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METHOD FOR ISOLATION AND DETERMINATION OF PYRIDOSTIGMINE AND METABOLITES IN URINE AND BLOOD

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SUMMARY

A procedure is presented for the unique isolation and determination by liquid chromatography of pyridostigmine and its metabolites in urine and blood. Isolation is accomplished by an extension of paired-ion theory using a reversed-phase chromatographic column. The assay of pyridostigmine is linear in concentrations from 40 to 5000 ng/ml. Separation and quantitation of pyridostigmine and metabolites in urine and blood are rapid. An analysis can be performed in 15 min. The method has been applied to the determination of urinary excretion and plasma levels of pyridostigmine administered intramuscularly in rats and to the isolation of acetylcholine, neostigmine, and edrophonium from aqueous solutions.

INTRODUCTION

Pyridostigmine is a drug used extensively in the treatment of patients with myasthenia gravis. Recently pyridostigmine, in combined therapy with atropine and an oxime, was suggested as an antidote in organophosphate poisonings [1]. In cases where patients received oral doses of pyridostigmine, considerable inter-subject variation in blood levels was reported [2—7]. Maximum plasma levels after similar oral doses administered to man range from 1 to 200 ng/ml. The quantitative isolation and accurate determination of the drug in biological fluids would result in establishing pharmacokinetic constants for pyridostigmine administered alone and in combination with other drugs.

Pyridostigmine is generally separated from plasma by extraction of an ion-pair complex with organic solvent. Quantitation has been reported by spectrophotometric [4], gas or liquid chromatographic analysis [5, 7—10]. Spectrophotometric methods are not sensitive enough to measure pyridostigmine in plasma after administration to man of 30- to 60-mg doses. Pohlmann and

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Cohan's method [8], based on electron-capture gas chromatography, measures picogram amounts, but lacks selectivity. Chan et al. [9], using an on-column gas chromatographic dequaternization method, report selective and sensitive detection of nanogram amounts of pyridostigmine in plasma. Recently two liquid chromatographic (LC) procedures have been introduced for determining pyridostigmine [10, 11]. The LC method used in this report is essentially similar. The major contribution of this report is the introduction of a new and unique isolation procedure which provides rapid, selective, and quantitative recoveries of pyridostigmine and metabolites in urine and plasma. The procedure has been applied to studies in which rats received intramuscular doses.

EXPERIMENTAL

Apparatus and reagents

Analyses were carried out using a Waters Assoc. (Milford, MA, U.S.A.) Model 244 liquid chromatograph equipped with two Model 6000A high-pressure pumps, a U6K loop injector, a Model 450 variable-wavelength detector, a Model 440 dual-wavelength absorbance detector, a Houston Instruments Omni-Scribe A-5000 dual-pen recorder, and a Shimadzu data processor. Hamilton syringes, 1—100 μl, were used to inject samples into the chromatograph. A bench-top centrifuge (Clay-Adams) was used to spin down precipitates.

Solvents

Spectroquality solvents were used: methanol, acetonitrile, chloroform, carbon tetrachloride from Waters Assoc., heptane, hexane from Burdick & Jackson Labs., Muskegon, MI, U.S.A., amyl alcohol and cyclohexane from Fisher, Fairlawn, NJ, U.S.A. Distilled, deionized, and charcoal-filtered water was used for all solutions.

Chemicals

Pyridostigmine bromide, neostigmine bromide, and methyl-*p*-aminobenzoic acid (methylparaben) were obtained from U.S. Pharmacopeia Convention (Rockville, MD, U.S.A.); acetylcholine was from Sigma (St. Louis, MO, U.S.A.); 3-hydroxy-N-methyl pyridinium bromide, RO-1-5237, dimethylcarbamyl ester of 3-hydroxypyridine, RO-1-5142, and edrophonium hydrochloride, RO-2-3198 were from Hoffmann-La Roche (Nutley, NJ, U.S.A.); Pic B-8 reagent containing 1-octanesulfonic acid buffers was from Waters Assoc.; sodium carbonate, potassium carbonate, and sodium phosphate. All reagents were analytical grade or better.

Columns and packing material

A prepacked 300 × 4 mm I.D. μBondapak C_{18} column, particle size 10 μm, Sep-Pak™ cartridge, C_{18} on porous silica, particle size 50 μm; Corasil Type II, particle size 31—50 μm were all obtained from Waters Assoc. Millex 0.45-μm filter units were from Millipore (Bedford, MA, U.S.A.) and B-D Yale syringes from Becton Dickinson (Rutherford, NJ, U.S.A.).

Procedure

A sample of pyridostigmine bromide was dried in vacuum according to specifications in USP XIX. A dilute standard containing 1 mg/ml of pyridostigmine bromide was prepared in $10^{-3}N$ hydrochloric acid and stored in a refrigerator at 4°C. Subsequent dilutions were prepared from this standard. A Sep-Pak cartridge was washed with 5 ml of methanol followed by 5 ml of water. Flow-rates were manually controlled to 5—10 ml/min of solvent.

Volumes of urine (1—10 ml) were brought to pH 10.2—10.5 with sodium hydroxide. Resulting precipitates were centrifuged for 1 min in a bench-top centrifuge. Supernatants were placed in a glass syringe and passed through a Millex filter. The syringe and filter unit were attached to the Sep-Pak cartridge and the urine passed through the Sep-Pak cartridge. The cartridge was washed with 5 ml of water, then 5 ml of methanol. About 3 ml of air were introduced into the Sep-Pak to eliminate excess methanol. A glass syringe containing 2 ml of 0.1 *N* acetic acid in methanol was attached to the Sep-Pak. One or two 1-ml fractions were collected in separate test tubes and 25 μl of internal standard solution added. Each tube was taken to dryness over nitrogen in a water bath set at 40°C and reconstituted with microliter volumes of 50% methanol. A volume equivalent to 1 ml of urine could be injected into the liquid chromatograph for quantitative analysis. This procedure was carried out in a similar manner with plasma except that the Millex filtration procedure was unnecessary.

Liquid chromatography

For LC analysis, a 300 × 3.9 mm I.D. μBondapak column prepacked with octadecylsilane bonded to 10-μm silica was used in the chromatography of all samples. The mobile phase consisted of 0.005 *M* 1-octanesulfonic acid (Pic B-8) in water and acetonitrile (prepared by mixing the contents of a prepackaged reagent bottle with 800 ml of water, 200 ml of acetonitrile, and 5 ml of acetic acid). The solvent was pumped through the column at a flow-rate of 2.5 ml/min. Column pressures were generally around 13.8 MPa. All separations were performed at ambient temperatures. Samples were introduced into the column through a continuous flow loop injector. The volumes of sample injected were between 5 and 100 μl. Absorbances were measured in the variable-wavelength detector at 270 nm. As reference standards individual solutions containing 0.05, 0.12, 0.25, 1.0, 2.5, or 5.0 μg of pyridostigmine per ml of water were prepared. Similarly, concentrations of pyridostigmine bromide were prepared by adding aqueous concentrates to human urine or plasma. Internal standard solutions contained methyl-*p*-aminobenzoic acid (methylparaben), 2.5—5.0 mg in 100 ml of water. Peak areas were measured and concentrations determined by an on-line computing integrator.

Animal studies

For animal studies, rats weighing 175—225 g were placed in metabolic cages and deprived of solid food for 14 h. For urine studies they were hydrated by the administration of 2.5 ml per 100 g of warm water by stomach tube. This was repeated after 1 h. An 80-μg aliquot of pyridostigmine in 0.2 ml water was injected intramuscularly into a hind limb. Urine free from feces was collected

over a period of 24 h. In blood studies a similar dose was administered intramuscularly. At selected intervals over a period of 1 h blood specimens were collected from excised hearts.

RESULTS

A typical liquid chromatogram of pyridostigmine recovered from the drug added to human urine is shown in Fig. 1. Retention time for pyridostigmine is

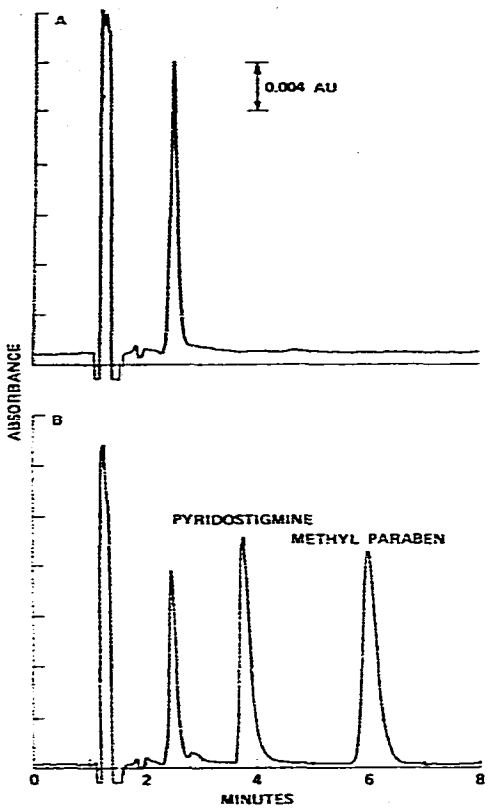

Fig. 1. Liquid chromatograms of (A) urine blank passed through Sep-Pak and (B) pyridostigmine isolated from urine by a Sep-Pak cartridge. Methylparaben is the internal standard.

3.68 min, for methylparaben 5.93 min. Concentration levels ranging from 49 to 4900 ng/ml were assayed. Urine samples (5 ml) were generally used and volumes equivalent to 1 ml could be injected in the liquid chromatograph for analysis. Only when volumes greater than 5 ml were used were small interferences from urine noted. Two separate concentrations of internal standard were used: 2.5 μg/ml for concentrations of pyridostigmine up to 250 μg/ml, and 5 μg/ml for concentrations greater than 250 μg/ml. A linear regression analysis of the data is shown in Fig. 2. The following values were obtained: r (coefficient of correlation) is 0.996; slope is 0.9914; y-intercept at 0.0432; standard error of the slope, 0.0207. To demonstrate the suitability of this procedure and method for pyridostigmine in blood, pooled plasma was obtained

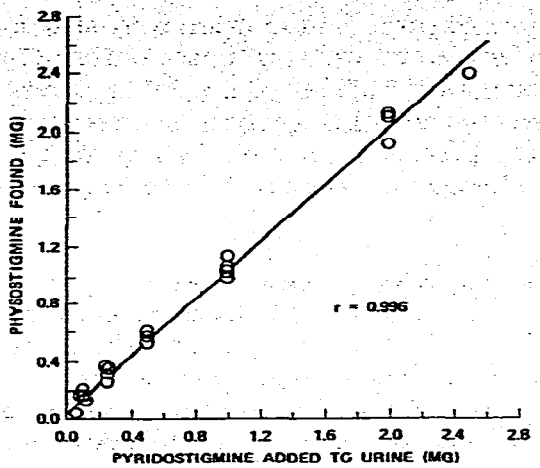

Fig. 2. Standard curve for recoveries of concentrations of pyridostigmine bromide in urine.

from normal blood samples. Pyridostigmine was added to human plasma in concentrations of 40 ng/ml to 20 μg/ml. Recoveries from 5-ml plasma samples are shown in Table I. Recoveries were 90—95% and 95—100% in the 40—100 and 100—5000 ng/ml levels, respectively. Chromatograms of pyridostigmine isolated from plasma had no interfering endogenous peaks. A correlation coefficient of 0.999 was obtained from linear regression data, with a slope of 0.9754 and the *y*-intercept at 0.0700. The standard error of the slope was 0.00739. Similar results occurred with pyridostigmine added to rat urine and plasma.

TABLE I

RECOVERY OF PYRIDOSTIGMINE BROMIDE ADDED TO 5-ml HUMAN PLASMA SAMPLES

Sample No.	Pyridostigmine added (ng/ml)	Pyridostigmine found* (ng/ml)
1	39	35
2	78	71
3	100	107
4	156	153
5	313	305
6	400	390
7	625	625
8	1250	1312
9	4000	4330

*Average of 3 determinations.

Liquid extraction of pyridostigmine bromide at pH 10.5

Pyridostigmine bromide was added to 0.05 *M* carbonate buffer (pH 10.5) to

a concentration of 40 μg/ml. Equal volumes of carbonate solution were shaken with five separate organic solvent systems saturated with carbonate buffer: (1) chloroform, (2) dichloromethane, (3) hexane, (4) 15% butyl alcohol in chloroform and (5) 20% amyl alcohol in cyclohexane. LC analysis showed that negligible amounts of pyridostigmine were present in any of the organic solvents and that essentially all of the pyridostigmine remained in the aqueous phase.

Effect of pH on the isolation of pyridostigmine bromide from urine

Urine samples are adjusted separately with 4 *N* sodium hydroxide to pH values 8.55, 9.02, 9.51, 10.02, and 10.60. Control samples containing no pyridostigmine were passed through Sep-Pak cartridges and analyzed by LC. Urine samples containing pyridostigmine at the aforementioned pH values were passed through cartridges, then quantitated by LC analysis. At the above pH values, recoveries were 52, 60, 80, 98, and 100%, respectively.

Effect of pH on the isolation of pyridostigmine bromide from aqueous solutions

Experiments, isolations and analyses similar to the ones mentioned above were carried out with pyridostigmine bromide in 0.02 *M* carbonate buffer solutions at pH values 8.88, 9.51, 10.0, and 10.52. Recoveries of pyridostigmine from the aqueous solutions were quantitative at all pH values.

Stability of pyridostigmine in alkaline solution

Since the isolation of pyridostigmine bromide from biological samples is carried out in basic solution, and carbamates are known to have varying degrees of instability in basic solution, the stability of pyridostigmine bromide at pH values of 8.45 and 10.1 over periods of time was determined in 0.02 *M* carbonate buffer. Results showed that after 20 h at pH 8.45 the decomposition of pyridostigmine was negligible. At pH 10.1, pyridostigmine bromide solution degraded 0.7, 1.0, 1.6, and 8% after 1.2, 2.2, 3.5, and 20 h, respectively. As the total assay time for pyridostigmine is about 15 min, the amount of pyridostigmine decomposing during the isolation and assay time periods would be negligible.

Controls for recovery of pyridostigmine

Concentrations of pyridostigmine in water, urine, and plasma at pH 10.2 were assayed (a) after passing solutions through a Millex filter, through a Sep-Pak and through both a Sep-Pak and a Millex filter, (b) after evaporation and reconstitution of an acetic acid—methanol eluate. Results showed 100% recovery at concentrations above 50 ng/ml.

Separation of metabolites of pyridostigmine

Chromatograms of pyridostigmine and two reported metabolites [6], RO-1-5237 and RO-1-5142, in aqueous solution are shown in Fig. 3. Retention times are 2.85 min for RO-1-5237, 3.54 min for RO-1-5142 and 3.63 min for pyridostigmine. As seen in Fig. 3, solutions containing both pyridostigmine and RO-1-5142 could produce overlapping peaks and would not be quantitated by

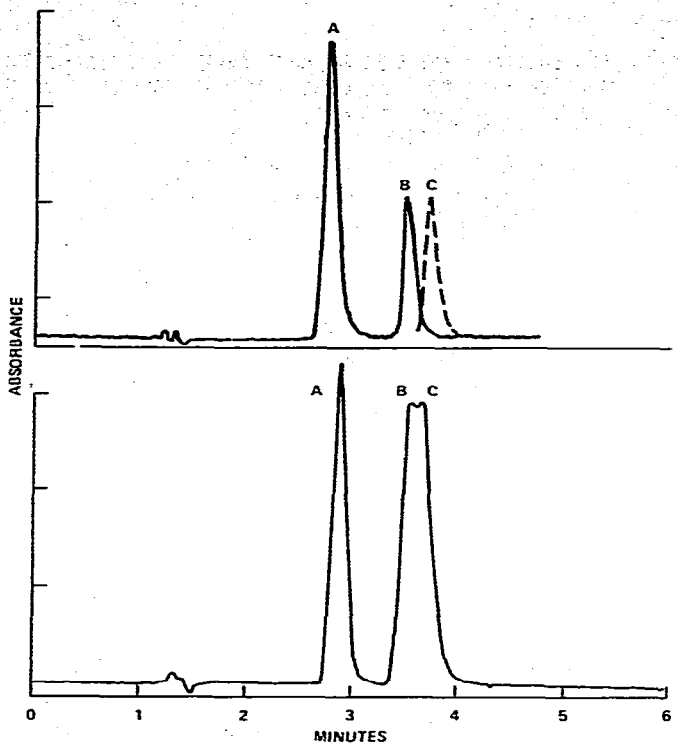

Fig. 3. Liquid chromatograms of pyridostigmine and metabolites. Peaks: (A) 3-hydroxy-N-methyl pyridinium bromide (RO-1-5237), (B) dimethylcarbamyl ester of 3-hydroxypyridine (RO-1-5142), and (C) pyridostigmine bromide. Top: low concentrations of components B and C are resolved; bottom: components B and C are unresolved at high concentration levels when assayed only by LC.

LC analysis. However, were these two products present together in a biological sample, they would be separated quantitatively by the Sep-Pak isolation procedure. The pyridostigmine would be retained on the cartridge, while the RO-1-5142 is eluted in the methanol wash. Were the RO-1-5237 metabolite and pyridostigmine present together in a sample, the former would be eluted in the water wash, the pyridostigmine would be retained. Additionally, the two could be separated and quantitated during the LC analysis.

Influence of inorganic ions on the binding and isolation of pyridostigmine from a Sep-Pak cartridge

Solutions of 0.1 *M* sodium chloride, 0.1 *M* sodium bromide, and 0.02 *M* phosphate buffer were spiked with pyridostigmine bromide to make final concentrations of 20 μg/ml. A 3-ml aliquot of each solution was assayed separately by the procedure described in this paper. Results are presented in Table II. The isolation and quantitation of pyridostigmine in aqueous solutions of sodium hydroxide and sodium carbonate at pH 10—10.5 have been shown to be quantitative and nearly quantitative. Lower recoveries were obtained from sodium chloride and sodium bromide.

TABLE II

RECOVERIES (%) OF PYRIDOSTIGMINE FROM AQUEOUS SOLUTIONS OF SODIUM CHLORIDE, SODIUM BROMIDE, AND PHOSPHATE BUFFER AFTER SEPARATION BY SEP-PAK AND ANALYSIS BY LIQUID CHROMATOGRAPHY

	Sodium chloride solution	Sodium bromide solution	Phosphate buffer
Amount pyridostigmine bromide added (μg)	59.2	58.8	58.2
Sample eluate (3 ml)	1.6	0	0
Water wash (5 ml)	1.7	0	0
Alcohol wash (5 ml)	30.9	25.9	0
Acetate—methanol (1 ml)	23.4	18.7	57.1
Acetate—methanol (1 ml)	4.0	3.7	22
% Recovery, total	103.1	82.1	101.9
acetate—methanol	46.3	38.1	101.7

Determination of a mechanism for the isolation of pyridostigmine by Sep-Pak cartridge

Since the contents of Sep-Pak cartridges become basic when pyridostigmine bromide at pH 10 is added, the resulting retention of pyridostigmine can be postulated to be due to binding to negatively charged siloxy groups present on the silica, or binding as an ion-pair to the octadecylsilane phase attached to the silica. To resolve this, it is necessary to compare silica to which octadecyl groups were bound to a silica, preferably with the same number of binding sites, and also which did not have octadecyl bound to it. Corasil II met this requirement. Corasil II has approximately the same number of free siloxy groups as the octadecyl-bound silica used in the Sep-Pak.

Glass cartridges, patterned to approximately the same size and shape as the Sep-Pak cartridges, were filled with Corasil II and also C_{18} silica from the Sep-Pak. Experiments were run with 45 μg pyridostigmine bromide in 0.05 *M* aqueous carbonate buffer (pH 10.5). Results shown in Table III indicate that pyridostigmine is strongly retained on silica which is not coated with organic phase.

Application of the method to biological specimens

The assay was applied to the quantitation of pyridostigmine in urine and plasma of rats following the oral administration of 450 μg of pyridostigmine bromide per kg of body weight. One ml of urine and 1.5 ml of plasma samples were used for an assay. The urinary excretion of pyridostigmine in three rats over a 24-h period was 60.0, 69.3, and 65.4% of the total dose injected. Less than 5% was excreted between 24 and 48 h. Initial plasma concentrations, using an average of three experiments for a single time point, were about 1/25 that of urine. Following a dose of approximately 80 μg per rat, average plasma levels of 250 ng/ml were determined after 7 min. Concentrations declined rapidly to less than 50 ng/ml within 1 h.

TABLE III

RECOVERIES OF PYRIDOSTIGMINE BROMIDE FROM GLASS CARTRIDGES CONTAINING CORASIL II AND SEP-PAK C_{18}-SILICA

	Corasil II (pH 10.5)	Sep-Pak (pH 10.5)	Corasil II (aqueous)
Amount pyridostigmine bromide added (μg)	45	45	45
Sample eluate (5 ml)	0	0.2	0
Water wash (5 ml)	0.5	0.9	0
Methanol wash (5 ml)	0	0.4	0
Acetate—methanol (1 ml)	6.8	31.8	0
Acetate—methanol (1 ml)	4.1	12.7	0.4
Acetate—methanol (1 ml)	2.6	1.7	0.6
% Recovery	30	103	2

Isolation of other types of aprotic compounds

In separate experiments, 2-ml aqueous solutions containing 25 μg/ml of acetylcholine, neostigmine, and edrophonium were adjusted to pH 10 and added to a Sep-Pak. Quantitative recoveries of these quaternary nitrogen compounds were obtained after elution with 2 ml of acetic acid in methanol.

DISCUSSION

A method is presented for the isolation and LC determination of pyridostigmine and metabolites in human urine and plasma. The drugs are separated at pH 10 with the aid of a Sep-Pak cartridge. The cartridge consists of a small plastic cylinder (6 × 7 mm) containing octadecylsilane bonded to porous 50-μm silica. Recoveries of pyridostigmine and metabolites added to human and rat urine and plasma are quantitative. Samples of urine or plasma (10 ml) can be extracted in less than 5 min with small volumes of solvents within a small laboratory space. Interferences from endogenous peaks from plasma were negligible and of minor nature from urine samples. In the ensuing analyses by LC a detection limit of 40 ng was obtained.

The method was applied to the determination of urinary excretion and plasma levels of pyridostigmine in rats following intramuscular administration. Levels were consistent with those reported by Birtley et al. [12] in which radiolabeled drug was used. A study of the method in humans will be the subject of a future report.

The procedure was also used to isolate aqueous solutions of aliphatic and aromatic quaternary nitrogen compounds such as acetylcholine, neostigmine, and edrophonium.

Possible mechanisms involved in the isolation of pyridostigmine in the reversed-phase cartridge are discussed below. In classical paired-ion reversed-phase chromatography the retention of an ionic species is enhanced by the addition of an organic counter-ion in the mobile phase. In the present work we found that polar inorganic counter-ions such as hydroxide, carbonate, and phosphate caused quantitative binding of pyridostigmine but not its metabolites on the

Sep-Pak reversed-phase column. Chloride and bromide ions caused lower affinities for binding. Either ion-pair formation, binding to siloxy groups present in the Sep-Pak, or possibly both effects could account for the binding of pyridostigmine. Organic solvents of various polarities could not extract pyridostigmine from basic solutions. Corasil II, containing a similar number of free binding sites as the Sep-Pak strongly retained pyridostigmine. Here, pyridostigmine could not be eluted with acetate—methanol. Thus it is likely that the isolation mechanism involves both ion-pair formation with hydroxide and the binding of the quaternary compound to siloxy groups. It is not clear why pyridostigmine in aqueous solutions, but not in urine, at pH values 8—9.5, can be recovered quantitatively. A possible explanation is that siloxy sites are not as available as pH decreases, and that compounds in urine may compete for these sites. Resulting ion-pair affinities of these inorganic counter-ions used in the isolation of pyridostigmine by the Sep-Pak are in reverse order to those reported for similar counter-ions in the basic efforts of Modin and Schill [13] and Borg [14].

A practical biological consideration of this work could apply to the way polar organic molecules are able to pass into lipid membranes. Results in this paper provide a mechanism as to how polar compounds can cross such barriers. Aprotic polar compounds could form an ion-pair with inorganic ions present in the body and be absorbed by a lipid membrane in a manner similar to that by which they are bound to the reversed-phase silicone system. The amount that is bound would depend both on the structure of the compound as well as the concentration of the inorganic counter-ion. Perhaps only small amounts of a drug would cross lipid barriers, but then only a small amount of drug entering a cell may be all that is required to sustain a therapeutic effect.

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