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Fluorimetric Determination of Tissue Distribution and Differences Between the Activity of Aspirin Esterases I and II in Mice and Rats

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Abstract

A modification of available fluorimetric methods, based on the native fluorescence of salicylic acid, has been used to assay the activity of aspirin esterases I and II in small amounts of several tissues from rats and mice.

The results obtained showed that aspirin esterase I from mouse liver and kidney had the lowest activity and that the activity of aspirin esterase II from these organs was greater than that from the same organs from rats. Liver enzyme kinetics and tissue distribution in rats was shown to be similar to previous data obtained using a spectrophotometric method.

This fluorimetric method, which is more sensitive and rapid than the spectrophotometric method, may be useful in studies on aspirin metabolism.

The metabolism of aspirin (acetylsalicylic acid) in tissues or body fluids is initiated by hydrolysis of aspirin to salicylic acid. This hydrolysis is promoted by esterases, called aspirin esterases, present but unevenly distributed in several tissues (Harris & Riegelman 1967; Howes & Hunter 1968; Menguy et al 1972; Builder et al 1977; Ali & Kaur 1982, 1983; Kaur & Ali 1983). Aspirin and salicylic acid do not seem to share the same pharmacological properties (Atkinson & Collier 1980) and changes in aspirin esterase activity in tissues have been shown to occur after treatment with different drugs (Howes & Hunter 1968; Ali & Kaur 1982; Kaur & Ali 1983). Thus, measurements of changes in aspirin esterase activity seems pharmacokinetically important.

Aspirin esterase activity has been measured by photometric or fluorimetric estimation of the salicylic acid formed. Fluorimetric methods developed to measure aspirin esterase activity are based on the native fluorescence of salicylic acid which is strong and stable over a wide pH range (Udenfriend 1962; Potter & Guy 1964). These methods use extraction of the salicylic acid formed into ether and back extraction into buffer, or direct reading of the sample without any salicylic acid purification (Harris & Riegelman 1967; Builder et al 1977; Aarons et al 1982). Fluorimetric procedures available for assay of aspirin esterases employ blood and its derivatives as the enzyme source and are performed on the macro scale. On the basis of the fluorimetric procedures available we have developed and standardized a very simple, reliable and sensitive procedure for the measurement of aspirin esterases in small amounts of tissue. Based on the native fluorescence of salicylic acid and its stability over a wide pH range, the procedure avoids organic extraction, estimates of salicylic acid being obtained from a clear supernatant solution.

This paper describes the methodology developed for assay of aspirin esterase activity and its use for the determination of enzyme activity in different tissues from rats and mice. The paper also describes differences between aspirin esterase activity in the species used.

Materials and Methods

Animals

Adult male Wistar rats and Swiss albino male mice, 3 months old, from our own breeding programme were used to obtain the tissues for biochemical assay. The animals had free access to water and food before they were killed by decapitation.

Reagents and drugs

All reagents used were analytical grade and the best commercially available. Double-distilled water was used in the preparation of solutions. Aspirin and salicylic acid were synthesized and kindly donated by Nova Química Laboratórios, São Bernardo, SP-Brazil.

Tissue obtention

After decapitation, trunk blood was collected in glass tubes by use of plastic funnels. After blood coagulation at room temperature (22°C) serum was obtained by centrifugation at 900 *g* for 15 min. For plasma samples, blood was treated with anticoagulant; after centrifugation plasma was transferred to glass test tubes. Serum and plasma samples were kept at −20°C until they were assayed. Liver, kidneys, adrenal glands and brain were dissected rapidly and washed with cold 1·15% KCl solution. After excess of KCl had been absorbed with filter paper, the tissue was wrapped in aluminium foil and immediately frozen over dry ice. Tissues were kept frozen at −20°C until they were assayed.

Tissue preparation

Serum or plasma samples were used in the assays without modification. Homogenates from liver, kidneys, adrenal glands and brains were prepared with cold 1·15% KCl. Portions of liver and kidneys, whole adrenal glands and brains were used to obtain the homogenates. Tissue was weighed while frozen and an appropriate volume of cold 1·15% KCl solution was

added. Homogenates were prepared in a motor-driven, teflon pestle, glass homogenizer (20 strokes). Homogenates were centrifuged for 15 min at 2250 *g* and the supernatant liquid was collected for enzyme assay. During all manipulations samples were kept at 0–4°C.

Buffer concentrations and pH, incubation times and substrate concentrations were based on data from Ali & Kaur (1983).

Enzyme assay

Homogenates of liver and kidneys (5% w/v) and whole brains and adrenal glands (10% w/v) were prepared in cold 1·15% KCl. After centrifugation at 2250 *g* for 15 min at 0°C, the supernatant was collected and used on the same day for esterase assay. Liver, kidney and adrenal homogenates were incubated for 10 min, whereas brain homogenates, serum and plasma were incubated for 20 min. The general procedure consisted of incubating tissue homogenates, serum or plasma (20 μL) in glass test tubes (52 mm length × 4 mm, i.d.) with aspirin (80 μL, 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) in duplicate, at 37°C in a shaking water bath. The reaction was stopped by adding 50 μL of cold 10% (w/v) trichloroacetic acid. Blanks for monitoring the spontaneous hydrolysis of aspirin were prepared by adding acid (50 μL) before incubation and were run in duplicate.

After centrifugation at 2250 *g* at 0°C for 15 min clear supernatant (20 μL) was transferred to a glass test tube containing buffer (380 μL) used for incubation. After vortex mixing, samples were read in round quartz microcells in a Hitachi-Perkin-Elmer model 203 spectrofluorimeter, at excitation and emission wavelengths of 305 and 405 nm (uncorrected), respectively. During the assays the samples and reagents were kept at 0–4°C in a crushed ice-bath, except for the incubation and reading steps.

Salicylic standard curves and calculation of salicylic acid formed enzymatically

Salicylic acid standard curves were obtained from standard solutions prepared by diluting 5 mM salicylic acid stock solution to concentrations ranging from 0·125 to 5 mM. Each salicylic acid standard solution (20 μL; 2·5 to 100 nmol) was mixed with buffer (80 μL) and trichloroacetic acid (50 μL). After vortex mixing, 20 μL was transferred to a test tube containing the same buffer (380 μL) and the solution (400 μL total volume) was vortex-mixed and read at 305–405 nm. The amount of salicylic acid formed enzymatically (nmol) was calculated by subtracting the blank relative fluorescence units reading (spontaneous hydrolysis) from the total relative fluorescence units reading (enzymatic plus spontaneous hydrolysis). The resulting relative fluorescence units reading resulting from enzymatic hydrolysis was measured by interpolation from the salicylic acid standard curve, and the amount of salicylic acid formed enzymatically was determined. The salicylic acid standard curve was linear throughout the entire range of salicylic acid concentrations used.

Protein measurement

Proteins were measured by the method of Lowry et al (1951) using bovine albumin as standard.

Statistical analysis

Student's two-tailed *t*-test, was used for comparisons and $P \leqslant 0.05$ was taken as an indication of significance.

Results

Protein concentration and incubation time

Figs 1 and 2 show the results obtained for aspirin esterase I activity from liver homogenates used to determine the dependence of reaction linearity on protein concentration and incubation time. The results obtained from aspirin esterase II activity assays followed the same pattern as those obtained for aspirin esterase I activity (data not shown).

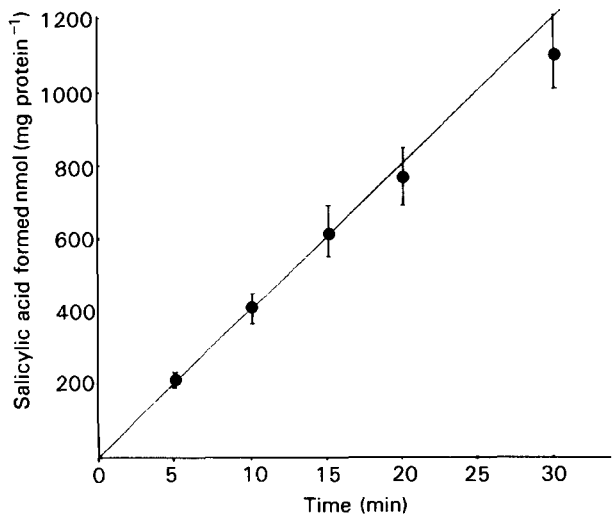

FIG. 1. Aspirin esterase I activity after different incubation times. The assays used a final aspirin concentration of 8 mM. Liver homogenates (5% w/v) were used and the amount of protein was 106 ± 5 μg (n = 3). Points on the curve represent the mean from three liver homogenates assayed separately.

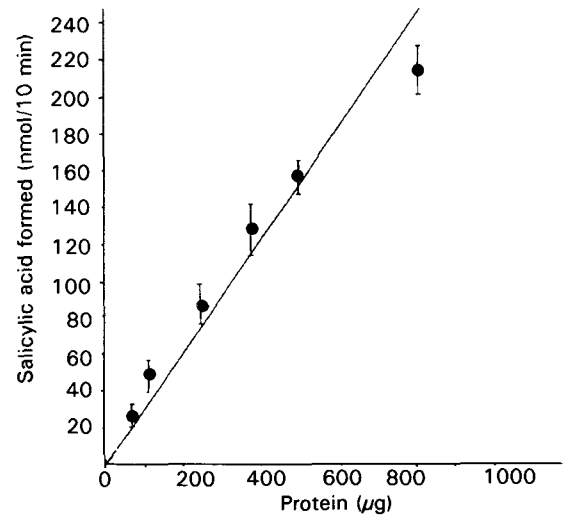

FIG. 2. Results obtained for aspirin esterase I activity by use of different amounts of protein. The final concentration of aspirin in the assay was 8 mM. Samples were incubated for 10 min. Points in the figure represent the mean ± s.d. from 3 different liver homogenates assayed separately.

The results showed that for both protein concentration and incubation time the reaction was linear over a broad range of the parameters studied. These data were submitted to linear regression analysis and Pearson's correlation statistics and were shown to be highly significant for both slopes and correlation coefficients ($P < 0.000001$).

Enzyme kinetics

Samples of liver homogenate supernatant were incubated in the presence of aspirin concentrations ranging from 0·1–16 mM for aspirin esterase I and 0·8–16 mM for aspirin esterase II. Table 1 shows the results obtained for apparent K_m and V_{max} for both enzymes using the procedure described by Wilkinson (1961). Data show lower K_m and V_{max} for aspirin esterase I than that for aspirin esterase II, indicating higher substrate affinity and lower tissue concentration for aspirin esterase I.

Figs 3 and 4 (aspirin esterase I) and Fig. 5 (aspirin esterase II) show typical results obtained from one of the liver homogenate assays. Fig. 3 (aspirin esterase I) shows the salicylic acid enzymatic reaction product readings in fluorescence units after enzymatic and spontaneous hydrolysis and Figs 4 (aspirin esterase I) and 5 (aspirin esterase II) show the saturation curves and the Lineweaver–Burk plots.

Table 1. Kinetic parameters of aspirin esterases I and II from rat liver homogenates.

Enzyme	K_m (mM)	V_{max} (nmol salicylic acid formed (mg protein)$^{-1}$ min^{-1})
Aspirin esterase I	1·33 ± 0·14 (3)	53·91 ± 8·64 (3)
Aspirin esterase II	8·85 ± 1·06 (3)	80·40 ± 9·38 (3)

Liver homogenate supernatant was incubated for 10 min at 37°C in the presence of aspirin (0·1–8 mM for aspirin esterase I and 0·8–16 mM for aspirin esterase II). Protein concentration was 139 ± 4 μg/20 μL. Data are expressed as mean ± s.d. The number of animals is given in parentheses. K_m is the Michaelis–Menten constant and V_{max} is the maximum rate.

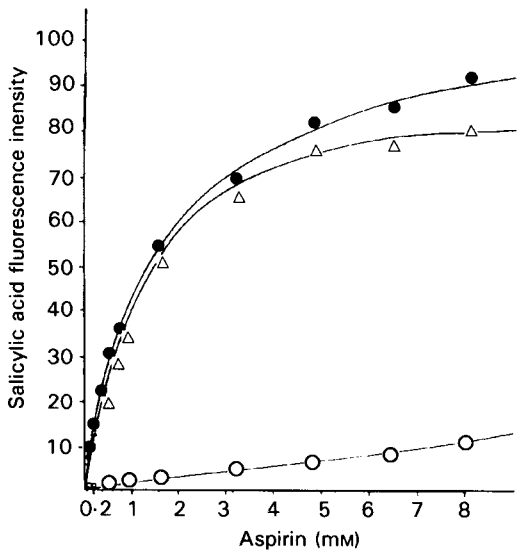

FIG. 3. Salicylic acid fluorescence readings after aspirin hydrolysis: (○) aspirin spontaneous hydrolysis, (●) aspirin spontaneous plus enzymatic hydrolysis, (△) enzymatic hydrolysis.

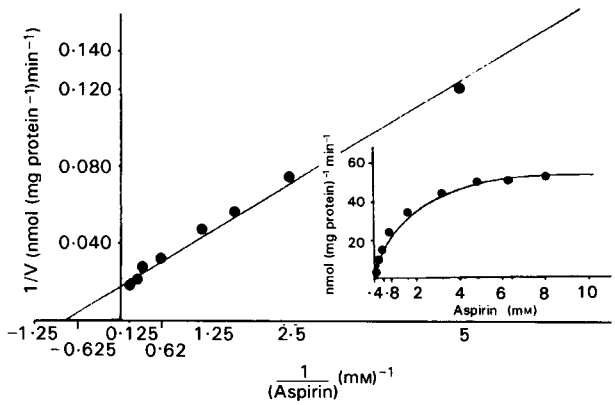

FIG. 4. Kinetic characteristics of aspirin esterase I. The figure shows the Lineweaver–Burk plot and the inset shows the saturation curve. The assay was performed using liver homogenate (5% w/v; 137 μg protein) and an incubation time of 10 min.

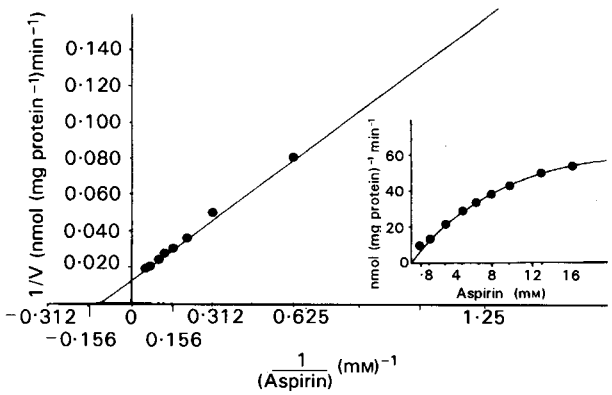

FIG. 5. Kinetic characteristics of aspirin esterase II. The figure shows the Lineweaver–Burk plot and the inset shows the saturation curve. The assay was performed using liver homogenate (5% w/v; 146 μg protein) and an incubation time of 10 min.

Activity of aspirin esterases I and II in rat and mice tissues

The results are shown in Table 2. The data showed that enzyme activity in rat and mouse liver and kidney was greater than that

Table 2. Activity of aspirin esterases I and II in several types of male rat and mouse tissue.

Species	Tissue	Aspirin esterase I	Aspirin esterase II
Rat	Liver	49·0 ± 4·50 (8)	41·4 ± 3·80 (8)
	Kidney	36·1 ± 4·50 (8)	35·4 ± 2·90 (8)
	Brain	4·50 ± 0·34 (8)	2·30 ± 0·31 (8)
	Adrenal gland	NA	6·69 ± 0·13 (6)
	Serum	0·84 ± 0·06 (6)	1·03 ± 0·13 (6)
	Plasma	NA	0·78 ± 0·03 (7)
Mouse	Liver	38·5 ± 7·90* (5)	77·3 ± 17·40‡ (5)
	Kidney	25·2 ± 6·30† (5)	79·8 ± 10·60‡ (5)

Aspirin esterase activity is expressed as nmol salicylic acid formed (mg protein)$^{-1}$ min^{-1}. The final concentration of aspirin in the assay was 8 mM. Data are expressed as mean ± s.d. The number of animals is given in parentheses. NA = Not assayed. *$P < 0.025$, significantly different from rat liver; †$P < 0.005$, significantly different from rat kidney; ‡$P < 0.001$, significantly different from rat liver and kidney.

in other tissues. Data from several tissues assayed showed uneven distribution of enzyme activity. Aspirin esterase I activity was lower in mouse liver and kidney and aspirin esterase II activity was higher in these tissues than in the corresponding rat tissues (Table 2).

Discussion

This study was based on data from Ali & Kaur (1983) who used a photometric method to assay salicylic acid formed after enzymatic hydrolysis of aspirin. On the basis of several criteria (such as pH range and response to inhibitors/activators) they described the existence of at least two different enzymes, namely aspirin esterases I and II. Rats of both sexes were used in that study, whereas the present study used male rats only. Although this can be an important difference between the two methodologies, our results, obtained by use of a fluorimetric method, are comparable with those of Ali & Kaur (1983). Measurements of liver, kidney and brain enzyme activity are comparable in the range nmol of salicylic acid formed (mg protein)$^{-1}$ min^{-1} or (mg tissue)$^{-1}$ min^{-1}.

Substrate kinetic studies were performed using liver homogenates with only slight purification (use of the supernatant from 2250 *g* centrifugation). Ali & Kaur (1983) showed that aspirin esterases I and II are unevenly distributed in different subcellular fractions in the rat liver. Their kinetic study was performed on the liver microsomal fraction only, where they detected the highest activity for both enzymes. The different methods of tissue preparation used may explain the differences between both studies. Although our results for both K_m and V_{max} (mM and nmol of salicylic acid formed (mg protein)$^{-1}$ min^{-1}) are in the same range of magnitude as theirs, they obtained a K_m of 2 mM for aspirin esterase I and 3·5 mM for aspirin esterase II whereas our values were 1·33 and 8·85 mM, respectively (Table 1). With regard to V_{max}, the authors obtained values of 90 nmol of salicylic acid formed (mg protein)$^{-1}$ min^{-1} for aspirin esterase I and 71·1 nmol (mg protein)$^{-1}$ min^{-1} for aspirin esterase II whereas our values were 53·9 and 80·4, respectively (Table 1). It is important to mention that in addition to the different tissues investigated, sex differences, as already pointed out, might also have accounted for the discrepancies between the two sets of results.

Our results for organ enzyme distribution are also comparable with those of Ali & Kaur (1983). We also found higher enzyme activity in liver and kidney than in brain tissue. The data obtained for aspirin esterase I and II activity showed that they are unevenly distributed in the rat tissues assayed. Ali & Kaur (1983) showed that liver and kidney enzymes have comparable properties. In the liver, aspirin esterase II has a higher K_m and V_{max} than aspirin esterase I. Because we did not perform kinetic studies we do not know if the pattern is the same in the other tissues assayed. At the substrate concentration used in the assays, aspirin esterase II activity from mouse liver and kidney tissue was much higher than that from the respective rat tissues. The activity of mouse liver and kidney aspirin esterase I is lower than that of the corresponding rat tissue enzyme. This difference, although statistically significant, is much lower in magnitude than that observed for aspirin esterase II. These data are in accordance with those of Ali & Kaur (1983).

The method described here provides an alternative for the assay of aspirin esterase activity in different tissues, and provides results comparable with those obtained from the photometric procedure.

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