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SHORT COMMUNICATIONS

Enzymatic hydrolysis of leucylaminoantipyrine in human serum*

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4-L-Leucylaminoantipyrine was synthesized to decrease a toxicity of amidopyrine (dimethylaminoantipyrine) [1, 2]. Toxicity of this frequently used analgesic–antipyretic drug depends upon its metabolism to dimethylnitrosoamine [3] and 4-formylaminoantipyrine [4], the very reactive species probably responsible for toxic effects attributed to the parent compound. The chemical structure of 4-L-leucylaminoantipyrine, i.e., the absence of dimethylamino group, excludes the possibility of formation of this kind of toxic metabolites.

Previously we have shown that the first step of biotransformation of 4-L-leucylaminoantipyrine in laboratory animals involves hydrolysis to give 4-aminoantipyrine, which is further metabolized ot acetylaminoantipyrine and rubazonic acid [5]. Enzymatic hydrolysis of this drug occurs in various tissues and subcellular fractions of rat liver and kidney [6]. On the other hand, the metabolic fate of 4-L-leucylaminoantipyrine in humans was not studied.

The present papter summarizes our recent findings concerning the enzymatic hydrolysis of 4-L-leucylaminoantipyrine and its structural analogs in human blood serum, as well as the effects of some factors on this process. This information could be important for a better understanding of metabolic fate of 4-L-leucylaminoantipyrine.

The 4-L-leucylaminoantipyrine-hydrolizing activity of the human serum is calculated from amount of 4-aminoantipyrine formed as determined by the spectrophotometric method, which is based on the formation of orange coloration by the reaction of 4-aminoantipyrine with phenol and potassium ferricyanide [7].

0.2 ml of serum was added to 2.8 ml of 0.2M Tris–HCl buffer pH 8.0 and preincubated at 37°. After 30 min, 1 ml of 4-L-leucylaminoantipyrine solution in the same buffer was added to a final concentration of 10^{-2} M and incubation was carried out at 37° for 30 min. The reaction was stopped by the addition of ammonia to a final concentration of 2 per cent. Next 3 mmoles of phenol and 1.5 mmoles of potassium ferricyanide were added to a final volume of 5 ml and after 2 min the extinction of coloured product formed was read at 500 nm. The amount of blood serum added to the incubation mixture contained about 1.5 mg of proteins and provided optimum conditions at which the rate of drug hydrolysis was the rate-limiting factor in the assays.

Protein measurement. Protein was determined by the biuret method with bovine serum albumin as a standard [8].

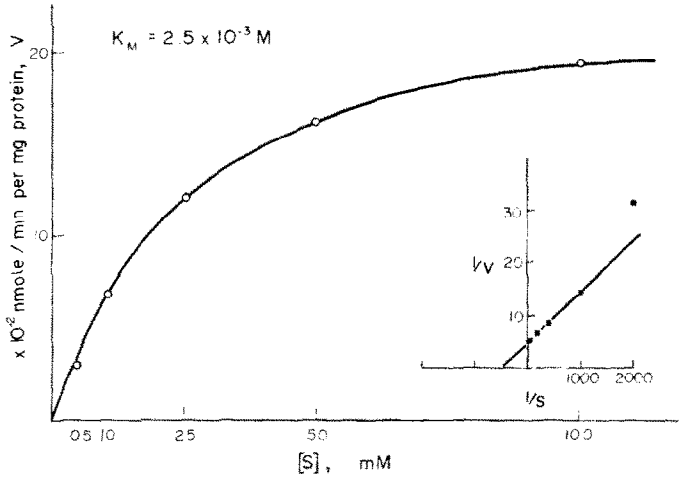

Fig. 1. 4-L-Leucylaminoantipyrine hydrolyzing activity in the presence of different concentration of substrate. Left, Michaelis–Menten plot, right Lineweaver–Burk plot. For other experimental details see text.

Human blood serum was obtained from the Central Laboratory of the Clinical Hospital of Medical Academy in Warsaw. 4-L-leucylaminoantipyrine, 4-D-leucylaminoantipyrine and 4-L-dimethylleucylaminoantipyrine were synthesized in the Department of Pharmaceutical Chemistry, Medical Academy in Warsaw. Other reagents were of analytical grade and were obtained from POCh, Gliwice, Poland.

Statistical analysis. All results are presented as the mean ± standard deviation. Significance of difference was estimated by the Student's *t*-test.

One of the objectives of these investigations was to obtain a survey of enzymatic hydrolysis of 4-L-leucylaminoantipyrine derivatives in human serum. Among the drug tested 4-L-leucylaminoantipyrine, but not 4-D-leucylaminoantipyrine or 4-L-dimethylleucylaminoantipyrine undergoes enzymatic hydrolysis in human serum, thus proving high stereospecificity of the enzyme, as well as the importance of a free amino group of amino acid moiety of 4-L-leucylaminoantipyrine for enzymatic hydrolysis of the drug.

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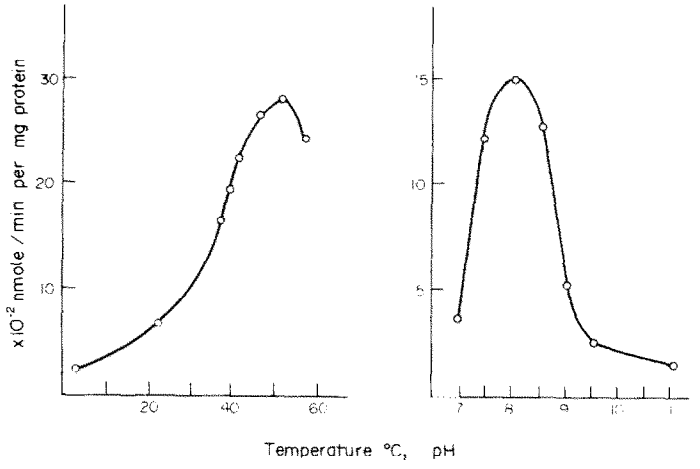

Fig. 2. The effects of temperature and pH on the activity of the drug-hydrolyzing enzyme. The final substrate concentration was always 10 mM. The effect of pH was studied at 37°, the effect of temperature was studied at pH 8.0.

It is interesting to note that 4-D-leucylaminoantipyrine at equimolar concentration (1 mM) had no effect on the enzymatic hydrolysis of 4-L-leucylaminoantipyrine.

Figure 1 shows the Lineweaver–Burk and Michaelis–Menten plots for 4-L-leucylaminoantipyrine hydrolyzing enzyme at pH 8.0. The saturation of enzyme by substrate is observed at a concentration of the drug of 10^{-2} M. The apparent K_M and V_{max} values are $2.5\ 10^{-3}$ M and 0.24 nmole/min/mg serum protein, respectively. The rate of 4-L-leucylaminoantipyrine hydrolysis was proportional to the incubation time up to 40 min. In these conditions nonenzymatic hydrolysis of 4-L-leucylaminoantipyrine was negligible.

Data presented in Fig. 2 indicate that the optimum temperature for enzymatic hydrolysis of 4-L-leucylaminoantipyrine is 51° and that the optimum pH is 8.0. To maintain constant activity of the drug hydrolyzing enzyme during the assay the reaction was carried out at 37°.

Table 1. The influence of some metal ions and metabolic inhibitors on the rate of enzymatic hydrolysis of 4-L-leucylaminoantipyrine

	Rates of hydrolysis (nmoles/min/mg serum protein)
None	0.216 ± 0.065
Cu^{2+}	0.197 ± 0.019
Ba^{2+}	0.171 ± 0.026*
Ca^{2+}	0.141 ± 0.047*
Mg^{2+}	0.131 ± 0.030*
Cd^{2+}	0.054 ± 0.013*
EDTA	0.169 ± 0.038
PCMS†	0.139 ± 0.006*
PCMB‡	0.127 ± 0.060*
Jodoacetamide	0.042 ± 0.011*
Jodoacetic acid	0.034 ± 0.005*

The final concentration of metal ions (used as chlorides) and metabolic inhibitors were 1 mM and 5 mM, respectively. After princubation of blood serum with these compounds at 37° for 30 min, the enzymatic reaction was initiated by the addition of a substrate to a final concentration of 10 mM. The reaction was carried out at 37°, pH 8.0. Data represent mean ± S.D. from 5 experiments.

* $P < 0.05$ or better.

† PCMS, *p*-chloromercuribenzenesulfonic acid.

‡ PCMB, *p*-chloromercuribenzoic acid.

The effects of various metal ions, as well as of typical inhibitors of enzymes activities on the 4-L-leucylaminoantipyrine hydrolysis rate are shown in Table 1. The drug hydrolysis is inhibited by Cd^{2+}, Ca^{2+}, Ba^{2+}, Mg^{2+}, as well as iodoacetamide and iodoacetic acid. On the contrary, none of the compounds tested had a stimulatory effect on this enzymatic reaction.

The 4-L-leucylaminoantipyrine hydrolyzing enzyme is tentatively referred to as 4-L-leucylaminoantipyrine hydrolase; however its properties and mechanism of action are not yet known. The high K_M value for 4-L-leucylaminoantipyrine indicates that the affinity of this drug to the hydrolyzing enzyme is quite low. Kinetic characteristics, optimum temperature, as well as the effects of some cations, inhibition by cadium ion and sulphydryl group reagents indicate that 4-L-leucylaminoantipyrine hydrolase differs in some properties from leucine aminopeptidase (EC 3.4.1.1). This suggestion is confirmed by the fact, that a pure preparation of leucine aminopeptidase isolated from hog kidney does not hydrolyse 4-L-leucylaminoantipyrine (unpublished results).

The results presented in this paper indicate that, similar to the rat tissues [6], the enzymatic hydrolysis of 4-L-leucylaminoantipyrine but not 4-D-dimethylleucylaminoantipyrine occur in human blood serum. It could be suggested that 4-L-leucylaminoantipyrine administered to the body is rapidly hydrolyzed to 4-aminoantipyrine and that the pharmacological effects of this drug is due to the action of this metabolite.

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Crossed digoxin immunoreactivity in chromatographic fractions of rat adrenal extract

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In a series of previous communications we have shown that in the serum of rats with cardiac overload produced by a ligature of abdominal aorta [1, 2] or by thyroid hormone-induced hypermetabolism [3] and accompanied by myocardial hypertrophy an "apparent" presence of digoxin can be demonstrated by homogenous enzymoimmunoassay. There is some indirect evidence [1, 4] that an endogenous cardiotropic hormone is produced by the adrenal cortex, and that this steroid is identical with neither corticosterone [1] nor with aldosterone [5]. In the present experiments we tried to detect "apparent" digoxin immunoreactivity in chromatographic fractions of rat adrenal extract.

In a typical experiment adrenals of 24 normal male Wistar rats (Velaz, Prague) with a wet weight of 571 mg were homogenized in 5.7 ml 70% acetone and the homogenate was extracted 3 times with 5 ml 70% acetone. Then it was filtered through Whatman Phase Separator and the acetone was evaporated. The remaining water phase was mixed with 10 ml of methanol and extracted 3 times with 10 ml Petroleumbenzin Merck (b.p. 60–80°) and the extracts were discarded. The remaining water phase was extracted 3 times with 12 ml dichloromethane and the pooled extracts were evaporated. The dry residues were dissolved in 20 μl of a mixture of chloroform–methanol (1:1) and applied to a thin layer plate (Merck Kieselgel 60F–254, 0.2 mm). The chromatogram was developed 3 times in cyclohexane–isopropanol (7:3) and then once in chloroform–ethanol–water (92:8:0.5). Ten spots were visualized in u.v. light (254 nm), extracted with chloroform–methanol (1:1) and dried, as well as the zones between the spots, where some tailing (especially in zones B, C and D) was present. Halves of the residues were dissolved in physiological saline and the aliquots in duplicate (corresponding each to a quarter of the original weight) were examined for the presence of digoxin immunoreactivity by homogenous enzymoimmunoassay [6] using the EMIT-cad kit (Syva, USA). The spots and zones containing digoxin-like immunoreactivity were applied to the same t.l.c. plate and rechromatographed in the same way. The spots were visualized in u.v. light, extracted and subjected to digoxin enzymoimmunoassay. The experiment was repeated several times with essentially identical results.

A typical chromatogram is shown in Fig. 1. The spots are numbered and the zones between them marked with letters. The numbers on the right represent relative digoxin-like immunoreactivity, expressed here as ΔA (absorbance change) of the enzyme reaction in the homogenous enzymoimmunoassay. Maximum digoxin-like immunoreactivity was present in the sub-aldosterone zone (B) and in the aldosterone spot (No. 4). When these materials were rechromatographed (Fig. 2) with hormonal as well as cardenolide standards the highest digoxin-like immunoreactivity was found in a spot produced by zone B and corresponding to digitoxigenin. The intensity of the spot was quite variable, but maximum digoxin-like immunoreactivity was always here. In some experiments another spot developed from the zone B eluate on rechromatography, with an R_f similar to digoxigenin and again displaying relatively high digoxin immunoreactivity.

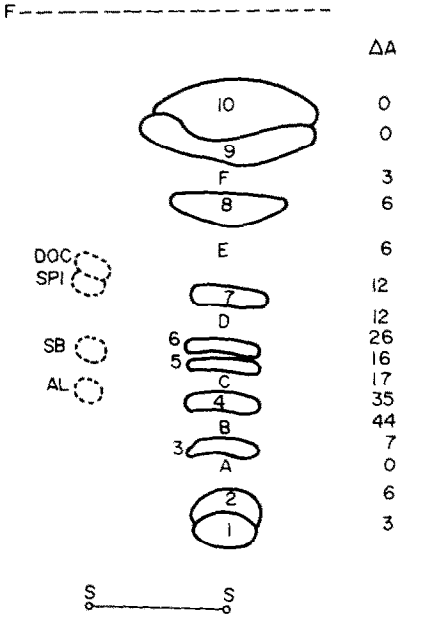

Fig. 1. Scheme of a typical chromatogram of crude extract from 571 mg of rat adrenal tissue. The spots were visualized by quenching (absorption) in u.v. light (254 nm). Abbreviations: S = start, F = front, A = aldosterone standard, SB = corticosterone standard, SPI = spironolactone standard, DOC = deoxycorticosterone standard (all 10 μg). ΔA = absorbance change in the enzyme reaction in homogenous digoxin enzymoimmunoassay (relative value of digoxin-like immunoreactivity): its maximum is in the sub-aldosterone zone B and in the aldosterone spot (No. 4). Some tailing was present in zones B, C and D, which produced the spots seen on rechromatography of these zones (Fig. 2).