = 2 min				t = 10 min			
		ng Px per brain part	ng Px per g tissue	AChE inhib. (%)	*n*	ng Px per brain part	ng Px per g tissue	AChE inhib. (%)	*n*
550	M	5076±386	3487±265	100±1	5	2054±356	1314±228	99±1	6
	P	2232±102	4501±206	100±1	5	964±174	1944±350	99±1	6
	H	1668±106	3691±235	99±1	5	415± 88	976±206	99±1	6
275	LM	937±164	1207±225	99±1	4	197± 24	270± 33	96±1	4
	RM	979±191	1332±260	98±1	4	203± 15	276± 20	96±2	4
	LP	463± 87	1907±358	98±1	4	105± 8	434± 32	95±1	4
	RP	446± 92	1763±364	97±1	4	107± 5	422± 19	94±1	4
	H	611±172	1352±380	98±1	4	127± 10	281± 22	95±1	4
150	LM	311± 97	427±133	86±4	4	n.d.**	—	—	
	RM	289± 74	292±100	85±4	4	n.d.	—	—	
	LP	95± 43	389±175	84±3	4	n.d.	—	—	
	RP	89± 33	352±132	82±4	4	n.d.	—	—	
	H	151± 43	335± 95	85±4	4	n.d.	—	—	

*M = medulla oblongata; P = pons; H = hypothalamus; LM = left side of medulla oblongata; LP = left side of pons; RM = right side of medulla oblongata; RP = right side of pons.
**n.d. = not detectable.

both sides of the pons and medulla. This is in agreement with previous observations that upon systemic application, drug distribution is uniform within the pontomedullary region [12]. Thus, this finding illustrates the validity of the method. The hydrolysis of paraoxon is demonstrated by the significantly smaller amounts measured 10 min after dosing. However, because of the detection limit, paraoxon could not be determined quantitatively 10 min after the infusion of 150 μg of paraoxon per kg. The presence of paraoxon was also established by the determination of acetylcholinesterase activity. The decrease of paraoxon concentration is accompanied by a reduction of the inhibitory effect on acetylcholinesterase activity. Conclusively, the developed method for the quantitative analysis of low amounts of paraoxon is a sensitive and accurate one. Its application to pharmacological experiments in vivo has been proven to be highly satisfactory. In a separate study [13] paraoxon concentrations in cat brain were measured also after central administration of the drug, and drug concentrations, enzyme inhibition and cardiovascular effects were compared.

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