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A highly sensitive HPLC method for the assay of propantheline used to measure its uptake by rat intestinal brush border membrane vesicles

HIROSHI SAITOH, YUKARI KOBAYASHI, KATSUMI MIYAZAKI AND *TAKAICHI ARITA

Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, Japan

A simple, sensitive, high-performance liquid chromatographic method for propantheline has been developed. Propantheline was quantitatively hydrolysed into xanthene-9-carboxylic acid in neutral or alkaline medium and the hydrolysate assayed by reversed phase high-performance liquid chromatography. This method measured to 2 pmol per injection and was used to investigate the uptake of the drug by rat intestinal brush border membrane vesicles. Propantheline was highly bound to the membrane and this binding was inhibited to varying extents by several quaternary ammonium compounds. Mepenzolate and methylbenactyzium inhibited it significantly, and neostigmine, butylscopolamine and *N*-methylnicotinamide inhibited it moderately. Choline, acetylcholine and thiamine had no effect.

Quaternary ammonium compounds (QAC) are completely ionized over the pH-range in the gastrointestinal tract and generally have low lipid solubility, but it is well known that they are absorbed to varying extents (Levine 1959; Levine & Steinberg 1966; Roberts et al 1966; Waldeck 1969; Gaginella et al 1973; Friedman & Wang 1972). The absorption behaviours cannot be explained by Brodie's pH-partition theory alone. Levine (1966) and Levine & Spencer (1961) reported that a phosphatide-peptide fraction derived from the small intestine formed an absorbable complex with QAC, while passive diffusion through aqueous pores in the surface of the membrane was suggested for pralidoxime (Crone & Keen 1969) and neostigmine (Kunze et al 1971). Some investigators have held that several QAC could be absorbed from stomach and intestine as lipophilic ion-pairs with endogenous anions (Irwin et al 1969; Gibaldi & Grundhofer 1973; Newburger & Kostenbauder 1977). As has been pointed out by Ruifrok & Mol (1983), paracellular transport could not be excluded for small molecule QAC. Despite these implications, details of the permeation characteristics of QAC across the lipoidal brush border membrane remain unknown.

Therefore, as one approach to clarify the absorption mechanism of QAC, we have examined the uptake and binding behaviour of propantheline, an anti-acetylcholine QAC, by using rat intestinal brush border membrane vesicles.

* Correspondence.

A simple and sufficiently sensitive analytical technique for propantheline is needed for the study of the uptake behaviour by membrane preparations. Several methods for propantheline determination have been reported. Ion-pair extraction followed by fluorescence spectroscopy has been proposed (Westerlund & Karset 1973), but requires multiple extraction and is lengthy and complex. Although Ford et al (1977) reported a highly sensitive gas chromatography–mass spectrometry method, it requires an expensive and specialized instrument. We have, therefore, developed a simple and sensitive high performance liquid chromatographic method for the determination of propantheline in biological fluids.

MATERIALS AND METHODS

Materials

Propantheline bromide (Dainippon Pharmaceutical Co., Osaka, Japan), methylbenactyzium bromide and butylscopolamine bromide (Yamanouchi Pharmaceutical Co., Tokyo, Japan), and mepenzolate bromide (Fujisawa Pharmaceutical Co., Osaka, Japan) were kindly donated. Thiamine hydrochloride, neostigmine bromide, acetylcholine bromide and choline chloride were purchased from Wako Pure Chem. Ind., Osaka, Japan. *N*-Methylnicotinamide chloride and xanthene-9-carboxylic acid were obtained from Sigma (St Louis, MO) and Aldrich Chem. Co. (Milwaukee, Wis.), respectively. Cephalexin was kindly supplied by Shionogi and Co. (Tokyo, Japan). All other reagents were of the highest grade available commercially.

Preparation of brush border membrane vesicles

Brush border membrane vesicles were isolated from the intestines of male Wistar rats (200–250 g) according to the calcium chloride precipitation technique of Kessler et al (1978). The membrane vesicles were suspended in a final concentration of 3–4 mg protein mL^{-1} with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (Hepes)/Tris buffer (pH 7·5) and 100 mM D-mannitol. The purity of the membrane was routinely evaluated by the enrichment of alkaline phosphatase (E.C.3.1.3.1.), an enzyme specific to the intestinal brush border membrane. The specific activity of this enzyme increased 12-fold in the final membrane suspension compared with concentrations found in the homogenate of intestinal scrapings. The membrane vesicles were equilibrated for 1 h at 4 °C before use.

Uptake experiments

The uptake of a substrate into the isolated membrane vesicles was measured by a rapid filtration technique using 0·45 μm Millipore membrane filters (HAWP 02500). The specific conditions for each experiment are given in the figure legends. In the assay, the reaction was initiated by an addition of 100 μL of buffer, containing propantheline bromide, to 100 μL of membrane vesicle suspension at 25 °C. At stated times, it was stopped by dilution of an incubation sample with 10 mL of ice-cold buffer (150 mM NaCl, 1 mM Tris/HCl, pH 7·5) in the reaction tube followed by rapid filtration through a membrane filter. The filter was washed once with 15 mL of the same ice-cold buffer. Non-specific adsorption onto the Millipore filter was determined using the incubation medium instead of brush border membrane suspension. This value was subtracted from the uptake data.

Procedures for the assay of propantheline

A membrane filter to trap the vesicles was placed in a test tube containing 4 mL of phosphate buffer solution with pH 7 (1/15 M KH_2PO_4–1/15 M Na_2HPO_4) and heated for 40 min in a boiling water bath. After the tube contents had been cooled in an iced water bath and the filter removed, 0·5 mL of 1 M HCl was added to acidify the medium and 5 mL of chloroform then added. The mixture was gently shaken for 15 min and centrifuged at 1500*g* for 5 min. Four mL of chloroform was taken to dryness under vacuum. The residue was dissolved in 200 μL of the internal standard (n-butyl-*p*-amino benzoate) methanol solution and an aliquot (15–20 μL) of the solution was injected into the HPLC.

The conditions of HPLC were as follows: a liquid chromatograph (Hitachi 635A) equipped with a variable wavelength UV monitor (Hitachi 638-41) was used. The column, 25 cm × 4 mm i.d. stainless steel, was packed with Hitachi gel 3053 (ODS, 5 μm, Hitachi Ltd., Tokyo, Japan) and warmed at 55 °C using a water bath circulator. The mobile phase consisted of 0·05 M KH_2PO_4 (pH 2·5) and acetonitrile (64 : 36). The flow rate was 0·8 mL min^{-1} and the wavelength was 200 nm.

Cephalexin was determined by HPLC according to Miyazaki et al (1983). Protein was determined by the method of Lowry et al (1951) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Determination of propantheline

It is well known that propantheline is unstable in alkaline medium, and xanthene-9-carboxylic acid, which is a strong UV-absorbable compound, is formed as its hydrolysate. The hydrolysis reaction of propantheline was fast in alkaline medium (pH 11) and it took only 10 min to complete in a boiling water bath. In acidic medium (pH 2), on the other hand, it was very stable, and only 2% was hydrolysed even after 60 min. In neutral medium, the hydrolysis was completed after about 30 min (Fig. 1). From these results, we examined the analytical conditions such as the extractability and conditions of HPLC of xanthene-9-carboxylic acid.

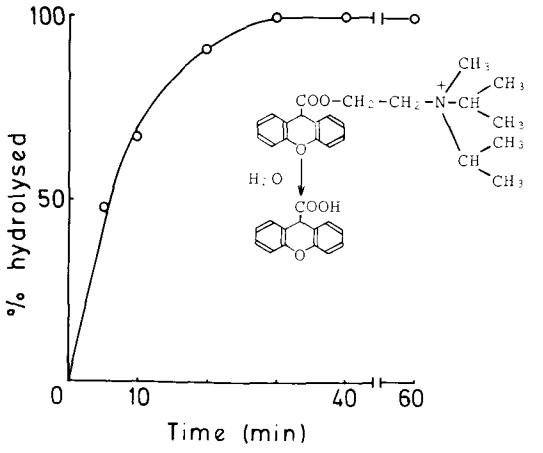

FIG. 1. Hydrolysis profile of propantheline at pH 7. Propantheline bromide (10 μM) was heated in a boiling water bath.

The extraction ratio of xanthene-9-carboxylic acid into chloroform from acidic medium below pH 2 was approximately 100%. Its retention time and that of

the internal standard were 10 min and 12 min, respectively. Sharp and symmetrical peaks were obtained for both compounds and the linear relationship between propantheline concentration and peak-height ratio was derived. The coefficient of variation at 5 nmol was 2% (n = 5), and minimum detectable amount was 2 pmol per injection.

To estimate the accuracy of the determination in the medium containing filter paper, a filter in alkaline medium was heated in a boiling water bath. The filter was dissolved and the medium became coloured, which disrupted the HPLC assay. In neutral medium, the filter was not affected and the recovery of propantheline was approximately 100%. Moreover, the presence of the membrane preparation did not affect the peak of the xanthene-9-carboxylic acid or the internal standard.

Time course of propantheline uptake

Fig. 2 shows the time course of uptake at various concentrations of propantheline. At 1 min, it was clear that the uptake was dependent on the concentration, but thereafter the difference became gradually less and tended to an equilibrium value. Furthermore, under the NaCl or KCl gradient (outside to inside), the time course of propantheline uptake was not affected (data not shown). In these experiments we confirmed that the hydrolysis of propantheline did not occur during uptake.

Binding to the brush border membrane

To distinguish between binding to the brush border membrane and transport into the intravesicular space,

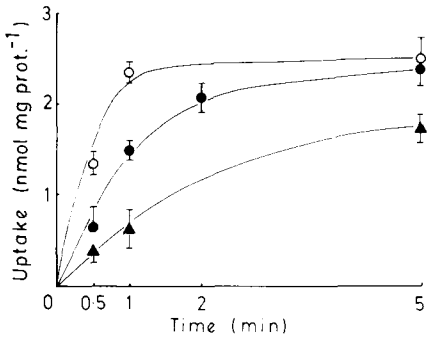

FIG. 2. Time course of propantheline uptake by brush border membrane vesicles at various concentrations of propantheline. Membrane vesicles were suspended in 20 mM Hepes/Tris buffer solution containing 100 mM D-mannitol (pH 7·5). Incubation medium was 20 mM Hepes/Tris buffer solution containing 100 mM D-mannitol and 1 mM (▲), 2·5 mM (●) and 5 mM (○) propantheline bromide, respectively (pH 7·5). Each point represents the means ± s.e.m. of five to six measurements with different preparations of vesicles.

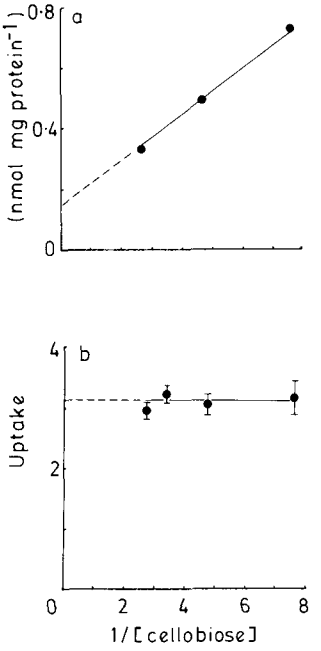

FIG. 3. Effect of medium osmolarity on cephalexin and propantheline uptake. Vesicles were suspended in 20 mM Hepes/Tris buffer solution containing 100 mM D-cellobiose instead of D-mannitol (pH 7·5). Incubation medium was 20 mM Hepes/Tris buffer solution containing various concentrations of D-cellobiose and 2·5 mM cephalexin (a) or propantheline (b). Uptake was determined after 30 min. Each point represents the mean of three measurements from a typical experiment in the case of cephalexin and means ± s.e.m. of four to six measurements with different preparations of vesicles in the case of propantheline.

the effect of extravesicular osmolarity on propantheline uptake was investigated and compared with that of cephalexin uptake. As shown in Fig. 3, for cephalexin the equilibrium uptake was decreased in inverse proportion to increase of extravesicular osmolarity. This meant that membrane vesicles were behaving as osmometers. For propantheline, on the other hand, the equilibrium uptake did not alter under the various medium osmolarities. Therefore it is suggested that propantheline is highly bound to the brush border membrane. This high binding property may partly explain the rapid arrival towards a steady state shown in Fig. 2.

Effects of other quaternary ammonium compounds on propantheline uptake

To clarify whether this binding property of propantheline was common for other QAC, the effects of various QAC on propantheline uptake were examined. As shown in Fig. 4, the equilibrium uptake of propantheline was significantly decreased when the

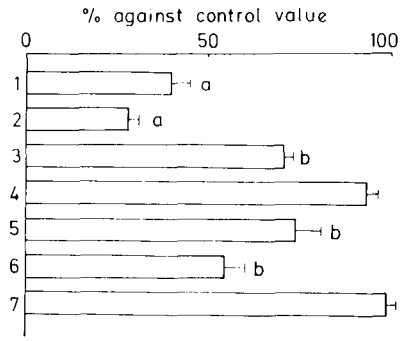

FIG. 4. Effects of various QAC on propantheline uptake by brush border membrane vesicles. Vesicles were preincubated in 20 mM Hepes/Tris buffer solution containing 100 mM D-mannitol and 5 mM various QAC (pH 7·5) at 25 °C for 3 min, and the mixture (200 μL) was incubated with 20 mM Hepes/Tris buffer solution (100 μL) containing 100 mM D-mannitol and 5 mM propantheline bromide (pH 7·5) at 25 °C for 10 min. Results are expressed as percent against the control value obtained from experiments without other QAC. Each column represents the mean ± s.e.m. of five to six measurements with different preparations of vesicles. (a) $P < 0{\cdot}01$, (b) $P < 0{\cdot}05$. Key: 1, methylbenactyzium; 2, mepenzolate; 3, butylscopolamine; 4, thiamine; 5, *N*-methylnicotinamide; 6, neostigmine; 7, choline.

vesicles were preincubated in the medium containing methylbenactyzium and mepenzolate, while neostigmine, butylscopolamine and *N*-methylnicotinamide inhibited moderately. Choline and thiamine, on the other hand, did not affect propantheline uptake.

Mepenzolate and methylbenactyzium have benzilic acid as the lipophilic side chain and the basal structure of acetylcholine. But both benzilic acid and acetylcholine had no effect on propantheline uptake. Therefore it was suggested that whole structures of both drugs may be essential for the inhibitory effect on propantheline uptake.

There was a large difference among QAC in their inhibitory effect on propantheline uptake. This suggests a specificity of binding to the brush border membrane. Although the binding mechanism of propantheline is unclear, this high binding to the membrane may play a role in the processes of transport through intestinal epithelium.

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