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QUANTITATIVE DETERMINATION OF THE CHOLINESTERASE INHIBITOR PHYSOSTIGMINE IN BRAIN TISSUE SAMPLES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method, based on a dynamic cation-exchange system was used for the determination of physostigmine in brain tissue extracts. The precision and detection limit of the method as well as the extraction efficiency were established. The distribution of physostigmine over several parts of the brain after intravertebral application is reported.

INTRODUCTION

Previous pharmacological experiments have demonstrated that physostigmine changes haemodynamic parameters in the anaesthetized cat. The cardiovascular effects evoked could be attributed to an action of the drug upon the pontomedullary region [1]. In order to localize the site of action and to study the kinetic behaviour of the drug within the central nervous system, a sensitive, precise and accurate analytical method is necessary for the measurement of drug concentrations. Moreover, such a method would make studies concerning concentration—activity relationships possible. Until now quantitative methods are only available to determine physostigmine in pharmaceutical preparations [2, 3]. The fluorimetric determination of physostigmine in tissues as described by Laverty et al. [4] and Taylor [5] was not sensitive enough for the detection of the low drug amounts present in brain tissue after the administration of pharmacologically relevant doses.

In the present study a suitable quantitative method for the determination of physostigmine in brain tissue of the cat using high-performance liquid chromatography (HPLC) is described. Moreover, the distribution of the drug within

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the brain following intravenous administration and infusion via the left vertebral artery is discussed.

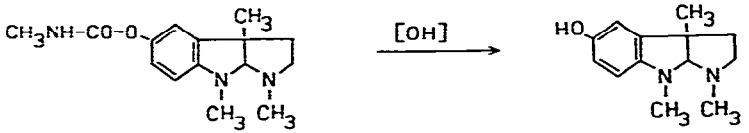
EXPERIMENTAL

Apparatus

The liquid chromatograph comprised a reciprocating pump (LC3-XP, Pye-Unicam), a high-pressure injection valve (Rheodyne 7105) equipped with a 175-μl sampling loop, and a variable-wavelength UV detector (Pye-Unicam LC3). The wavelength was set at 245 nm. All chromatograms were recorded on a linear potentiometric recorder (Servogor RE 542). In all experiments stainless-steel 316 columns, with the 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. Sodium dodecyl sulphate (SDS) was obtained from Merck (Darmstadt, G.F.R.). The column was packed with octyl-modified silica (LiChrosorb RP-8, Merck) with a mean particle size of 5 μm. Physostigmine sulphate was obtained from BDH (Poole, Great Britain). The structure of the hydrolysis product of physostigmine is represented in Fig. 1.



physostigmine eseroline

Fig. 1. Physostigmine and its degradation product.

Chromatography

The HPLC columns were packed by a pressurized balanced slurry method [6]. The slurry liquid consisted of a mixture of chloroform and tetrabromomethane of specific gravity 1.82 for the alkyl-modified silica (RP-8). After packing, the columns were washed with 100 ml of methanol and subsequently equilibrated with the eluent until constant retention of physostigmine was obtained. A standard solution of physostigmine sulphate was freshly prepared from a stock solution, containing 4.7 mg per 100 ml of methanol, and was stored at 4°C. The mobile phase used for the analysis of physostigmine in extracts of brain tissue consisted of 0.005 *M* NaH_2PO_4, 0.3% (w/v) SDS and 0.1 *M* $NaClO_4$ in 500 volumes of methanol and 500 volumes of water (pH 3.00). The flow-rate was 0.60 ml/min.

Administration of drugs

Mongrel cats of either sex (weight 2—4 kg) were anaesthetized with 60 mg of α-glucochloralose per kg, given intraperitoneally. After inserting a tracheal canula the animals were subjected to artificial respiration. The intravenous application of physostigmine was carried out by the infusion of the drug into a femoral vein in a volume of 140 μl during 1 min. For central application

physostigmine was infused into the left vertebral artery in a total volume of 140 μl during 1 min. For a detailed description of the vertebral artery model the reader is referred to Van Zwieten [7]. After administration via the left vertebral artery the drug will accumulate mainly within the right part of the brain stem [8, 9]. In order to obtain a more uniform distribution pattern within the pontomedullary region the opposite (right) vertebral artery was ligated [10]. After left-sided thoracotomy the aorta was ligated. At different intervals after dosing the circulation was arrested by occluding the aorta. Immediately afterwards, various brain regions were isolated on ice and weighed.

Sample preparation

Both the medulla oblongata and pons were divided into a left and a right part. Subsequently, the hypothalamus and a part of the cortex (gyrus marginalis) were isolated. After weighing, an homogenate 20% (w/v) was prepared in appropriate glass tubes with Teflon pads at 0°C, using the mobile phase as the homogenising fluid. The mixture was centrifuged at 68,000 *g* for 15 min at ca. 2°C. The clear supernatant was stored for 24 h at 4°C, filtered through a porous metal frit (2 μm) and 100 μl of the clear filtrate were injected.

RESULTS AND DISCUSSION

Chromatography

A cation-exchange system, dynamically generated by addition of SDS to the mobile phase, was chosen as the separation mode for the analysis of physostigmine in brain tissue extracts. Dynamic cation-exchange systems have been found to be very attractive for the separation of amino acids [11] and basic compounds such as tricyclic antidepressants [12], catecholamines [13], butyrophenones and diphenylalkylpiperidines [14]. Compared with a normal reversed-phase system the dynamic cation-exchange system shows a greater flexibility with respect to selectivity. In complex samples such as extracts of brain tissue this system has definite advantages for the analysis of small

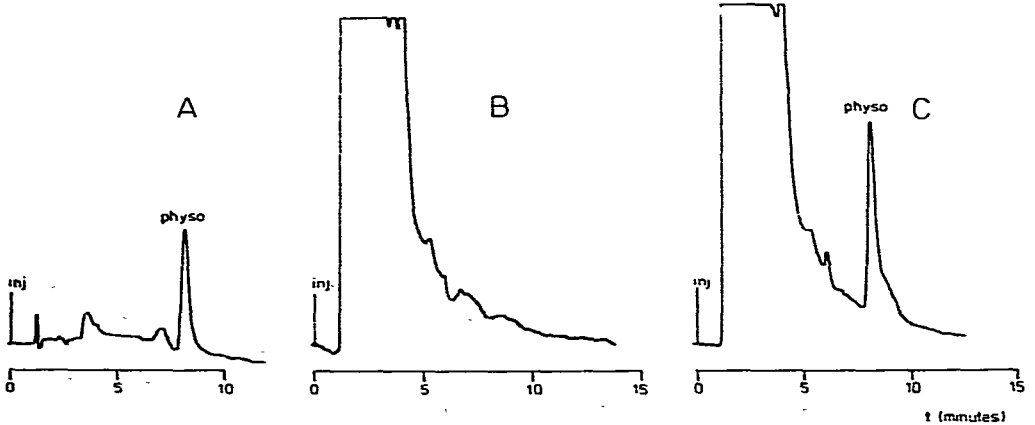


Fig. 2. Chromatograms of (A) a standard amount of physostigmine, (B) a blank brain tissue sample, and (C) a tissue sample obtained after a pharmacological experiment, containing physystigmine (10.7 ng/mg).

amounts of basic drugs. Moreover, columns with a greater theoretical plate height are obtained [12, 13]. Furthermore, the pH of the mobile phase can be adjusted to obtain minimal hydrolysis of drugs containing labile structures such as physostigmine (Fig. 1) without any loss of column efficiency. In general, minimal hydrolysis of ester-type drugs occurs often between pH 3 and 5 [15].

In order to determine the optimal chromatographic conditions for the separation of the solutes the capacity ratio of solutes as a function of a number of parameters, i.e. organic modifier, SDS, Na^+ and H_3O^+ concentrations in the mobile phase, were investigated. As was expected physostigmine behaves like a cationic solute in the chosen system. In Fig. 2 some typical examples of chromatograms are represented.

Quantitative aspects

The precision and linearity of the method were determined by injecting 50-μl or 100-μl solutions of physostigmine in different concentrations. The linear regression of peak height versus injected amount (10—500 ng) of physostigmine yielded a correlation coefficient of 0.9994, which indicates a high degree of linearity.

The precision of the method was estimated by repeated injections ($n = 5$) of 50 ng of solute dissolved in mobile phase. The standard deviation was found to be 1.8 ng (3.6%).

The peak-to-peak value of the baseline noise was determined to be $5 \cdot 10^{-5}$ a.u. This leads to a calculated limit of detection of 2 ng of physostigmine for a signal-to-noise ratio of 3. This corresponds to a theoretical, calculated limit of detection of 100 ng per gram of brain tissue for an injection volume of 100 μl.

Efficiency of the extraction

The recovery and reproducibility of the extraction have been determined. Thus, different amounts of physostigmine were added to blank brain tissue and subsequently extracted as described above. The efficiency of extraction was 89.9 ± 7.2%.

Distribution of physostigmine in different brain regions after intravenous administration and infusion into the left vertebral artery

The method described above for the quantitative analysis of physostigmine was applied to brain tissue samples of cats that had received physostigmine intravenously and via the left vertebral artery. The latter animal model is often used for the study of drug actions upon the pontomedullary region [7].

Table I represents drug concentrations in different brain regions following intravenous administration of $1 \cdot 10^{-6}$ mol kg^{-1} physostigmine (ca. 270 μg of physostigmine base per kg). Concentrations were established in the medulla oblongata, pons and in the higher brain structures like the hypothalamus and cortex, 5 min after dosing. At that time the pharmacological effect, i.e. the hypotensive effect, was maximal. The results indicate that the drug is equally distributed in the brain; similar amounts of drug per gram of tissue are found in the left and right parts of the pontomedullary region, hypothalamus and cortex. This observation is in agreement with distribution studies in cats after systemic application of clonidine [16], isoarecaidine propyl ester [17] and R28935 [18].

TABLE I

DISTRIBUTION OF PHYSOSTIGMINE IN DIFFERENT BRAIN REGIONS AFTER INTRAVENOUS ADMINISTRATION

Physostigmine, $1 \cdot 10^{-6}$ mol kg^{-1}, was administered intravenously. Results are expressed as the mean ± S.E.M., $n = 4$.

Brain part*	ng per brain part	ng per gram wet tissue	Percentage of the administered dose
R pons	168 ± 20	559 ± 51	0.022 ± 0.003
L pons	138 ± 9	510 ± 46	0.017 ± 0.001
R medulla oblongata	350 ± 31	542 ± 41	0.044 ± 0.003
L medulla oblongata	348 ± 29	546 ± 50	0.044 ± 0.003
Hypothalamus	148 ± 8	492 ± 35	0.018 ± 0.001
Cortex	—	543 ± 52	—
Brain stem (total)			0.127 ± 0.003

*R = right; L = left.

TABLE II

AMOUNTS OF PHYSOSTIGMINE IN VARIOUS BRAIN REGIONS AT DIFFERENT TIME INTERVALS FOLLOWING INFUSION INTO THE VERTEBRAL ARTERY

Amount of physostigmine infused was $3 \cdot 10^{-8}$ mol kg^{-1}. Values are expressed as the mean ± S.E.M., $n = 5$.

Brain part*	Time after infusion					
	2 min		5 min		30 min	
	ng per brain part	ng per gram wet tissue	ng per brain part	ng per gram wet tissue	ng per brain part	ng per gram wet tissue
R pons	193 ± 64	512 ± 188	166 ± 40	582 ± 185	n.d.	n.d.
L pons	255 ± 83	750 ± 292	176 ± 50	648 ± 214	n.d.	n.d.
R medulla oblongata	535 ± 115	791 ± 207	660 ± 129	1048 ± 238	n.d.	n.d.
L medulla oblongata	1932 ± 266	2638 ± 463	2241 ± 321	3349 ± 497	n.d.	n.d.
Hypothalamus	n.d.†	n.d.**	n.d.	n.d.***	n.d.	n.d.
Cortex	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

*R = right; L = left.
**120 ng per gram of tissue measured in one cat; in the other cats no detectable amounts of physostigmine.
***In two cats 192 ng and 137 ng of physostigmine per gram of tissue; no detectable amounts in the other cats.
†n.d. = not detectable (< 120 ng per gram of tissue).

In Table II the distribution of the carbamate is shown in the various brain parts after injection of the drug ($3 \cdot 10^{-8}$ mol kg^{-1}, ≡ 8.3 μg of base per kg) into the vertebral artery. The drug concentrations were determined 2, 5 and 30 min following application of physostigmine. Most of the drug accumulates in

the medulla oblongata and pons whereas the higher brain parts like the hypothalamus and cortex contain no detectable amounts or insignificant amounts of the drug. Similar observations have been described for other drugs [16, 19].

According to the results of El Sherbini-Schepers and Van Zwieten [10], we found a similar discrepancy in drug concentration between the left and the right part of the medulla oblongata (a ratio of between 3 and 4) after administration of the drug via the left vertebral artery. These findings illustrate the validity of the developed HPLC method for physostigmine. The drug could not be detected 30 min after application of physostigmine. This suggests a rapid elimination of the drug (possibly due to redistribution and hydrolysis) from the lower brain stem. In a separate paper (to be published) the brain concentrations are discussed in relation to the depression of acetylcholinesterase and the haemodynamic effects induced by physostigmine.

Finally, it is concluded that this newly developed HPLC method is sensitive, relatively simple and is suitable for the determination of physostigmine in brain tissue with respect to pharmacological experiments.

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