INTESTINAL ABSORPTION OF SALICYLAMIDE AND EFFECT OF ATROPINE ON IT

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(Received October 3, 1984)

The effect of atropine (ATR), a parasympatholytic agent, on the intestinal absorption of salicylamide (SAM) was studied using the absorption kinetic model proposed by Winne et al. The disappearance of SAM from perfusate and the appearance in intestinal blood were determined using perfused intestinal loop of the rat in vivo. The results showed that the absorption of SAM was simulated by the four compartment model consisting of luminal, interstitial, blood and serosal compartments. The model was assumed to have three rate determining factors, namely mucosal membrane permeability, clearance by blood flow and serosal membrane permeability. ATR decreased the absorption of SAM by decreasing the clearance factor relating to intestinal blood flow and increased the fraction of the transported amount of SAM from interstitial space to serosal compartment.

Keywords — salicylamide; atropine; intestinal absorption; intestinal blood flow; perfusion; everted sac

INTRODUCTION

Intestinal absorption is the first important factor which determines the availability of the administered drug. The factors affecting it are so many and so complicated that the precise description and prediction of the real absorption process are difficult.

For a practical purpose, the pharmacokinetic description rather than the precise mechanism of the real absorption process is good to determine the intestinal absorption characteristics of a drug. Winne et al. proposed intestinal absorption models consisted of three or four compartments including blood flow. These models indicated that the absorption of a drug which has considerably large membrane permeability may be controlled by intestinal blood flow.

It has been reported in the previous paper that intestinal absorption of sulfamethoxazole obeyed the three compartments model and was controlled by not only the membrane permeability but also the clearance by blood flow, and that chlorpromazine decreased the absorption of sulfamethoxazole (SMZ) by decreasing both factors. In this paper we studied the absorption kinetics of salicylamide (SAM) and the effect of atropine (ATR) on it. ATR was chosen because it is a famous parasympatholytic agent and is known to have effects on blood flow, blood pressure and motility of gastrointestinal tract.

MATERIALS AND METHODS

Materials — Salicylamide (SAM) and benzoic acid (BA) were purchased from Tokyo Kasei Chemicals, Tokyo, Japan. Atropine (ATR) was supplied by Nakarai Chemicals, Kyoto, Japan. β-glucuronidase/arylsulfatase was purchased from Boehringer Mannheim, West Germany. All other reagents were commercial products and of analytical grade.

Perfused Everted Sac in Vitro — Male Sprague Dawley rats, weighing 250—300 g were used without fasting. The rat was killed by a depletion of blood from the carotid artery. After an abdominal incision, the first 25 cm of small intestine distal to the pylorus was discard-
ed and the next 25 cm was removed into an ice cold physiological saline solution that was bubbled with 100% O₂. The intestine was washed with 30 ml of the same solution, everted inside-out and put into Soerensen buffer containing 120 mM NaCl, 30 mM K₂HPO₄, 30 mM KH₂PO₄, 1 mM CaCl₂, and 1 mM MgCl₂, adjusted to pH 6.5 and saturated with 100% O₂. The sac was cut into 10 cm long, cannulated with vinyl tubings at both ends and immersed in 80 ml of the same buffer which contained 10 mM SAM (mucosal solution). The inside of the sac was perfused with 8 ml of buffer (serosal solution) at a rate of 2 ml/min. Both solutions were bubbled with 100% O₂. In ATR experiment, ATR was added to both solutions at a concentration of 20 μg/ml. A sample of 0.1 ml serosal solution was taken at 10, 20, 30, 40, 50, and 60 min after the initiation of perfusion. After perfusion, the length of the sac was measured and converted to a wet tissue weight multiplied by 0.067 g/cm.³

The permeability (Pᵣ) was calculated from the next equation:

\[ Pᵣ = \frac{(dCₛ/dt) \cdot (Vₛ/Cₘ)}{\text{ml/min}} \]  (1)

where
\[ \frac{dCₛ}{dt} \]: slope of the plot of serosal concentration of SAM versus time
\[ Vₛ \]: volume of the serosal solution (8 ml)
\[ Cₘ \]: mucosal concentration of SAM (10 mM)

**Perfused Intestinal Loop in Vivo** — The method was described previously.³ Male Sprague Dawley rats, weighing 270—300 g, were fasted for 16—20 h prior to the experiments, but water was allowed at libitum. The rat was anesthetized with ethylcarbamate (25% w/v) by an intraperitoneal injection of 4.5 ml/kg. After an abdominal incision, an intestinal segment of about 10 cm long, situated in the proximal jejunul region at 25 cm distal to the duodenjejunal flexure was chosen. Both ends of the intestinal loop were cannulated with polyethylene tubings.

The intestinal loop was rinsed with 20 ml of 0.154 M NaCl solution and then all the solution was pushed out from lumen with 20 ml of air. The loop was perfused with 40 ml of Soerensen buffer containing 10 mM SAM at a flow rate of 2 ml/min. In ATR experiments, ATR (500 μg/kg) was injected from the femoral vein at the initiation of perfusion. In recovery experiments, 2 mM BA was added to mucosal solution. The intestine was covered by a wet gauze and a vinyl sheet to prevent dryness and the rat was warmed with an electric lump and a heating pad to keep the body temperature at 37 °C.

**Calculation of the Parameters** — The three parameters limiting the absorption, i.e. mucosal permeability \( k_mA_m \), serosal permeability \( k_sA_s \) and the effective blood flow ratio \( \alpha aV_b \), were calculated according to the four compartment absorption model proposed by Winne (Fig. 1).²

In this model, the appearance rate of a drug (\( \phi_b \)) into intestinal blood is given by the next equation;

\[ \phi_b = \frac{C_m}{k_mA_m} + \frac{k_mA_m + k_sA_s}{k_mA_m \cdot \alpha aV_b} \]  (2)

where
\[ C_m \]: drug concentration in intestinal lumen (mm)

![FIG. 1. Four Compartment Model](image)

For details, see text.
$k_m$ : permeability coefficient of mucosal membrane (cm/min)

$A_m$ : mucosal area (cm$^2$)

$k_s$ : permeability coefficient of serosal membrane (cm/min)

$A_s$ : serosal area (cm$^2$)

$\alpha$ : fraction of the total blood flow rate that is attributable to subepithelial capillaries

$a$ : ratio of the concentration in blood to that in plasma

$V_b$ : total blood flow rate in the intestinal segment (ml/min)

Rearrangement of Eq. 2 yields

$$C_m \phi_b = \frac{1}{k_m A_m} + \left[ 1 + \frac{k_s A_s}{k_m A_m} \right] \frac{1}{\alpha a V_b}$$ (3)

The mucosal membrane permeability ($k_m A_m$) was calculated from the intercept of the plot $C_m / \phi_b$ versus $1/V_b$ according to Eq. 3. Then serosal membrane permeability ($k_s A_s$) and $\alpha a$ were estimated using nonlinear least square method according to Eq. 2.

**Water Flux Determination** — The intestinal perfusion method *in vivo* was the same as described previously. 3) Perfusion solution of 80 ml Soerensen buffer contained 10 mM SAM, 1.46 mg/ml polyethylene glycol 4000 (PEG) and 0.0188% D$_2$O. A sample of 1 ml was pipetted out from the perfusate at 10, 15, 20, 25, 30, 35 and 40 min after the initiation of perfusion. Then all perfusate was removed from the intestinal lumen and the intestinal loop was rested for 10 min. The same perfusion procedure was restarted concomitantly with an intravenous injection of ATR (500 $\mu$g/kg) from the femoral vein.

The volume change of perfusate was calculated from PEG concentration as a non-absorbable marker.

$$V_n = \frac{117000 - \Sigma X_n}{C_n}$$ (4)

where

$V_n$ : volume of the perfusate before the $n$-th sampling (ml)

$C_n$ : PEG concentration of the $n$-th sampling (µg/ml)

$\Sigma X_n$ : sum of PEG amount removed before the $n$-th sampling (µg)

Initial PEG amount in 80 ml perfusion solution was 117 mg.

The absorption rate of H$_2$O ($J$) from perfusate was given by Eq. 5, and the apparent water flux was given by Eq. 6. 5)

$$J = W_i \frac{X_t}{V_o - (W_i - W_e) t} + W_e \frac{X_o - X_t}{V_d + (W_i - W_e) t}$$ (µg/min) (5)

$$W_n = W_i - W_e$$ (ml) (6)

where

$X_o$ : amount of D$_2$O at time 0 (µg)

$X_t$ : amount of D$_2$O at time $t$ (µg)

$V_o$ : volume of perfusate at time 0 (ml)

$V_d$ : distribution volume of D$_2$O, 0.56 × body weight (ml)

$W_n$ : water netflux (ml/min)

$W_i$ : water influx (ml/min)

$W_e$ : water efflux (ml/min)

The values of $W_i$ and $W_e$ were best estimated from the observed data by a combination of nonlinear least square method and Runge-Kutta-Gill method.

**Lymph Collection** — The fasted rat was administered with 2 ml of milk by a stomach tube 30 min before the operation. Under anesthesia with ethylcarbamate, a polyethylene tubing (PE 50, Clay Adams, U.S.A.) was cannulated to the thoracic duct. 6) Then the whole intestine, from 10 cm distal to the pylorus to 5 cm proximal to the cecum, was perfused with 80 ml of buffer solution containing 10 mM SAM at a rate of 2 ml/min for 2 or 3 h. During perfusion, whole lymph was collected.

**Protein Binding of SAM** — Two chambers were separated by a molecular sieve membrane (Spectrapor, Spectrum Med. Ind., U.S.A.). The one contained 1 ml of rat plasma and 1 ml of buffer solution containing SAM at a concentration of 2.0, 1.0 or 0.5 mM, and the other contained 2 ml of buffer solution. In ATR experiments, ATR was added to the plasma chamber.
at a concentration of 2 mM. The apparatus was shaken at 100 cycle/min in a 37 °C water bath for 3 h. In preliminary experiments, it was assured that 3 h incubation was enough for the concentration equilibrium and that the binding of SAM to the apparatus was negligible. A sample of 0.1 ml was transferred from the plasma free chamber for the SAM determination.

Analytical

SAM — Free and conjugated SAM were determined by the fluorometric method. A sample of 0.1 ml of the buffer solution was mixed with 0.2 ml of β-glucuronidase/arylsulfatase (0.026 u/0.013 u) mixture and 0.8 ml of water. After incubation at 37 °C for 24 h, 0.4 ml of KH2PO4, 0.5 ml of 0.1 N HCl and 5 ml of ethylenedichloride/cyclohexane (65/35) mixture were added. After shaking for 10 min and centrifugation for 10 min at 1000 × g, 4 ml of organic phase was taken and mixed with 5 ml of 0.2 N NaOH. The total SAM concentration in aqueous phase was determined photofluorometrically in a Hitachi 650-10 photofluorimeter (exciting 350 nm and emission 420 nm). Free SAM was determined as following. To 0.1 ml of buffer solution or diluted blood (2.5 times), 1.8 ml of water, 0.5 ml of 0.1 N HCl and 5 ml of ethylenedichloride/cyclohexane mixture were added. The fluorescence was determined as described above. Conjugated SAM was calculated by subtracting free SAM from total SAM.

BA — A 0.1 ml portion of perfusate was diluted to 3 ml and BA concentration was determined photometrically at 230 nm in a Hitachi 200-20 photometer. SAM has also absorbance at 230 nm, so a correction for it was done. The blood concentration of BA was determined in the same sample used for free SAM determination. The pH of final aqueous phase was adjusted to 6–7 by an addition of HCl and absorbance at 230 nm was measured.

PEG 4000 — To 0.1 ml of perfusate, 2 ml of 20% trichloroacetic acid was added. After 5 min, absorbance was measured at 650 nm.

D2O — The D2O concentration in perfusate was determined by the infrared absorption method reported by Thornton et al. using CaF2 cell (0.1 mm thick) at 2500 cm⁻¹ in a Hitachi 295 infrared spectrophotometer.

Statistical Analysis — All means are presented with their standard errors. Student’s t-test was utilized to determine the significance of the difference between the control and ATR-treated experiments, with p = 0.05 as the minimal level of significance.

RESULTS

In Vitro Perfused Everted Sac

The time courses of free and conjugated SAM concentrations in serosal solution are shown in Fig. 2. The intestinal wall permeabilities of free SAM (Pf) were calculated from the slope of the straight line (Table I). These were 0.089 and 0.077 ml/min/g for control and ATR experiments, respectively, and had no significant difference (p < 0.1). The conjugation clearances of SAM were calculated similarly, and are shown in Table I. These were 12.7 and 11.5% of the total clearances of SAM for control and ATR, respectively.

![Graph showing time course of SAM concentration in serosal solution](image_url)

**FIG. 2. Time Course of SAM Concentration in Serosal Solution of Everted Sac In Vitro and ATR Effect**

Each point and vertical bar represent the mean ± S.E. of three experiments.

Key: Free SAM for control (○) and for ATR (●), conjugated SAM for control (▽) and for ATR (▼).
TABLE I. Effect of ATR on Permeability of Free SAM (Pf) and Conjugation Clearance in Everted Sac Experiments in Vitro a)

<table>
<thead>
<tr>
<th></th>
<th>Pf (ml/min/g)</th>
<th>Conjugation (ml/min/g)</th>
<th>Percent of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.089 ±0.005</td>
<td>0.013 ±0.008</td>
<td>12.7</td>
</tr>
<tr>
<td>ATR</td>
<td>0.077 ±0.005</td>
<td>0.010 ±0.002</td>
<td>11.5</td>
</tr>
</tbody>
</table>

a) Results are given as the means ± S.E. of three experiments.

TABLE II. Effect of ATR on Recovery Percent of Drug Transported from Perfusate a)

<table>
<thead>
<tr>
<th></th>
<th>Recovery percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SAM b)</td>
</tr>
<tr>
<td>Control</td>
<td>44.6 ±4.3</td>
</tr>
<tr>
<td>ATR</td>
<td>26.8 ±3.2 c)</td>
</tr>
<tr>
<td>% of ATR/Cont</td>
<td>60.1</td>
</tr>
</tbody>
</table>

a) Results are given as the means ± S.E. of three experiments.
b) Percent of free SAM/total SAM.
c) Significantly different from the control.

FIG. 3. Effect of ATR on the Relationship between Appearance Rate of SAM in Blood and Blood Flow Rate

Data points were obtained from four (control) or five (ATR) experiments.

Recovery Experiments in Vivo Perfused Intestinal Loop

The recovery percent of the drug transported from perfusate into blood during perfusion for 10 min was shown in Table II. In case of SAM, percents of free SAM in blood/total SAM transported are shown. The percents in control were 44.6 for SAM and 97.4 for BA. In ATR the percents decreased to 26.8 and 59.3, respectively, these were 60% of those of control.

Relationship between Appearance Rate of SAM into Blood and the Blood Flow Rate

Fig. 3 shows the plot of appearance rate of free SAM into blood (ϕb) versus blood flow rate. The mean blood flow rate were 0.35 and 0.30 ml/min/g for control and ATR, respectively, and had no significant difference (Table III), while appearance rate of SAM was decreased significantly by ATR from 594 to 382 nmol/min/g.

Fig. 4 is a representative plot of the data according to Eq. 3 from one rat for control and ATR. The plot is well approximated by a straight line and the intercept of the ordinate gives the value of 1/kmA_m. Then αa and kSA_s were estimated and are shown in Table IV. Though k_mA_m and k_SA_s were not decreased by ATR, αa was significantly decreased to 60% of that of control.
**TABLE III. Effect of ATR on Appearance Rate of SAM (φ_b) and Blood Flow Rate (V_b)**
a)

<table>
<thead>
<tr>
<th></th>
<th>φ_b (nmol/min/g)</th>
<th>V_b (ml/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>594 ± 35</td>
<td>0.35 ± 0.09</td>
</tr>
<tr>
<td>ATR</td>
<td>382 ± 27</td>
<td>0.30 ± 0.09</td>
</tr>
</tbody>
</table>

*a) Results are given as the means ± S.E. of four (control) and five (ATR) experiments. b) Significantly different from the control.*

![Graph](image)

**FIG. 4. Effect of ATR on the Relationship between 1/V_b versus C_m/φ_b**

A representative data from each one rat was plotted according to equation 3 in the Text. Key: (○) control and (●) ATR.

**DISCUSSION**

SAM has been used in absorption experiments frequently and known to be conjugated partly in the intestinal wall during the absorption. In this study, the luminal concentration of SAM was sufficiently large so as to saturate the conjugation capacity and to minimize the conjugation effect on the behavior of free SAM. The following discussions were done in regard to free SAM excluding a part of SAM to be conjugated.

The recovery percents of total SAM in Table II are corrected to show that of free SAM using the percents of conjugated SAM/total SAM. The corrected percents were 51.1 and 30.3 for control and ATR, respectively. The conjugated SAM was not detected from mucosal solution. The mucosal membrane may be a barrier for polar conjugated SAM. Free SAM recovered in collected blood was only a half of SAM transported from perfusate without conjugation in control. The possibility that the collection of blood was not complete was denied by the fact that BA recovery was perfect in control. BA has been reported to be recovered completely from intestinal blood and to be absorption rate limited by both the membrane permeability and blood flow by Ochsenfahrt et al. The administration of ATR decreased the recovery in blood to 60% of control in both cases of SAM and BA. This fact showed that ATR increased flux to any routes other than collected blood. Considering these results, the four compartments model proposed by Winne was used to describe the absorption kinetic of SAM. This model has the fourth compartment named the serosal compartment. At first the data were plotted according to Eq. 2 to determine the mucosal membrane permeabili-
The plot is well approximated by a straight line and shows a propriety of this method resulting in $kma_m$ values of 0.130 and 0.131 ml/min/g for control and ATR, respectively. These were larger than permeabilities ($P_i$) of 0.089 and 0.077 ml/min/g obtained from $in vitro$

**TABLE IV. Effect of ATR on Clearance Parameters of Intestinal Absorption of SAM in Vivo**

<table>
<thead>
<tr>
<th></th>
<th>$kma_m$ (ml/min/g)</th>
<th>$\alpha a$</th>
<th>$kSA_s$ (ml/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.130 ± 0.006</td>
<td>0.597 ± 0.057</td>
<td>0.178 ± 0.038</td>
</tr>
<tr>
<td>ATR</td>
<td>0.131 ± 0.021</td>
<td>0.383 ± 0.049 $b)$</td>
<td>0.239 ± 0.093</td>
</tr>
</tbody>
</table>

$a)$ Results are given as the means ± S.E. of four (control) and five (ATR) experiments. $b)$ Significantly different from the control.

**TABLE V. Effect of ATR on Water Fluxes**

<table>
<thead>
<tr>
<th></th>
<th>$W_n$ (ml/min/g)</th>
<th>$W_i$ (ml/min/g)</th>
<th>$W_c$ (ml/min/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.134 ± 0.019</td>
<td>1.102 ± 0.164</td>
<td>0.968 ± 0.152</td>
</tr>
<tr>
<td>ATR</td>
<td>0.146 ± 0.023</td>
<td>1.013 ± 0.153</td>
<td>0.866 ± 0.134</td>
</tr>
</tbody>
</table>

$a)$ Results are given as the means ± S.E. of five experiments.

**TABLE VI. Appearance of SAM into Lymph**

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Time (h)</th>
<th>Disap. $a)$ (nmol)</th>
<th>Appea. $b)$ (nmol)</th>
<th>Recov. $c)$, $10^{-3} %$</th>
<th>Lymph. $d)$ (ml/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>0.186</td>
<td>21.8</td>
<td>11.7</td>
<td>0.258</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>0.150</td>
<td>13.1</td>
<td>8.7</td>
<td>0.350</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>0.116</td>
<td>88.8</td>
<td>76.0</td>
<td>0.567</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>0.151</td>
<td>41.2</td>
<td>32.1</td>
<td>0.391</td>
</tr>
<tr>
<td>S.E.</td>
<td></td>
<td>0.020</td>
<td>23.9</td>
<td>22.0</td>
<td>0.092</td>
</tr>
</tbody>
</table>

$a)$ SAM amount disappearing from perfusate. $b)$ SAM amount appearing into lymph. $c)$ Recovery in lymph. $d)$ Lymph flow rate.

**TABLE VII. Protein Binding of SAM**

<table>
<thead>
<tr>
<th>SAM Conc. $b)$ (mM)</th>
<th>Binding percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0.5</td>
<td>33.7 ± 4.2</td>
</tr>
<tr>
<td>1.0</td>
<td>24.8 ± 4.6</td>
</tr>
<tr>
<td>2.0</td>
<td>21.9 ± 5.0</td>
</tr>
<tr>
<td>Mean</td>
<td>26.7 ± 2.9</td>
</tr>
</tbody>
</table>

$a)$ Results are given as the means ± S.E. of seven experiments. $b)$ Initial concentration in the plasma free chamber.
Intestinal Absorption of Salicylamide

experiments.

If we assume that mucosal membrane permeability were the same in vivo and in vitro ($K_m A_m = P_m$), and that mucosal and serosal membranes made barriers in series in vitro, serosal membrane permeability ($P_s$) could be calculated from the next equation.

$$P_s = \frac{P_m P_t}{P_m - P_t},$$

$P_s$ were 0.282 and 0.187 ml/min/g for control and ATR, respectively. $P_s$ in vivo was thought to be the serosal muscularis layer permeability. These $P_s$ were compared to serosal membrane permeability ($k_s A_s$) obtained in vivo of 0.178 and 0.239 ml/min/g for control and ATR, respectively. Serosal permeabilities obtained in different experimental conditions did not differ so much, nevertheless the everted sac in vitro has no circulation systems while $k_s A_s$ in vivo were estimated using the data obtained from intestinal blood. The serosal compartment was suspected to have a relationship with lymph flow, but the amount of SAM recovered in lymph was too small to play a role in intestinal absorption. SAM in not absorbed from lymph because the flow rate of lymph is far smaller than that of blood and SAM in not so lipophilic as to be concentrated in lymph. The SAM transported to the serosal compartment may be a leak into the body fluids or the peritoneal cavity.

The administration of ATR significantly decreased the appearance rate of free SAM into blood and $\alpha$ while the parameters such as blood flow and membrane permeabilities were not affected. The value of $\alpha$ was confirmed to be affected by the addition of ATR in binding experiments, so only $\alpha$ was decreased by ATR. ATR was reported to decrease the intestinal absorption of water in vitro and was suspected to affect water fluxes resulting in the decrease of solvent drag and blood flow. Contrary to our expectation, water fluxes have not been affected by ATR. ATR is known to have many pharmacological effects. The inhibition of acetylcholine inducing increase of intestinal blood flow by ATR was reported. Chlorpromazine (CPZ), a major tranquilizer, also decreased $\alpha$ in intestinal absorption of sulfamethoxazole, and at the same time, decreased the mean blood flow rate ($V_b$) significantly. The difference of the effect on $V_b$ between ATR and CPZ may result from different pharmacological actions. Through the precise mechanism was not clear, ATR decreased $\alpha$ and increased the ratio of SAM that was transported to the serosal compartment.

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