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DETERMINATION OF AMBENONIUM IN BIOLOGICAL SAMPLES BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and selective analytical method for the determination of ambenonium ion in biological samples is described The procedure involves ion-pair extraction of the drug, followed by reversed-phase ion-pair chromatographic analysis with ultraviolet detection at 217 nm. The detection limits at a signal-to-noise ratio of 5 were 100 pmol/ml using 0.2 ml of plasma and bile, 250 pmol/ml using 0.2 ml of urine and 200 pmol/g using 1 ml of tissue homogenates containing 0.1 g/ml of each tissue. This assay procedure was used to study the pharmacokinetics of ambenonium ion after intravenous administration in rats.

INTRODUCTION

Ambenonium (AMB) chloride, a reversible cholinesterase inhibitor, is used for the treatment of myasthenia gravis, a neuromuscular disorder. There have been no reports on the pharmacokinetics or pharmacodynamics of AMB, since the measurement of AMB in biological samples is difficult.

For the measurement of other quaternary ammonium compounds with anti-cholinesterase activity, such as neostigmine, pyridostigmine and edrophonium in biological samples, gas chromatography [1–3] and liquid chromatography (LC) [4–9] are available. More sensitive and selective methods involving gas chromatography–mass spectrometry are also available for the determination of neostigmine [10] and pyridostigmine [11,12].

More recently, an ion-pair LC method for the determination of AMB in human plasma was reported [13]. It is the only method that can be used to measure AMB concentrations in biological samples. However, to study the pharmacokinetics and pharmacodynamics in detail, it is necessary to determine the drug concentration not only in plasma but also in other biological samples, such as bile, urine

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and tissues. Furthermore, only small amounts of plasma can be used for the measurement to avoid physical damage by frequent blood collection in a small animal such as a rat.

This paper reports a high-performance liquid chromatographic (HPLC) method that involves ion-pair extraction of AMB from biological samples, followed by reversed-phase separation and UV detection at 217 nm. The assay procedure was used to study the pharmacokinetics of AMB after an intravenous administration to rats.

EXPERIMENTAL

Chemicals and reagents

Ambenonium chloride was kindly supplied by Nippon Shoji Kaisha (Osaka, Japan) and benzilonium bromide (BZN), used as an internal standard, was from Sankyo (Tokyo, Japan). The chemical structures of these drugs are shown in Fig. 1. AMB chloride was used as a stock solution composed of 1 μmol/ml as AMB in physiological saline. Sodium alkylsulphonates were purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and were of ion-pair chromatographic grade. Acetonitrile was purchased from Wako (Osaka, Japan) and was of liquid chromatographic grade. All other solvents and reagents used were purchased from commercial sources and were of reagent grade. All materials were used without further purification.

Sample preparation

Blood samples were collected in heparinized containers through a polyethylene cannula inserted into a femoral artery of a rat, and centrifuged for 10 min at 2260 *g* to separate plasma. Bile and urine were collected through a polyethylene cannula inserted into the bladder or bile duct, respectively, and diluted as necessary with physiological saline.

To 0.2 ml of each sample, 1 nmol of BZN in 10 μl of water, 1 ml of 1 *M* hydro-

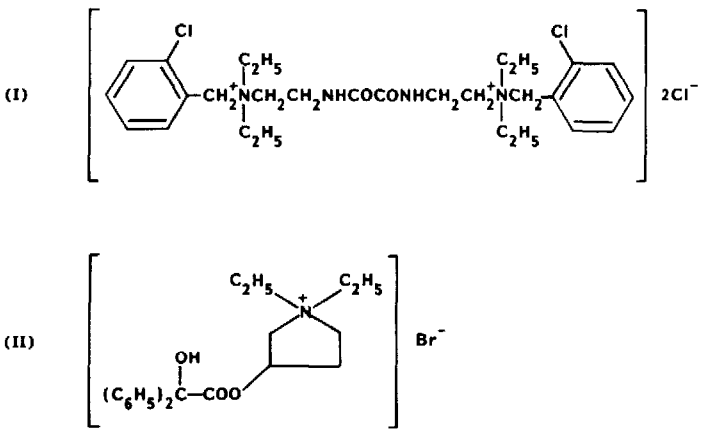

Fig. 1. Chemical structures of ambenonium chloride (I) and benzilonium bromide (II).

chloric acid and 4 ml of dichloromethane were added. The mixture was shaken with a mechanical shaker for 10 min and centrifuged at 1660 *g* for 5 min. A 1-ml volume of the upper aqueous phase was transferred to another tube, and 11 ml of dichloromethane and 0.2 ml of 1 *M* perchloric acid were added. The mixture was shaken for 10 min, then separated by centrifugation. After the upper aqueous phase had been removed with a pasteur pipette, 10 ml of the organic phase were transferred to another tube and 0.2 ml of 1 *M* sodium perchlorate–1 *M* sodium hydroxide (4:3) solution was added. The mixture was shaken for 10 min, then separated by centrifugation. Then 9 ml of the lower organic phase were transferred to another tube and evaporated to dryness at room temperature. The dried residue was dissolved in 50–100 μl of mobile phase, and 20 μl were injected into the HPLC column.

Liver, kidney, spleen, lung, skeletal muscle and brain were immediately removed from a rat killed by decapitation, then gently rinsed with physiological saline solution and blotted with filter paper to remove excessive moisture. A 0.3–1.0 g aliquot was homogenized with saline in an ice-bath, and prepared as a tissue homogenate sample containing 0.1 g of tissue in 1 ml. To 1.0 ml of each sample, 1 nmol of BZN, 1.5 ml of 1 *M* hydrochloric acid and 4 ml of dichloromethane were added. The mixture was shaken for 10 min, then separated by centrifugation. A 2-ml volume of the upper aqueous phase was transferred to another tube and subjected to the procedure described above.

Chromatography

The HPLC apparatus used in this study was composed of a Shimadzu LC-6A liquid chromatograph and a Shimadzu SPD-6AV spectrophotometer (Shimadzu, Kyoto, Japan). UV detection was at 217 nm. The column was a stainless-steel tube (250 mm×4 mm I.D.) packed with Senshu gel $7C_{18}H$ (Senshu Kagaku, Tokyo, Japan) by using a column-packing apparatus (Senshu Kagaku). The column temperature was maintained at 40°C by a column jacket connected to a water-bath.

The mobile phase was 35% acetonitrile in water containing 20 m*M* sodium octanesulphonate, 2.5 m*M* tetramethylammonium chloride and 10 m*M* sodium dihydrogenphosphate, and the pH was adjusted to exactly 3.0 with concentrated sulphuric acid. The mobile phase was degassed before use and pumped at a flow-rate of 0.7 ml/min.

Intravenous administration of AMB chloride to rats

Male Wistar rats weighing 280–330 g were used in all experiments. AMB chloride was administered intravenously to each rat in a single dose of 1 μmol/kg. Blood samples were drawn at 1, 3, 5, 10, 15, 20, 30, 45, 60, 90 and 120 min after administration. Urine and bile samples were collected for 0–1, 1–2, 2–3, 3–4, 4–6, 6–8, 8–24 and 24–48 h after administration. Tissue samples were collected at 24 h after administration. All samples were analysed within 24 h.

RESULTS AND DISCUSSION

Chromatographic conditions and extraction

Under the conditions described, AMB has a relatively long retention time compared with some other quaternary ammonium compounds, such as pyridostigmine and neostigmine. The only method for the determination of AMB in plasma, recently reported by Tharasse-Bloch et al. [13], was the absolute calibration method. To achieve further precision, the internal standard method was used. Of several compounds tested for use as an internal standard, BZN was found to be most suitable. The retention times of AMB and BZN are ca. 19 and 16 min, respectively.

Several kinds of mobile phase were investigated. Hexane-, heptane-, octane-, nonane- and decanesulphonate anions were tested for the ion-pairing reagent. Increasing the carbon chain-length of the alkyl group increased the retention times for AMB and BZN (Fig. 2). Hexane- and heptanesulphonate did not retain AMB sufficiently to separate it from endogenous substances, but octane-, nonane- and decanesulphonate were well suited for AMB analysis. Octanesulphonate was superior to the others in purity, availability and price. Perchlorate was also tested, but was found to be inappropriate as the pairing ion in the mobile phase.

When the concentration of pairing ion, octanesulphonate is increased the capacity factors of AMB and BZN are decreased (Fig. 3), because the retention of positively charged solute ion depends on the concentration of negatively charged pairing ion at the hydrophobic surface of the stationary phase. Thus, to achieve a good separation of AMB and BZN from endogenous substances, a relatively high concentration of octanesulphonate is desirable. However, little change in the retention was observed at concentrations above 20 m*M* (Fig. 3) so this concentration was selected.

The pH of the mobile phase had a slight effect on the retention of AMB and BZN in the range 2–4 (Fig. 4). In a usual ion-pairing system, the pH value of mobile phase is very important because the changing pH can convert the solute

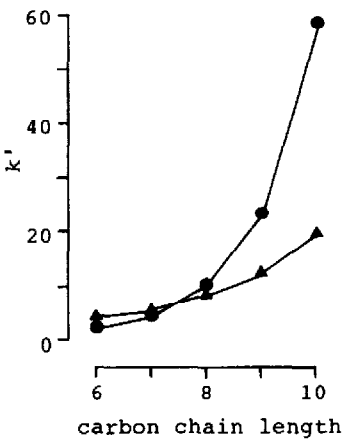

Fig. 2. Influence of carbon chain-length of the pairing alkylsulphonate anion on the retention of AMB (●) and BZN (▲). Other conditions are as described in the text.

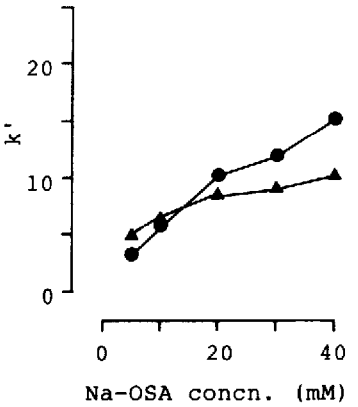

Fig. 3. Influence of sodium octanesulphonate (Na-OSA) concentration on the retention of AMB (●) and BZN (▲). Other conditions are as described in the text.

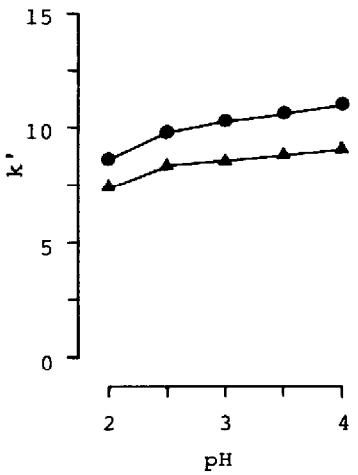

Fig. 4. Influence of pH on the retention of AMB (●) and BZN (▲). Other conditions are as described in the text.

into a non-ionized form. Quaternary ammonium compounds, however, cannot be in the non-ionized form, and thus the effect of the pH on their chromatographic behaviour is usually limited. Therefore, the pH of the mobile phase was set at 3.0, as in the method of De Ruyter et al. [5].

As shown in Fig. 5, a small change in the proportion of acetonitrile yielded a relatively large change in the retention of AMB and BZN. A slight change in the acetonitrile concentration in the mobile phase would be fatal for the AMB analysis, because an increase results in incomplete separation between AMB and BZN, and a decrease causes too long a retention and too low a sensitivity.

Perchlorate [4] and picrate [5,12,13] anions have frequently been used as ion-pair extraction reagents for the extraction of quaternary ammonium compounds. In this study, perchlorate anion was chosen, since it gave a higher extraction recovery than picrate anion in a preliminary study.

Prior to ion-pair extraction, biological samples were washed with dichlorome-

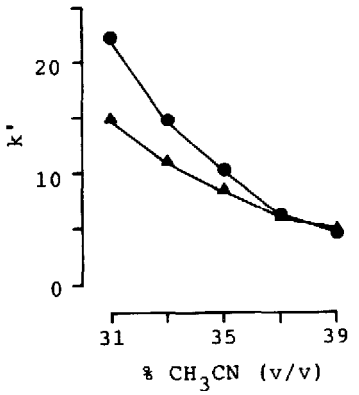

Fig. 5. Influence of acetonitrile concentration on the retention of AMB (●) and BZN (▲). Other conditions are as described in the text.

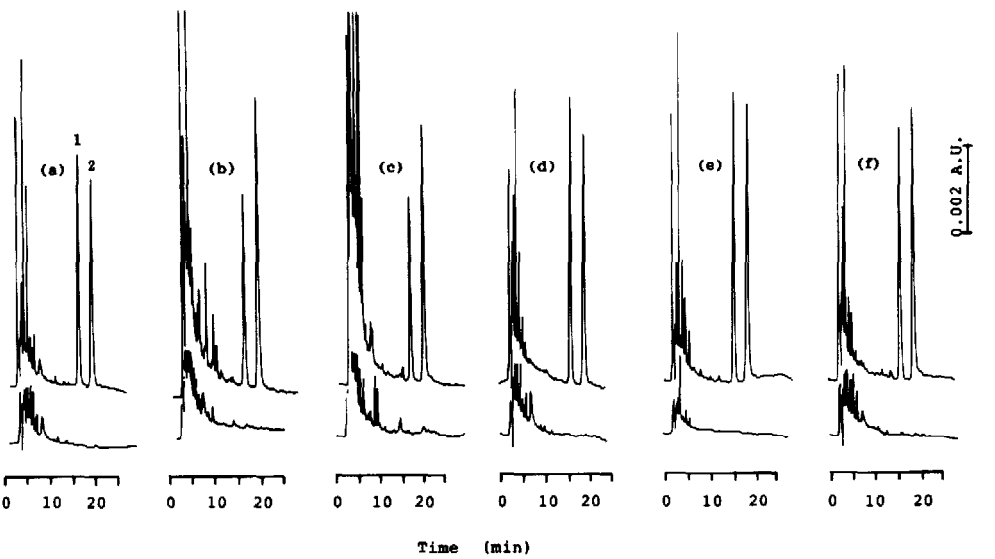

Fig. 6. Chromatograms of plasma (a), bile (b), urine (c), liver (d), kidney (e) and spleen (f) from rats, spiked with 500 pmol of AMB chloride and 1 nmol of BZN bromide (upper chromatograms) and drug-free (lower ones). Peaks: 1 = BZN; 2 = AMB. Chromatographic conditions are as described in Experimental.

thane to remove oil-soluble endogenous substances. Further clean-up under basic conditions decreased the amounts of endogenous substances.

Typical chromatograms obtained from each blank biological sample and the samples spiked with AMB chloride are shown in Fig. 6. In the blank chromatogram, a slight interference with the AMB peak was found, but only in the urine sample. The detection limits for AMB were 100 pmol/ml in plasma and bile, 250 pmol/ml in urine and 200 pmol/g in each tissue, respectively, at a signal-to-noise ratio of 5.

A series of AMB standards containing 20, 50, 200, 500 and 2000 pmol in 0.2 ml

of plasma, bile, urine, and in 1 ml of each tissue homogenate was prepared, and these samples were analysed by the described procedure. A calibration curve for each sample was obtained by plotting the peak-height ratio against the known amount of AMB in each sample. The calibration curve for each biological sample (except urine) was linear in the range 20–2000 pmol (e.g. plasma: $y=0.00235x-0.00116$, $r=1.0000$; liver: $y=0.00205x-0.00063$, $r=0.9999$), and that for urine was linear in the range 50–2000 pmol ($y=0.00238x+0.09661$, $r=0.9998$). Furthermore, the calibration curves for bile and urine diluted with 9 volumes of saline were also linear in the range 50–2000 pmol (e.g. diluted bile: $y=0.00243x+0.01543$, $r=1.0000$).

The reproducibility of the analysis was determined by repeating the procedure five times for each sample. The coefficients of variation (C.V.) of the peak-height ratio were sufficiently small, as shown in Table I.

The extraction recovery of AMB from each sample was determined by comparing the peak height obtained from the extract of each sample with that resulting from direct injection of a known amount. Extraction recoveries from each sample at four different amounts (50, 200, 500 and 2000 pmol) were substantially constant, as shown in Table II.

It is well known that some quaternary ammonium cholinesterase inhibitors are readily hydrolysed in blood [10,11], plasma [7,9–11,14] and even buffer solutions [3,6]. This decomposition is probably related to the action of plasma cholines-

TABLE I

REPRODUCIBILITY OF AMBENONIUM ANALYSIS IN BIOLOGICAL SAMPLES FROM RATS

Amount added (pmol)	Coefficient of variation ($n=5$) (%)								
	Plasma	Bile	Urine	Liver	Kidney	Brain	Muscle	Lung	Spleen
20	3.4	3.1	–	5.5	4.2	4.0	7.0	5.0	4.7
50	6.0	2.5	7.8	5.5	5.0	4.5	4.7	4.7	5.5
200	2.5	3.1	6.2	6.0	4.2	4.3	4.7	5.0	6.2
500	5.4	5.4	5.6	4.6	3.4	7.8	5.8	3.1	4.3
2000	5.2	3.2	5.4	4.8	4.5	6.7	4.6	4.4	2.8

TABLE II

EXTRACTION RECOVERIES OF AMBENONIUM FROM BIOLOGICAL SAMPLES FROM RATS

Amount added (pmol)	Recovery (mean ± S.D., $n=5$) (%)								
	Plasma	Bile	Urine	Liver	Kidney	Brain	Muscle	Lung	Spleen
50	80.2±6.3	87.8±6.3	93.0±4.2	87.8±3.3	103.4±11.9	86.6±2.3	95.7±4.2	94.3±9.3	96.9±5.5
200	86.2±1.3	90.2±3.8	79.9±4.6	81.1±3.5	90.8± 9.1	82.2±3.8	82.6±3.8	97.9±2.3	100.1±4.1
500	77.9±4.2	87.1±6.6	86.5±3.1	86.1±8.2	99.7± 7.1	78.7±4.7	85.4±5.8	97.4±3.8	97.6±4.2
2000	80.8±2.4	89.3±2.3	82.2±6.9	86.2±7.4	95.0± 4.2	84.1±6.7	92.5±2.9	94.4±6.6	101.2±2.9

terase, so, blood sampling and plasma separation should be performed under cool conditions. In a preliminary study, we examined the stability of AMB in stock solutions and in rat plasma. The concentration of AMB in stock solutions remained constant for more than a year at room temperature. In plasma, a sample containing 2.5 nmol/ml AMB was incubated at 37 °C, and analysed. No decrease in the AMB concentration was found within 48 h. Consequently, the decomposition of AMB in a biological sample at room temperature will be negligible.

Application to pharmacokinetics

The analytical method described above was applied to a study of the disposition of AMB in rats. The time course of the mean plasma concentration and the cumulative urinary and biliary excretion of AMB after intravenous administration of 1 μmol/kg AMB chloride to three rats are shown in Fig. 7 and Fig. 8, respec-

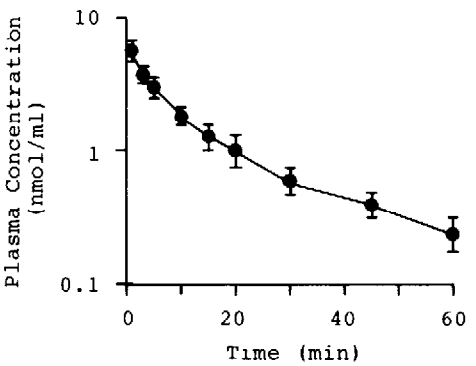

Fig 7. Time course of AMB concentration after intravenous administration of 1 μmol/kg AMB chloride to rats. Each point and vertical bar indicate the mean and standard deviation

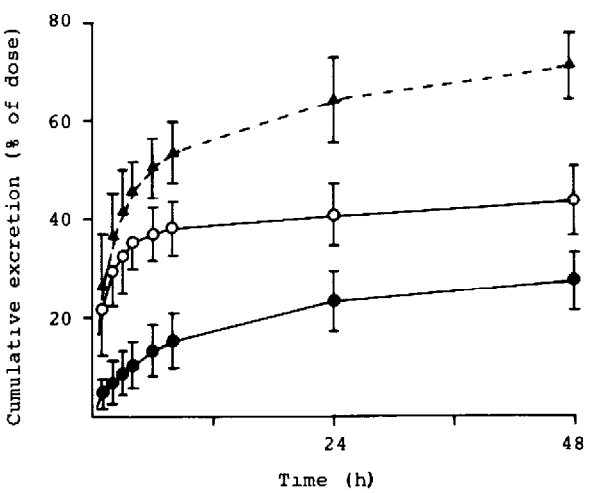

Fig. 8. Cumulative amount of AMB excreted into bile (●), urine (○) and bile + urine (▲) after intravenous administration of 1 μmol/kg AMB chloride to rats. Each point and vertical bar indicate the mean and standard deviation.

TABLE III

AMBENONIUM CONCENTRATION IN RAT TISSUES

Each value represents the mean ± S.D. of five experiments 24 h after intravenous administration of 1 μmol/kg AMB chloride.

Tissue	Concentration (nmol/g)
Liver	3.51 ± 0.62
Kidney	2.77 ± 1.63
Brain	Not detected
Lung	Trace
Spleen	0.55 ± 0.13
Muscle	Not detected

tively. The plasma concentration of AMB decreased rapidly, and was less than 100 pmol/ml 90 min after administration. The apparent elimination half-life from plasma was ca. 20 min. Up to 24 h after administration, 40.9 ± 6.3% of the given dose of AMB was excreted in urine and 23.3 ± 6.2% in bile.

Contrary to its rapid elimination from plasma, AMB is excreted in urine and bile throughout 48 h. Therefore, we applied this method to the analysis of AMB in tissues 24 h after intravenous administration of AMB chloride. The results are shown in Table III. Although the concentration of AMB in plasma was low, it was high in liver and kidney.

CONCLUSION

A sensitive, specific and precise method for the determination of AMB in plasma, bile, urine and tissue homogenates by ion-pair HPLC was established. It would be useful in pharmacrkinetic studies of AMB, since the ambenonium ion concentration in these samples could be precisely determined in the range 20–50 pmol. Further studies of the disposition of AMB will be reported elsewhere.

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