

# Gender differences in the activities of aspirin-esterases in rat tissues

M.A.C. Benedito

Departamento de Psicobiologia, Escola Paulista de Medicina,  
Universidade Federal de São Paulo, São Paulo, SP, Brasil

## Abstract

The activities of aspirin (acetylsalicylic acid)-esterases were measured in several tissues (liver, kidney, adrenal glands, brain and serum) from adult male and female Wistar rats. In males, both aspirin-esterase I (assayed at pH 5.5) and II (assayed at pH 7.4) activities were higher in liver homogenates when compared to females (aspirin-esterase I: males  $48.9 \pm 4.8$  (N = 8) and females  $29.3 \pm 4.2$  (N = 8) nmol of salicylic acid formed  $\text{min}^{-1}$  mg protein $^{-1}$ ; aspirin-esterase II: males  $41.4 \pm 4.1$  (N = 8) and females  $26.1 \pm 4.5$  (N = 8) nmol of salicylic acid formed  $\text{min}^{-1}$  mg protein $^{-1}$ ,  $P < 0.001$ ). In serum, enzyme activity was higher in females than in males (aspirin-esterase I: males  $0.85 \pm 0.06$  (N = 6) and females  $1.18 \pm 0.11$  (N = 6) nmol of salicylic acid formed  $\text{min}^{-1}$  mg protein $^{-1}$ ; aspirin-esterase II: males  $1.03 \pm 0.13$  (N = 6) and females  $1.34 \pm 0.11$  (N = 6) nmol of salicylic acid formed  $\text{min}^{-1}$  mg protein $^{-1}$ ,  $P < 0.001$ ). In the other tissues assayed, no statistically significant difference between males and females was found. There were no statistically significant differences when the enzymes were assayed in different phases of the estrous cycle in liver and serum. These results show that the differences in aspirin-esterase activity observed between males and females are not due to the estrous cycle. The gender difference obtained in our study may indicate an involvement of gonadal hormones in the control of the hydrolysis of aspirin. This possibility is currently under investigation.

## Key words

- Aspirin-esterases
- Sex differences
- Rats
- Liver
- Serum

## Correspondence

M.A.C. Benedito  
Departamento de Psicobiologia  
EPM, UNIFESP  
Rua Botucatu, 862, 1º andar  
04023-062 São Paulo, SP  
Brasil  
Fax: 55 (011) 572-5092

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## Introduction

Aspirin (acetylsalicylic acid) (ASA) is the drug most frequently used worldwide. Several pharmacological actions have been attributed to aspirin, e.g. analgesic, antipyretic, anti-inflammatory activities, etc. Aspirin metabolism starts by hydrolysis promoted by esterases (aspirin-esterases) found in several tissues (1-5). Salicylic acid (SA), the product of aspirin hydrolysis, does not have the same pharmacological actions as aspirin (6-11). Some investigators have proposed that the rate of aspirin hydrolysis has

an important role in the pharmacological action and toxicity of salicylates (1,12,13).

Several enzyme activities have been shown to differ between males and females (14-18). These sex differences were shown to occur mostly in liver tissue and they seem to be determined early in life (15). In adults they are under control of sex hormones and the pituitary (15-17). Some reports have demonstrated a sex difference in the action of aspirin, e.g., in rats the inhibitory effect on  $\text{Ca}^{2+}$  mobilization in platelets produced by aspirin is higher in males than in females (19), in bulls the aspirin toxicity ( $\text{LD}_{50}$ ) is

higher in females than in males (20), in rabbits aspirin decreases thrombin size in males but not in females (21), in humans aspirin is effective in reducing the extent of platelet-subendothelium interaction in males but not in females (22) and inhibition of platelet aggregation is higher in females than in males (23).

A gender difference in aspirin hydrolysis in human plasma was demonstrated and aspirin hydrolysis was shown to be higher in males (3). A similar sex difference in aspirin hydrolysis was also shown in serum, i.e., a higher enzymatic activity in males than in females, and aspirin-esterase activity in rat liver homogenates has been reported to be lower in females than in males (1). However, these authors did not present any data nor did they provide a reference where these results could be found. Therefore, we may assume that they did not perform a systematic study of aspirin-esterase activity comparing males to females.

To our knowledge, the present paper is the first to report a difference in the activity of aspirin-esterases between male and female rats in a systematic study of different tissues.

## Material and Methods

### Subjects

Adult male and female Wistar rats, 4 months old, from our breeding colony were used in the experiments. Rats had free access to tap water and Purina® lab chow before sacrifice. After weaning they were kept in wire cages (3 rats/cage) in a room with controlled temperature (22°C) and a 12-h light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.).

The phases of the estrous cycle were determined daily by standard laboratory procedures before sacrifice. Only those animals presenting a well-defined estrous cycle were used in the experiment.

### Homogenate preparation

All animals were sacrificed by decapitation in the morning. Trunk blood was collected into test tubes using plastic funnels. After coagulation, blood was centrifuged for 15 min at 900 g at 0°C and serum transferred to glass test tubes. Serum was kept frozen at -20°C until assayed. Whole brains, liver, kidneys and adrenal glands were excised rapidly, wrapped in parafilm followed by aluminum foil and frozen at -20°C until assayed. Serum was used in the assays without any modification. Homogenates from liver, kidneys, adrenal glands and brains were prepared with cold 0.15 M KCl. Fragments of liver and kidneys, whole adrenal glands and brain were used to obtain the homogenates. Tissue was weighed while frozen, and cold 0.15 M KCl was added in appropriate volumes. Homogenates (5% liver and kidney and 10% brain and adrenal glands) were prepared in a motor driven teflon pestle glass homogenizer (20 strokes). Homogenates (liver and kidney) were centrifuged for 15 min at 2250 g and the supernatants were collected for enzyme assays run on the same day the homogenates were obtained. Samples were kept at 4°C over crushed ice during all manipulations.

### Aspirin-esterases determination

Based on several criteria, two aspirin-esterases were determined and named aspirin-esterase I and II (2). These esterases can be assayed using different buffers. We measured the two aspirin-esterases in our assays using a method based on the native fluorescence of SA (24). Tissue samples (20 µl) were incubated in duplicate with 80 µl of ASA (diluted in 0.2 M Tris/HCl buffer, pH 7.4, for aspirin-esterase II or 0.2 M acetate buffer, pH 5.5, for aspirin-esterase I) at 37°C in a shaking water bath. The final concentration of aspirin in the incubations was 8 mM. The reaction was halted by adding 50 µl of cold 10% trichloroacetic acid (w/v). Blanks

for monitoring the spontaneous hydrolysis of aspirin were obtained by adding 50  $\mu\text{l}$  of trichloroacetic acid before incubation and were run in duplicate. After centrifugation at 2250  $g$  at  $0^\circ\text{C}$  for 15 min a 20- $\mu\text{l}$  aliquot from the clear supernatant was transferred to a test tube containing 380  $\mu\text{l}$  of buffer used for incubation. After vortexing the samples, SA fluorescence was read in a Hitachi-Perkin Elmer fluorometer at 305 (excitation)-405 (emission) nm. SA standard curves were obtained by diluting a 5-mM SA stock solution. SA concentrations ranged from 0.125 mM to 5 mM. Twenty microliters (2.5 to 100 nanomoles) of each SA concentration was mixed with 80  $\mu\text{l}$  of incubation buffer and 50  $\mu\text{l}$  of trichloroacetic acid. Then, after vortexing, a 20- $\mu\text{l}$  aliquot was transferred to a test tube containing 380  $\mu\text{l}$  of the same buffers as used previously. The solution was vortexed and read at 305-405 nm. The amount of SA formed enzymatically was calculated as nmol by subtracting the blank relative fluorescence unit reading (spontaneous hydrolysis) from the total relative fluorescence unit reading (enzymatic plus spontaneous hydrolysis). The resulting relative fluorescence unit reading due to enzymatic hydrolysis was interpolated in the SA standard curve and the amount of SA formed enzymatically was determined. The SA standard curve was linear throughout the entire range of SA concentrations used. The enzyme assays were run in the linear range for both incubation time and protein concentration. Enzyme activity is reported as nmol SA formed  $\text{min}^{-1}$  mg protein $^{-1}$ .

All reagents used were of analytical grade and the best commercially available. Twice-distilled water was used to prepare the solutions. Aspirin and SA were synthesized and kindly donated by Novaquímica Laboratórios, São Bernardo, SP, Brazil.

### Protein measurement

Proteins were measured by the method of

Lowry et al. (25) using bovine serum albumin as standard.

### Statistics

Results were analyzed statistically by one-way analysis of variance (one-way ANOVA) and by the two-tailed Student *t*-test, with the level of significance set at  $P \leq 0.05$ , two-tailed.

Table 1 - Aspirin-esterase I specific activity in tissues from adult male and female rats.

Serum and brain homogenates were incubated for 20 min and liver and kidney homogenates were incubated for 10 min at  $37^\circ\text{C}$  at pH 5.5. Aspirin-esterase I activity was assayed at a final aspirin concentration of 8 mM. Results are reported as nmol SA formed  $\text{min}^{-1}$  mg protein $^{-1}$  (mean  $\pm$  SD). The number of animals is given within parentheses. \* $P < 0.001$  vs respective value for males (Student *t*-test).

	Males	Females
Liver	48.9 $\pm$ 4.8 (8)	29.3 $\pm$ 4.2 (8)*
Kidney	36.1 $\pm$ 4.8 (8)	38.1 $\pm$ 3.5 (8)
Brain	4.44 $\pm$ 0.33 (8)	4.39 $\pm$ 0.39 (8)
Serum	0.85 $\pm$ 0.06 (6)	1.18 $\pm$ 0.11 (6)*

Table 2 - Aspirin-esterase II specific activity in tissues from adult male and female rats.

Serum, adrenal glands and brains were incubated for 20 min, and liver and kidneys were incubated for 10 min at  $37^\circ\text{C}$  at pH 7.4. Aspirin-esterase II was assayed at a final aspirin concentration of 8 mM. Results are reported as nmol SA formed  $\text{min}^{-1}$  mg protein $^{-1}$  (mean  $\pm$  SD). The number of animals is given within parentheses. \* $P < 0.001$  vs respective value for males (Student *t*-test).

	Males	Females
Liver	41.4 $\pm$ 4.1 (8)	26.1 $\pm$ 4.5 (8)*
Kidney	35.3 $\pm$ 3.0 (8)	37.4 $\pm$ 3.5 (8)
Adrenal glands	4.99 $\pm$ 0.18 (4)	4.69 $\pm$ 0.21 (4)
Brain	2.24 $\pm$ 0.32 (8)	2.08 $\pm$ 0.33 (8)
Serum	1.03 $\pm$ 0.13 (6)	1.34 $\pm$ 0.11 (6)*

## Results

Tables 1-4 show the results obtained in this study for aspirin-esterases I and II. As can be seen in the Tables, liver homogenate presented the highest enzyme specific activity for both aspirin-esterases I and II, whereas serum showed the lowest specific activity. The statistical comparison between males and females showed that for both aspirin-esterase I and II males presented a higher specific activity in liver tissue ( $P < 0.001$ ). In

serum the opposite was observed with females showing higher specific activity than males for both enzymes ( $P < 0.001$ ). Statistical analysis of brain, kidney and adrenal gland data showed no difference between males and females.

Tables 3 and 4 show the results of enzyme activity during the estrous cycle of females. There were no statistically significant differences in liver aspirin-esterase II ( $F_{3,24} = 0.83$ ,  $P > 0.05$ ) or serum aspirin-esterase I ( $F_{3,24} = 1.18$ ,  $P > 0.05$ ) and II ( $F_{3,24} = 0.91$ ,  $P > 0.05$ ) activities.

Table 3 - Rat serum aspirin-esterase I and II specific activity at different phases of the estrous cycle.

Serum was incubated for 20 min. Aspirin-esterase I and II activities were assayed at a final aspirin concentration of 8 mM. Results are reported as the mean  $\pm$  SD. The number of animals is given within parentheses. Overall mean for aspirin-esterase I specific activity was  $1.24 \pm 0.18$  (N = 28) and for aspirin-esterase II was  $1.09 \pm 0.15$  (N = 28).

Estrous cycle phases	Aspirin-esterase specific activity (nmol SA formed $\text{min}^{-1}$ mg protein $^{-1}$ )	
	I	II
Diestrus I	$1.27 \pm 0.11$ (7)	$1.02 \pm 0.13$ (7)
Diestrus II	$1.27 \pm 0.19$ (7)	$1.06 \pm 0.12$ (7)
Proestrus	$1.30 \pm 0.26$ (7)	$1.17 \pm 0.20$ (7)
Estrus	$1.15 \pm 0.12$ (7)	$1.10 \pm 0.12$ (7)

Table 4 - Rat liver aspirin-esterase II specific activity during the estrous cycle.

Liver homogenate was incubated for 10 min at 37°C, pH 7.4. Aspirin-esterase II activity was assayed at a final aspirin concentration of 8 mM. The results are reported as the mean  $\pm$  SD. Overall enzyme mean activity was  $30.3 \pm 4.0$  (N = 28). The number of animals is given within parentheses.

Estrous cycle phases	Aspirin-esterase II specific activity (nmol SA formed $\text{min}^{-1}$ mg protein $^{-1}$ )
Diestrus I	$31.7 \pm 2.4$ (7)
Diestrus II	$32.5 \pm 5.4$ (7)
Proestrus	$28.3 \pm 2.9$ (7)
Estrus	$28.8 \pm 3.6$ (7)

## Discussion

The present results show that male and female rats differ in the rate of aspirin hydrolysis promoted by pH-dependent aspirin-esterases. This difference was specific since it was observed in liver and serum but not in brain, adrenal glands or kidneys. Males showed higher rates of aspirin-esterase activity in liver when compared to females, whereas females showed higher enzyme activity in serum. The possible influence of the estrous cycle on this sex difference in aspirin-esterase activity can be ruled out since our data (Tables 3 and 4) showed no difference in enzyme activity throughout the estrous cycle of females. Plasma and serum aspirin-esterase activities are higher in human males than in females (1,3) and in rats the sex difference is the opposite, with females showing higher rates. In contrast, male rats showed higher aspirin-esterase activity in the liver. The difference in enzyme activity in liver demonstrated here in rats suggests that this phenomenon should be explored in humans.

Besides having a major role in xenobiotic metabolism, the liver is also the site of synthesis for blood proteins, and therefore it is possible that the aspirin-esterases measured in serum are synthesized in the liver. According to our results, the specific activity of aspirin-esterases is much lower in serum

than in liver. This suggests that although aspirin metabolism may start in the blood after absorption, the liver would be responsible for most of its hydrolysis. In the liver of male rats there is a higher aspirin-esterase activity than in the liver of females, whereas the serum of females has higher activity. Whether this difference is due to a different amount of enzymes released into the blood still needs to be determined.

We have measured aspirin-esterase activities in liver homogenate supernatants obtained by 2250 g centrifugation. This preparation allows only to discard cell nuclei, vessels and large cell debris. Aspirin-esterases correspond to several enzymes, both particulate and soluble (4,5). Since we have assayed both particulate and soluble enzymes further experiments are necessary to determine if the differences reported here are due to a specific isozyme, soluble or particulate, or to all aspirin-hydrolyzing enzymes.

Since the pharmacokinetics of aspirin involves other important steps (26,27), it is necessary to determine if male and female

rats also differ in the overall aspirin metabolic pathway to fully understand possible pharmacological differences in aspirin action between genders.

Most of the gender differences in drug metabolism are under hormonal control and androgens seem to play a pivotal role in this difference (15-17). This possibility is currently under investigation in our laboratory and preliminary results show that at least in males androgens are responsible for the liver sex difference reported here.

In conclusion, we have shown that aspirin-esterase activities in rat tissues differ between males and females. These data may have implications in the differences in the pharmacokinetics and pharmacological effects of aspirin between males and females.

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