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Effects of piretanide on plasma fibrinolytic activity, platelet aggregation and platelet factor-4 release in man

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Abstract. Piretanide, 4-phenoxy-3-(pyrrolidinyl)-5-sulphamoyl benzoic acid, apart from being an efficient diuretic, enhances endogenous plasma fibrinolytic activity after a single dose of 6 mg administered by oral route. After ingestion of the drug, acceleration of fibrinolytic acitivity became manifest within 1 h, reached its peak in 3 h and was associated with a fall in fibrinogen and diminished urokinase excretion. Piretanide did not cause lysis of fibrin *in vitro.* Primary platelet aggregation, induced by adenosine-diphosphate, was inhibited by piretanide. In *in vitro* experiments piretanide led to effective inhibition of adenosine-diphosphate-induced platelet aggregation with complete inhibition at 5 mM concentration. Piretanide led to a highly significant decrease of platelet factor-4 release.

Keywords. Piretanide; synthetic fibrinolytic agent; anti-platelet agent; PF-4 release; diuretic.

Introduction

A great deal of interest is involved in search of newer synthetic agents with antithrombotic and fibrinolytic properties to exploit the natural system of thrombolysis and to gain long-term fibrinolytic prophylaxis against degenerative vascular disorders (Davidson and Walker, 1979). Several combinations of thrombolytic and anti-platelet agents have been tried, including heparin, plasminogen with streptokinase, urokinase and ancrod, to achieve better results by promoting fibrinolysis and inhibiting platelet aggregation and/or release reaction, with variable results and attendant immunological problems. However, the attraction of such combinations is that smaller doses of thrombolytic agents may be effective, thus reducing episodes of bleeding complications (Kwaan, 1979). In our experience, furosemide (Lasix®) effectively reverses the adverse changes in blood coagulation in high altitude pulmonary oedema which is associated with diminished fibrinolytic activity and hypercoagulability (Singh and Chohan, 1973, 1974). At present, smaller doses of 20 mg furosemide, administered orally or intravenously, are advocated to restore fibrinolytic activity and to achieve inhibition of platelet aggregation and release reaction in this disorder (Chohan, 1980, 1984). A close similarity in structure and function, between furosemide and piretanide, as diuretics, prompted the present study to elucidate effects of the latter on plasma

Abbreviations used: PF-4, Platelet factor-4; ADP, adenosine-diphosphate; PRP, platelet rich plasma; PAT, platelet aggregation times; f.c., final concentration; PGG_2, prostaglandin endoperoxides.

fibrinolytic activity, platelet aggregation and release of platelet factor-4 (PF-4). Piretanide, 4-phenoxy-3-(1-pyrrolidinyl)-5-sulphamoyl benzoic acid, is a new diuretic whose effects are similar to those of furosemide in man and animals (Lawrence *et al.,* 1978) but its effects on blood coagulation are not known.

Materials and methods

Subjects, drug and reagents

Twenty two males with an average age of 25·8 ± 3·8 years were studied with their informed consent. Parameters estimated, before and at different intervals after ingestion of 6–12 mg piretanide included plasma euglobulin fibrinolytic activity (Astrup and Mullertz, 1952), urokinase activity (Chohan *et al.,* 1977a), fibrinogen contents (Ratnoff and Menzie, 1951), PF-4 availability (Harada and Zucker, 1971; Chohan *et al.,* 1977b) and platelet aggregation and platelet aggregation times (Born, 1964; Chohan *et al.,* 1977b). Piretanide (molecular weight, 362·4) in powder form was dissolved in 100 mM Tris buffer (pH 11·0) containing 95 mM NaCl and 3 mM KCl and pH was adjusted to 7·5 with HCl. A stock solution of 50 mM was diluted when desired for *in vitro* studies. All concentrations were expressed as final concentration (f.c). Piretanide in powder as well as in tablet form, dispensed in 3-12 mg, was used (Farbwerke Hoechst, A. G., Frankfurt, Germany, designated as HOE-118).

Other reagents used in the study were Adenosine-diphosphate (Sigma Chemical Co., St. Louis, Missouri, USA), Fibrinogen (Poviet, N. V., Amsterdam, The Netherlands), Heparin (Liquemine, Roche, Basel, Switzerland) and Bovine Thrombin (Parke Davis, Detroit, Michigan, USA) as per directions of the manufacturers.

Fibrinolytic activity and fibrinogen

Thirteen subjects formed part of this study, who were administered 6 mg of piretanide orally. Euglobulin fibrinolytic activity was measured as areas of lysis in mm^2 on blood samples collected from them at intervals of 0 h to 1, 3, 6, 12 and 24 h. Simultaneously, plasma fibrinogen contents were determined in 9 subjects.

Urokinase activity

Urokinase activity in urine samples collected at different intervals was observed qualitatively in the above subjects.

PF-4

PF-4 activity was assayed in 10 subjects on blood samples collected before and after 3 h and 24 h of administration of piretanide and values were expressed in units per ml of plasma.

For the above parameters, the test subjects served as their own control.

Platelet aggregation

Nine subjects were studied for *in vitro* and *in vivo* experiments. For *in vitro* studies the final concentration of piretanide varied from 0·1–5 mM in which PRP was incubated at 37°C for 1 min prior to induction by ADP for aggregation. For *in vivo* studies, 6–12 mg piretanide was administered orally, blood was collected at intervals of 0 h to 1,6,12 and 24 h. A final concentration of 1×10^{-6} M ADP was used to induce platelet aggregation in all experiments. Platelet aggregation times (PAT) were recorded automatically along with the aggregation curves.

Statistical analysis of the results were obtained by using the Student's *'t'* test.

Results

Changes brought about in plasma fibrinolytic activity, fibrinogen and PF-4 by piretanide after 3 h of administration are depicted in the table 1. Following a single dose of 6 mg piretanide there was a significant rise in the mean euglobulin lytic activity, expressed as areas of lysis in mm^2, the peak being attained after 3h ($P < 0{\cdot}005$). The effect became evident within 1 h and persisted upto 24 h. The mean areas of lysis, from the 0 h to 1, 3, 6, 12 and 24 h were 222, 251, 312, 289, 267 and 249 mm^2, respectively. With rising fibrinolytic activity, plasma fibrinogen showed a mild fall which was not significant statistically. The mean plasma contents of fibrinogen, from 0 h to 1,3,6, 12 and 24 h were 291, 285, 256, 261, 265 and 289 mg/dl, respectively. Diuresis became manifest within 30–40 min and amounted to an average 300–500 ml. Urokinase activity in urine diminished within 30 min and it was gradually restored after $1\frac{1}{2}$ -3 h. This effect of piretanide was similar to that of furosemide (Chohan *et al.,* 1977a).

Table 1. Changes in plasma fibrinolytic activity, fibrinogen and PF-4 3 h after a single oral dose of 6 mg piretanide.

Parameters	Initial		After piretanide		Significance
	Mean	SE	Mean	SE	
Fibrinolytic activity, area of lysis—mm^2 (13)	221·7	39·9	311·6	38·8	$P < 0{\cdot}005$
Fibrinogen, mg/dl (9)	290·7	17·1	255·9	18·8	$P < 0{\cdot}10$
Platelet factor—4, PF-4 units/ml (10)	0·164	0·028	0·118	0·014	$P < 0{\cdot}001$

Numbers in parentheses indicate number of subjects.

PF-4 activity in plasma was significantly diminished 3 and 24 h after administration of piretanide compared to basal levels. PF-4 values, from 0 h to 3 and 24 h were 0·164, 0·118 ($P < 0{\cdot}001$), and 0·148 ($P < 0{\cdot}01$) units/ml plasma, respectively.

Primary platelet aggregation induced by ADP was progressively inhibited by prior addition of piretanide (f.c. ranged from 0·1–1·0 mM in the PRP) (figure 1A). However, 5 mM f.c. of the agent led to complete inhibition of platelet aggregation (figure 1B). When 5 mM piretanide was added just after platelet aggregation had been initiated by

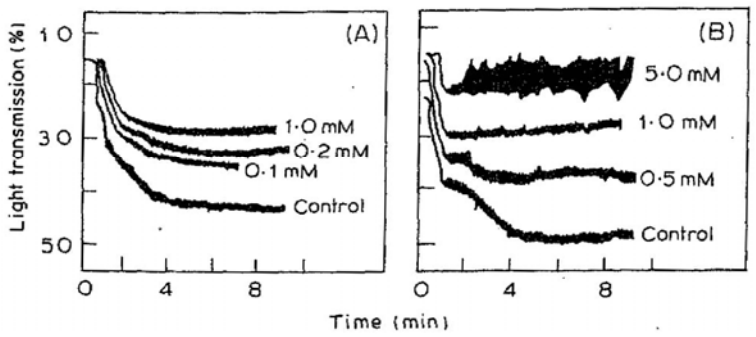

Figure 1. A. Inhibition of ADP-induced platelet aggregation by increasing concentrations of piretanide (0·1–1·0mM) *in vitro.* **B.** Complete inhibition of ADP-induced platelet aggregation by 5 mM piretanide *in vitro.* The final concentration of ADP used is 10 μM.

ADP, disaggregation resulted immediately and secondary wave of aggregation did not occur (figure 2).

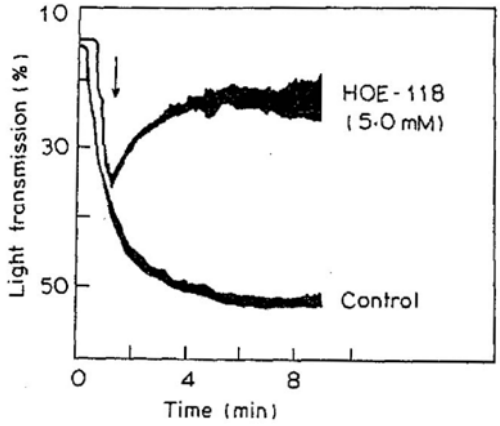

Figure 2. Reversal of ADP-induced platelet aggregation by piretanide (HOE-118,5 mM) *in vitro.* The arrow indicates the moment of addtion of piretanide. The final concentration of ADP used is 10 μM.

After oral administration of 6 mg piretanide, ADP-induced platelet aggregation was inhibited generally within 1 h and this effect lasted beyond 12 h and in some subjects the effect persisted upto 24 h. Figure 3 shows a typical curve of inhibition of platelet aggregation in a subject after 1 h and 45 min of administration of 6 mg piretanide. Secondary wave of aggregation did not result. Platelet aggregation time was prolonged from 29 to 39 s in this experiment. Figure 4 depicts inhibition of platelet aggregation which persisted beyond 12 h after administration of 12 mg piretanide in this subject. The PAT prolonged from 20 to 25 s (left side curve). The inset on the right upper side in this figure shows an *in vitro* experiment in which PRP was obtained from the same subject prior to administration of the drug. It was incubated with 2·5 mM piretanide

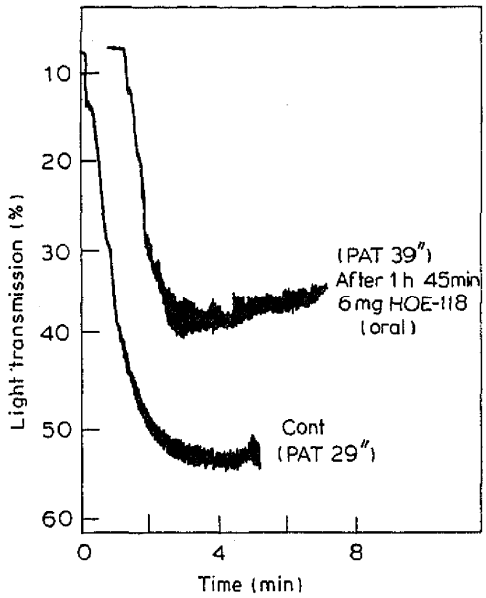

Figure 3. Inhibition of ADP-induced platelet aggregation following administration of a single oral dose of 6 mg piretanide (HOE-118). PAT prolonged from 29s (control) to 39s (test). The final concentration of ADP used is 10 μM.

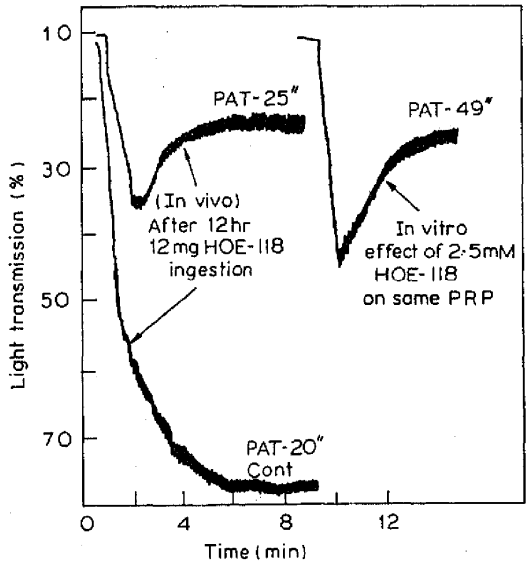

Figure 4. Left side curves: Persistence of inhibition of ADP-induced platelet aggregation beyond 12 h following administration of a single oral dose of 12 mg piretanide (HOE-118). Right upper side curve shows *in vitro* inhibition of platelet aggregation by 2·5mM concentration of piretanide on the PRP obtained prior to ingestion of piretanide in the same subject. The f.c. of ADP used is 10 μM in both the situations.

(f.c.) and this led to an inhibition of ADP-induced platelet aggregation. This curve compared to the curve of inhibition obtained after ingestion of piretanide (left upper curve) shows that the *in vivo* inhibitory effects of 12 mg piretanide are more potent than that of 2·5 mM piretanide *in vitro.*

Discussion

The present study shows that piretanide, a potential diuretic, on oral administration in smaller doses of 6 mg, leads to a significant increase of plasma euglobulin fibrinolytic acitivity, a mild fall in fibrinogen, diminished urinary urokinase activity initially, and a decrease of PF-4 release from platelets in circulation. Piretanide also inhibits primary platelet aggregation both *in vivo* and *in vitro.* The rapid reversal of ADP-induced platelet aggregation by piretanide suggests induction of platelet relaxation. The platelet contractility, aggregation and release reaction are events of the same process and are governed by cAMP. cAMP inhibits the synthesis of prostaglandin endoperoxides (PGG_2) and thromboxane A_2, which induce aggregation and secretion of human platelets (Malmsten *et al.,* 1976). Furosemide, like several inhibitors of platelet aggregation, achieves these results by its cAMP-phosphodiesterase inhibitory and adenyl cyclase stimulatory properties (Chohan, 1980, 1984). A close parallelism between furosemide and piretanide would suggest a similar mechanism of action, though this remains to be elucidated for the latter.

The increase in plasma fibrinolytic activity by piretanide seems to have resulted from a spill over of a plasminogen activator or urokinase from renal parenchyma into general circulation.

Significant inhibition of platelet aggregation was achieved with 12 mg of piretanide. This effect persisted beyond 12 h and proved far better than that of 2·5 mM piretanide in *in vitro* experiment (figure 4). Assuming a plasma volume of 3,000 ml for an average person, this low dose would amount to a maximal concentration of 12 μM of piretanide in circulation. Since the *in vitro* concentration of the drug required to achieve similar degree of inhibition was 100 fold higher, it may be assumed that certain metabolic products of piretanide are involved in eliciting these inhibitory effects *in vivo.*

Activation of fibrinolysis and restriction of platelet activity and limiting release of PF-4 (possessing heparin-neutralising capacity) are of great importance to ensure patency of the microvasculature and prevent platelet thrombus formation. Defective fibrinolytic activity has been demonstrated in a number of vascular diseases, involving both veins and arteries, including the coronary vessels (Almer and Nilsson, 1975; Walker *et al.,* 1977) and invariably it is associated with increased platelet aggregation/adhesion, as observed in cardiopulmonary disorders, pulmonary oedema and hypertension of high altitude (Singh and Chohan, 1972a,b, 1973, 1974). An increased amount of PF-4 activity has been encountered after myocardial infarction (Holger-Madsen, 1960; Cotton *et al.,* 1968; Farbiszewski *et al.,* 1968; O'Brien *et al.,* 1975), in peripheral arteriosclerosis (Cotton *et al.,* 1972), in rheumatoid arthritis (Cotton and Johnson, 1968), in metastatic cancer (Gjesdal and Abrahamsen, 1976), and after surgery (Gjesdal and Sorlie, 1975; Godal and Fichera, 1961). In the above vascular disorders, therefore, a therapeutic approach of combining fibrinolytic and anti-platelet

agents, would be logical, to achieve dissolution of platelet microthrombi and facilitate better tissue perfusion.

A large number of compounds have been used to enhance fibrinolytic activity (von Kaulla, 1975), either with transient benefits or immunological side effects (Davidson and Walker, 1979). No synthetic agents have been reported to possess a combination of properties of activating the natural fibrinolytic system and inhibiting the platelet aggregation and PF-4 release at the same time, except furosemide (Chohan, 1980,1984) and the presently studied drug piretanide (Chohan, 1982). Effectiveness with smaller dose and the ease of oral administration of piretanide are additional advantages. Apart from its usefulness as a potential diuretic, piretanide would be worthy of trial in conditions where fibrinolytic activity is reduced and platelet aggregation and PF-4 are increased.

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