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Studies on the Absorption of Pyridostigmine: the Application of a Spectrophotometric Method for the Determination of Pyridostigmine in Plasma

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A method is described for the quantitative determination of pyridostigmine in blood, based on the principle of ion pair extraction. With the aid of this method it was shown that pyridostigmine is absorbed relatively slowly and irregularly, following its oral application. In order to achieve the same concentration in the blood of rats, the oral dose must be 50 times higher than the intraperitoneal dose. Orientation studies on humans confirmed that the absorption is low and irregular after oral application.

Es wird ein Verfahren zur quantitativen Bestimmung von Pyridostigmin im Blut beschrieben, das auf dem Prinzip der Ionenpaarextraktion beruht. Mit dieser Methode konnte festgestellt werden, daß Pyridostigmin nach oraler Gabe relativ gering und unregelmäßig resorbiert wird. Um dieselbe Konzentration im Blut der Ratte zu erreichen, wird bei oraler Gabe eine 50fach höhere Dosis benötigt als bei intraperitonealer Verabreichung. Orientierende Versuche am Menschen haben die Ergebnisse der geringen und unregelmäßigen Resorption bestätigt.

The therapy of Myasthenia gravis with pyridostigmine (Mestinon) often fails to produce the required result. Even in favourable cases, the effective dosage varies considerably between individuals (1). One reason for this could be a poor and/or irregular intestinal absorption of the pyridostigmine. Little is known so far about the uptake and distribution of this substance in the whole organism. The present work was therefore undertaken to determine the relationship between the pyridostigmine concentration in the blood and the quantity administered orally or intraperitoneally in experimental animals. In addition, the lethal dose was determined for the different modes of application. A method was also developed for the quantitative determination of pyridostigmine in plasma.

Pyridostigmine is a quaternary ammonium base, which can be transferred to organic solvents from aqueous solution as an ion pair with the aid of hexanitrodiphenylamine (dipicrylamine). The ion pair can then be dissociated by shaking the organic phase with diluted hydrochloric acid, whereby the pyridostigmine is transferred to the aqueous phase, being finally purified, isolated and identified by chromatography (3, 4).

This rather laborious and time consuming procedure can be avoided by following the acid treatment with an ion pair extraction of the amine with I_3. The resulting I_9-complex in the organic phase possesses two typical absorption maxima at 365 and 293 nm, which can be measured directly in a spectrophotometer (5).

The determination procedure can be summarized as follows:

1. Pyridostigmine is mixed with dipicrylamine in a neutral buffer. The resulting stable ion pair is extracted with dichloromethane.
2. The dichloromethane phase is evaporated to dryness, the ion pair cleaved with HCl and the dipicrylamine removed by extraction again with dichloromethane.
3. The iodine complex of pyridostigmine is formed with the aid of KI_3. This complex is extracted with dichloromethane, and determined spectrophotometrically by measuring the absorbance at 293, 329 and 365 nm.

Materials and Methods

Reagents

1. Mestinon (solid pure substance) from Hoffmann–La Roche[1])
2. Dipicrylamine (moistened with an equal part of water) p. a. Merck 3089
3. Sodium dihydrogen phosphate 1-hydrate p. a. ($NaH_2PO_4 \cdot H_2O$) Merck 6346
4. Sodium hydroxide flakes, reinst. p. a. Merck 6498
5. Iodine doubly sublimed p. a. Merck 4761
6. Potassium iodide neutral p. a. Merck 5043
7. Sodium sulphate, anhydrous p. a. Merck 6649
8. Dichloromethane p. a. Merck 6050, distilled
9. Heparin (solid substance) Hoffmann–La Roche[1])

[1]) Mestinon and heparin were generous gifts from Hoffmann–La Roche.

Solutions

1. Dipicrylamine solution 10 mmol/l: 440 mg dipicrylamine are dissolved in 8 ml 1 mol/l NaOH; 20 ml 0.5 mol/l NaH_2PO_4 are added and the volume is adjusted to 50.0 ml with distilled water. The pH of this solution is 7.0–7.15.
2. Iodine solution.
   15.7 g iodine and 20.0 g KI are dissolved in 100.0 ml distilled water and shaken mechanically for 40 min. This solution is stable indefinitely at 4° C (2). The stock solution is diluted 1:10 with water. This solution is stable for about 3 weeks.

Experimental animals

The experimental animals were female albino rats, wistar strain, from the Hoffmann animal breeding farm, Berlin. They weighed 180–220 g, and received water and Altromin R 10 dry food ad libitum.

Determination of pyridostigmine

Rats were decapitated and their blood collected in centrifuge tubes containing a small amount of heparin (solid substance). After centrifugation at 3,000 rpm, 3 ml of the supernatant plasma were used for the determination.

Analytical method

1. 3 ml plasma are mixed with 1 ml dipicrylamine solution (10 mmol/l), followed by 25 ml dichloromethane, and the mixture, in a glass stoppered centrifuge tube, is shaken vertically on a mechanical shaker for 30 min at 20 rpm. After 10 min centrifugation at 1,500 rpm, 20 ml of the dichloromethane phase are removed. The most favourable extraction of the pyridostigmine complex from the plasma into the organic phase occurs at pH 7.2. The extraction yield from rat plasma or whole blood is 93%, as determined in serial experiments in which pure pyridostigmine was determined with or without prior extraction. Variations in the ratio of volumes of dipicrylamine solution and dichloromethane, in the concentration and quantity of dipicrylamine, or in the shaking time offered no advantages. The results were not improved by deproteinization of the rat blood, by acid precipitation, or boiling.
2. The dichloromethane phase (20 ml), in a ground glass centrifuge tube, is evaporated to dryness in a stream of air. The residue can be stored overnight in the refrigerator without loss. It is dissolved in 0.5 ml 0.05 mol/l HCl and the residual dipicrylamine, which is still present at this stage, is removed by washing first with 6 ml, then with 3 ml dichloromethane.
3. The aqueous phase is mixed with 20 µl KI_3 solution plus 3 ml dichloromethane, then shaken for 10 min at 40 rpm. After centrifugation to separate the phases, the organic phase is transferred to a glass stoppered tube containing 10 mg anhydrous sodium sulphate. The solution is centrifuged and its absorbance measured at 293, 329 and 365 nm against dichloromethane. The quantity of pyridostigmine can be determined from the difference in the absorbance at 293 and 329, or 293 and 365 nm (Fig. 1b), using a calibration curve prepared with pure material in 3 ml of plasma from an untreated animal. In the concentration range encountered in the animal experiments and in humans, i. e. 0.5–5 µg/3 ml plasma, the individual calibration values show a scatter of 10–20% around the average.

Preliminary investigation of the method showed that light causes a linear decrease in the extinction after the addition of the KI_3 solution. The spectrum also shows qualitative and quantitative changes (Fig. 1, a and b). After shaking, all the samples must therefore be stored in the dark.

Choline occurs naturally in plasma and it is precipitated from aqueous solution with I_3^- (2), but it does not interfere because, unlike pyridostigmine, it cannot be extracted as its I_3^- complex with dichloromethane or chloroform (5). Erroneous results can be obtained, however, when other pharmaceutical compounds are administered at the same time (unpublished results). Under the above conditions, the lower limit for the determination of pyridostigmine is 0.3 µg/3 ml plasma.

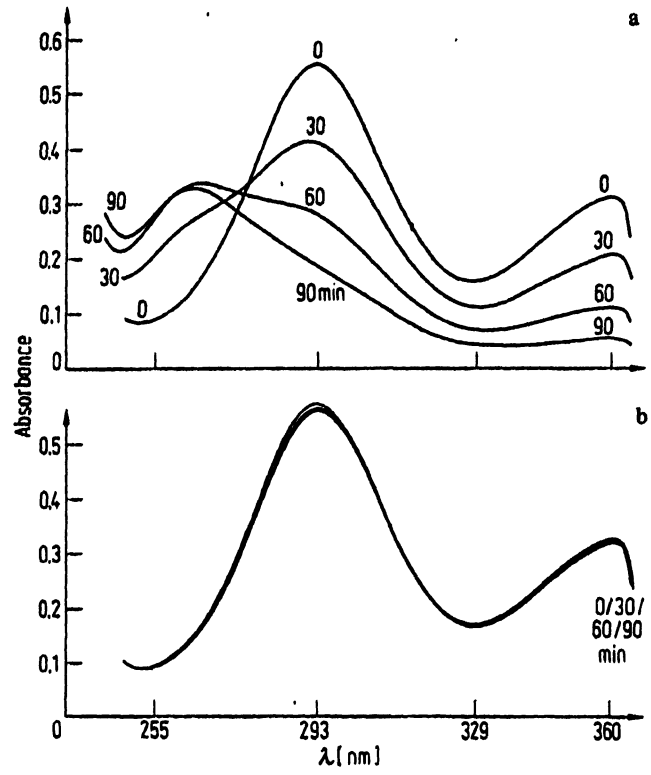

Fig. 1. Alteration with time of the UV absorption of the iodine complex of pyridostigmine with KI_3 in dichloromethane, a) in the light b) in the dark.

Application of pyridostigmine

In the first series of experiments, the relationship between the dose and the plasma concentration was determined at an arbitrarily fixed time after application. The pyridostigmine was administered in 1 ml aqueous solution per 100 g animal body weight. The rats were killed 5 min after the intraperitoneal injection of 1, 2, 3 and 4 mg/kg, and 15 min after the oral application of 50, 70, 90 and 110 mg/kg.

In the second series of experiments, the animals received 2 mg pyridostigmine per kg body weight i. p. (1 ml aqueous solution/100 g). Decapitation was 5, 10, 15, 20, 30 and 40 min after the application. For comparison, another group of rats received 50 mg pyridostigmine per kg orally, again in 1 ml per 100 g. These animals were killed 30, 60, 120, 180 and 240 min after the application.

Results

The LD_{50} for pyridostigmine after i. p. injection in rats is 3 mg/kg. A concentration range of 1–10 mg/kg was tested on 5 groups, each containing 6 rats, and the result was calculated according to Kärber (6). After oral application, however, the LD_{50} is 115 mg/kg (7 values, each determined on 6 rats for the range 10–180 mg/kg), with considerable scatter. This value alone indicates that the intestinal absorption is low; this is confirmed by experiments in which the pyridostigmine concentration in the plasma was determined after oral and i. p. application. Figure 2 shows that after the i. p. application of 1–4 mg/kg, the concentration of pyridostigmine in the plasma shows a relatively steep and linear increase. After oral dosage, however, the ratio, dose : plasma concentration, is much flatter. Thus, for the same plasma

concentration, the dose ratio for intraperitoneal : oral is about 1: 55.

Pyridostigmine appears rapidly in the blood and reaches a maximum concentration in the plasma 5 min after i. p. application (Fig. 3). Absorption from the peritoneal cavity is therefore very rapid. For the concentration range used in these experiments, the half life of the elimination, starting from the maximum, is 28 min. In contrast, it takes 30 min to attain the maximal concentration after oral application. At this time, the absorption is probably still incomplete, for, as shown in Figure 3, the subsequent elimination occurs much more slowly. Plasma pyridostigmine concentrations above 2 µg/3 ml give rise to easily recognizable muscular convulsions, shivering and chromodacryorrhea, etc. After an i. p. application of 2 mg/kg, these symptoms become apparent within the first 5 min, and they have largely died away after 15–20 min. After 50 mg/kg orally, however, these symptoms do not appear for 20–30 min, after which they continue for further 30 min. Investigations on humans principally confirmed that the absorption is low and irregular after oral application. This is demonstrated in Figure 4 for four patients who received 120 mg pyridostigmine in the form of Mestinon dragees. Since the determination method is not sensitive below 0.5 µg/3 ml and the scatter in this part of the calibration curve is greater than 20%, it is not known if absorption occured during the first hour in patients S. L. and E. J.

A detailed study of the relationship between the serum concentration of pyridostigmine and its therapeutic effect, or its possible toxic action will be published elsewhere.

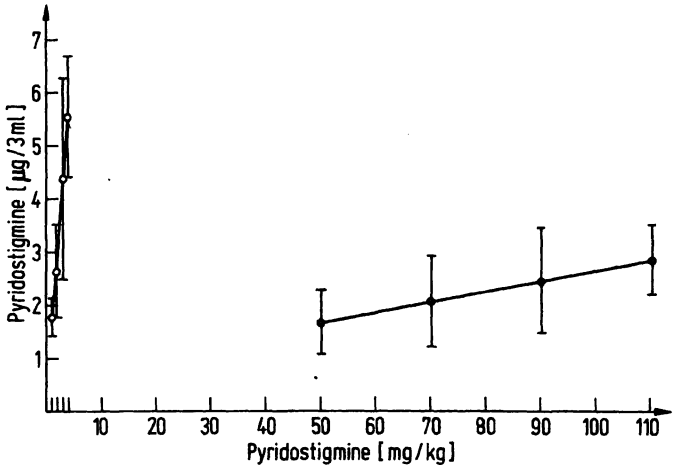

Fig. 2. Dose versus plasma concentration of pyridostigmine for intraperitoneal (o——o 5 min after 1–4 mg/kg) and oral (•——• 15 min after 50–110 mg/kg) application. Each point represents the average (± s) from 4–7 animals.

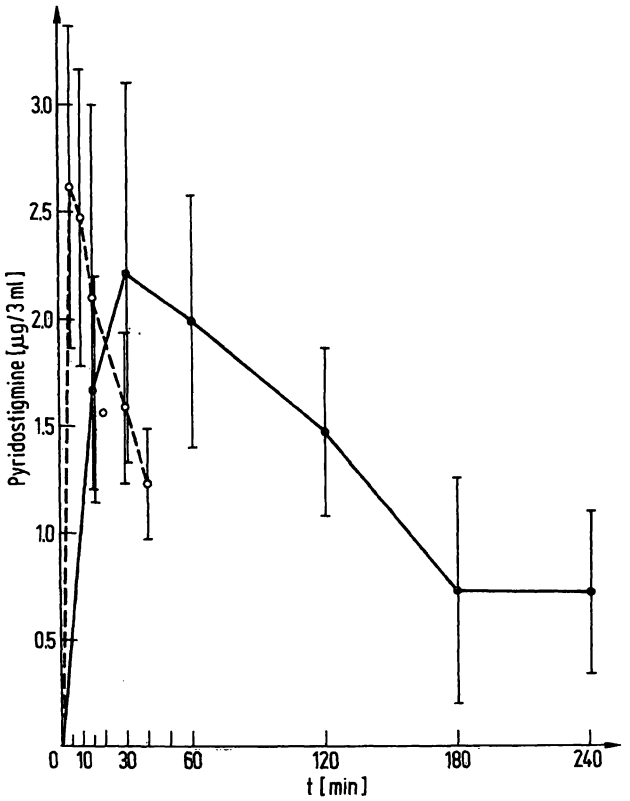

Fig. 3. Comparison of the plasma concentrations of pyridostigmine in the rat after intraperitoneal (o——o, 2 mg/kg) and oral (•——• 50 mg/kg) application. Each point represents the average (± s) from 10–11 animals.

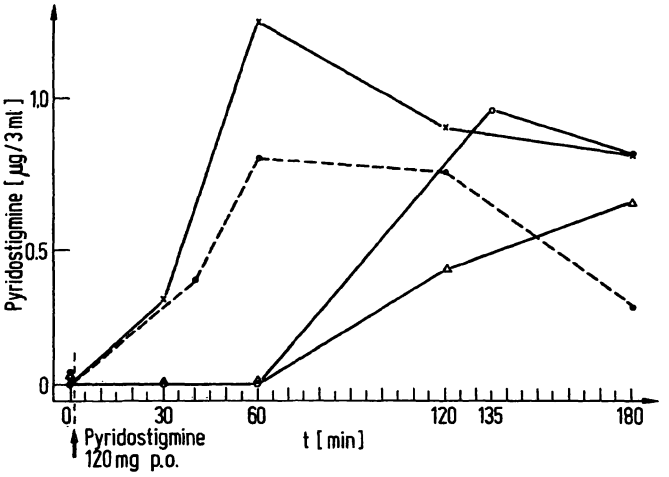

Fig. 4. Behaviour of the pyridostigmine concentration in the serum of patients after the oral application of 120 mg Mestinon.
X——X E. T.
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