Table I—Reproducibility of Results

Tablet Formulation, mg	*RSD*, %[a]
10	1.11
40	0.64
80	0.86
120	0.46

[a] Ten runs of each tablet formulation.

Fifteen minutes after mixing the last tube on the vortex mixer, add 0.20 ml of sodium arsenite solution with an automatic syringe and mix well on the vortex mixer. Five minutes after mixing the last tube on the vortex mixer, add 1.0 ml of 2,4-dinitrophenylhydrazine solution with a volumetric pipet and mix well on the vortex mixer. After 5 min, add 10.0 ml of chloroform to each tube and mix on the vortex mixer for 5 sec.

To each tube add 10.0 ml of 20% cupric chloride, cover the tubes with plastic caps, and shake mechanically for 15 min. Centrifuge the tubes at about 2000 rpm for 5 min. Aspirate completely and discard the top aqueous layer. If needed, aspirate some of the chloroform layer.

Measure the absorbance of the chloroform solutions of the sample and standard in 1-cm cells at the 352-nm maximum with the chloroform reagent blank in the reference cell. Nadolol in an average tablet is calculated from the concentration and absorbance of a nadolol standard.

RESULTS AND DISCUSSION

Experimental results for the oxidation of nadolol with periodate were given previously (2). The 2,4-dinitrophenylhydrazine reagent was optimized by using excess reagent and a proper hydrochloric acid concentration. When less than 0.5 ml or more than 4.0 ml of acid/100 ml of reagent was used, a lower absorbance was obtained. A novel method for eliminating excess 2,4-dinitrophenylhydrazine from the chloroform extracts with cupric chloride was introduced.

Several concentrations of cupric chloride were tried for the elimination of the excess 2,4-dinitrophenylhydrazine. From 10 to 30% cupric chloride solutions were used successfully. Higher than 30% cupric chloride solutions decomposed the oxidation reagent by producing a small amount of iodine, which slightly lowered the absorbance of the solutions and had an adverse effect on reproducibility. Elimination of 2,4-dinitrophenylhydrazine was rapid and occurred within a few minutes, but 15 min of shaking is recommended for reproducible results.

Nadolol 2,4-dinitrophenylhydrazone was stable in chloroform when shaken with cupric chloride solutions. No change in the absorbance was found when chloroform solutions were shaken with 20% cupric chloride solutions for up to 0.5 hr.

Table II—Nadolol in Tablet Formulations

Tablet Formulation, mg	Nadolol, mg/tablet Run 1	Run 2
10	10.04	9.97
40	40.00	40.20
80	80.20	80.00
120	120.80	120.10

The reaction of oxidized nadolol with 2,4-dinitrophenylhydrazine was completed in less than 1 min. The product was stable in the reaction mixture and in chloroform for at least 60 min.

The volume of sample or standard to be oxidized and then reacted with 2,4-dinitrophenylhydrazine should be 1 ml. When samples of more than 2 ml were used, lower absorbances were obtained. At least 30% methanol also was needed in the reaction mixture for maximum color development.

Various solvents were tried for the extraction of nadolol 2,4-dinitrophenylhydrazone from aqueous solutions. Methylene chloride, chloroform, and ethylene chloride produced the same reagent blank and extracted the color completely. No color was extracted with carbon tetrachloride, ether, ethyl acetate, butyl alcohol, and methyl ethyl ketone. Butyl acetate extracted the color completely, but incomplete destruction of 2,4-dinitrophenylhydrazine was obtained. Toluene extracted about 60% of the color.

Nadolol 2,4-dinitrophenylhydrazone–chloroform solutions had a maximum absorption at 352 nm and obeyed Beer's law at least between 0 and 200 μg of nadolol.

Recovery of nadolol was studied by adding the drug to the appropriate placebo tablet. Recoveries from 10-, 40-, 80-, and 120-mg tablets varied between 99.2 and 101.0%. Reproducibility of results was checked by analysis of each tablet formulation 10 times (Table I).

The method has performed reliably for a number of batches of nadolol tablets and, therefore, can be used for manufacturing control. Results from the analysis of some nadolol tablets are given in Table II.

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New 5-Hydroxy-2-indolecarbohydrazides as Platelet Aggregation Inhibitors in Ethylene Glycol

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Received May 2, 1977, from the *Departamento de Química Orgánica, Facultad de Farmacia, Universidad de Navarra, Pamplona, Spain.* Accepted for publication October 27, 1977.

Abstract □ The effect of ethylene glycol on blood platelet aggregation was examined using a previously described method. This method also was used to investigate several derivatives of 2-indolecarbohydrazide *in vitro*. All compounds inhibited platelet aggregation induced by collagen, epinephrine, or adenosine diphosphate at concentrations below 5×10^{-4} *M*.

Keyphrases □ Indolecarbohydrazides, various—effect on human platelet aggregation in ethylene glycol *in vitro* □ Platelet aggregation, human—effect of various indolecarbohydrazides in ethylene glycol *in vitro* □ Structure–activity relationships—effect of various indolecarbohydrazides on human platelet aggregation in ethylene glycol *in vitro*

The role of platelets in the formation of thrombi and arterial occlusions and the effect of adenosine diphosphate (ADP) on platelet aggregation are well documented (1). It has been suggested that the inhibition of platelet aggregation may be more useful than standard anticoagulant therapy (2, 3).

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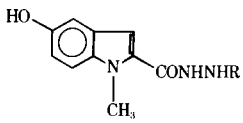

Table I—Effect of the Indolecarbohydrazides on Platelet Aggregation [a]

Compound	R	Final Concentration, *M*	Inhibition, % of Platelet Aggregation Induced by: Collagen	Epinephrine	Adenosine Diphosphate[b]	Adenosine Diphosphate[c]
I	H	5×10^{-4}	38	23	2	20
II	$(CH_3)_2CH$	5×10^{-4}	12	33	5	80–90
		5×10^{-5}				10
III	$CH_3CH_2(CH_3)CH$	5×10^{-4}	15	20	6	80–90
		5×10^{-5}				10
IV	$(CH_3)_2CHCH_2$	5×10^{-4}	30	10	0	90–100
		5×10^{-5}				90
		5×10^{-6}				85
		5×10^{-7}				10
V	$(CH_3)_2CHCH_2(CH_3)CH$	5×10^{-4}	0	11	0	50
		5×10^{-5}				20
VI	$CH_3(CH_2)_8CH_2$	5×10^{-4}	30	0	20	40
VII	$(CH_2)_4CH$	5×10^{-4}	25	30	30	20
VIII	$(CH_2)_5CH$	5×10^{-4}	37	16	34	30–35
IX	$C_6H_5CH_2$	5×10^{-4}	38	25	30	70

[a] The tests were carried out on eight different samples of plasma of apparently healthy humans without measuring maximum and minimum values. [b] Platelet-rich plasma incubated for 90 sec at 37°. [c] Platelet-rich plasma incubated for 5 min at 37°.

Various substances that inhibit platelet aggregation were investigated extensively (3), and several compounds that inhibit the adenosine diphosphate and/or collagen-induced platelet aggregation were described. An obvious relationship between chemical structure and inhibition of platelet aggregation has not been established (4–18).

The present paper describes an alteration in the ability of platelets to agglutinate with adenosine diphosphate, collagen, and epinephrine when a suspension of human thrombin was mixed with new 2-alkyl-1-(1-methyl-5-hydroxy-2-indole)carbohydrazides. Preparation of the hydrazides from ethyl 1-methyl-5-hydroxy-2-indolecarboxylate and their action as monoamine oxidase inhibitors were described previously (19).

EXPERIMENTAL

Samples—Blood was obtained by venipuncture in the forearms of apparently healthy humans. The blood was collected in polyethylene tubes containing a 1:9 volume of 3.8% sodium citrate.

Platelet-rich plasma was obtained by sedimentation and adjustment of platelet-poor plasma to 200,000 platelets/mm^3. Platelet-poor plasma was obtained by centrifugation of citrated blood at 4000 rpm for 30 min.

Compounds—Compounds I–IX (Table I) were described previously (19).

Solvent—Ethylene glycol was used as the solvent.

Aggregative Agents—Adenosine diphosphate (2×10^{-5} *M*), epinephrine (10^{-4} *M*), and collagen (0.2 mg/ml) in pH 7.4 phosphate buffer were used. The collagen solution was incubated at 37° for at least 30 min until opalescence was observed. It was then maintained in ice water at 0° until the end of the test.

Platelet Aggregation—Platelet aggregation was measured by the method of Born and Cross (20), using a platelet aggregation meter[1]. A 0.1-ml aliquot of the test sample dissolved in ethylene glycol was added to a polyethylene cell containing 0.8 ml of platelet-rich citrated plasma. It was incubated for 90 sec at 37°, after which 0.1 ml of adenosine diphosphate, epinephrine, or collagen in saline was added to the platelet-rich citrated plasma.

The final concentration of each sample was 5×10^{-4} *M*. If the product produced inhibition greater than 50% at 5×10^{-4} *M*, tests also were performed with lower concentrations (Table I).

The percent inhibition of aggregation by a test compound was calculated by comparison between the inhibition produced by the solvent (ethylene glycol) and that produced by the test compound.

[1]Evans EEL 169.

RESULTS AND DISCUSSION

Platelet aggregation tests were carried out on new indolecarbohydrazides to study their possible biological action in primary human hemostasis.

Since these compounds are not soluble in water, it was necessary to use a solvent that would not affect the polyethylene tubes, would be soluble in water, and would produce minimum antiaggregative effects. The use of dimethyl sulfoxide (13) was described for tests with male rabbits carried out in a parallel manner to those described here; this solvent, however, inhibited the adenosine diphosphate-induced platelet aggregation at 10^{-1} *M* up to 100% in human plasma.

The action of different solvents on platelet aggregation was determined before the aggregation test to ensure the utilization of a product capable of dissolving the compounds under study. Hexametapol, ethanol, 2-propanol, 1,4-dioxane, tetrahydrofuran, diethylene glycol, dipropylene glycol, and ethylene glycol were tested. The first six solvents were inhibitors, but the inhibitions elicited by dipropylene glycol and ethylene glycol were comparatively much lower.

Ethylene glycol was chosen. When it was added to plasma, the first of the two waves characteristic of epinephrine aggregation disappeared and only the second wave was observed. This second wave is related to the adenosine diphosphate of the platelets (Fig. 1).

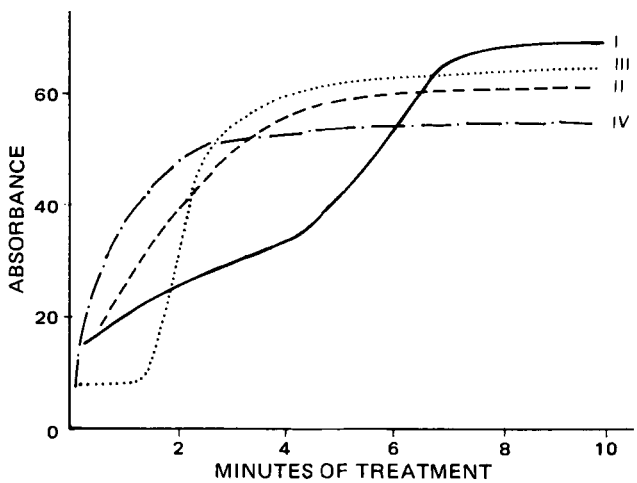

Figure 1—*Aggregation produced by: epinephrine (I) (a double wave is observed); epinephrine after the addition of ethylene glycol to plasma (II) (the disappearance of the double wave is observed without marked inhibition of the aggregation); collagen (III) (three phases are observed: a latent period, rapid aggregation, and then a stationary phase); and collagen after the addition of ethylene glycol to plasma (IV) (the disappearance of the latent period is observed).*

The aggregation curve due to collagen changed when ethylene glycol was added to plasma, and the time lag characteristic of this aggregant disappeared (Fig. 1).

In Table I, the action of indolecarbohydrazides in platelet aggregation is represented with reference to the aggregant adenosine diphosphate (2×10^{-5} *M*), epinephrine (10^{-4} *M*), and collagen (0.2 mg/ml). Platelet-rich plasma was incubated with the compounds for 90 sec at 37°. Inhibition of aggregation at 5×10^{-4} *M* of the compounds is significant (Table I).

When the incubations were carried out for 5 min at 37°, obvious differences were observed in the antiaggregant action of the tested compounds with adenosine diphosphate as the aggregant. Under these conditions, all of the compounds presented a higher capacity of aggregation inhibition. Compound IV at 5×10^{-6} *M* totally inhibited aggregation; *i.e.*, approximately 2 mg of the compound can produce complete platelet aggregation inhibition in humans.

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Assay of Mercaptopurine in Plasma Using Paired-Ion High-Performance Liquid Chromatography

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Abstract □ A sensitive, quantitative, and specific high-performance liquid chromatographic method for mercaptopurine in plasma is described. The analysis, in which mercaptopurine and the internal standard, 6-methylthio-2-hydroxypurine, are chromatographed as ion-pairs with heptane–sulfonic acid, employs a simple and rapid sample preparation based on deproteination using 60% trichloroacetic acid. Quantitation of plasma samples to 0.2 μg of mercaptopurine/ml is reported. The retention times of the major metabolites do not interfere.

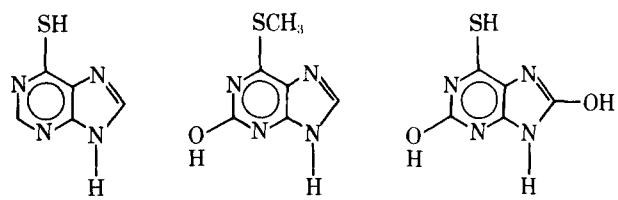
Keyphrases □ Mercaptopurine—high-performance liquid chromatographic analysis in plasma □ High-performance liquid chromatography—analysis, mercaptopurine in plasma □ Antineoplastic agents—mercaptopurine, high-performance liquid chromatographic analysis in plasma

To investigate plasma mercaptopurine (I) levels for pharmacokinetic studies, a sensitive and accurate assay was needed that would distinguish I from its metabolites. Several methods have been reported for I in biological fluids, but each has some disadvantages in specificity and sensitivity (1–3).

Recently, a GLC method was described that separates I from its metabolites (4). However, to achieve a sensitivity of 0.5 μg/ml, I must be extracted from a biological sample and then methylated to achieve a sample suitable for assay. The use of cation-exchange high-performance liquid chromatography (HPLC) for the detection, but not the quantification, of I in biological samples was reported (5, 6). The described method is specific and convenient and has sufficient sensitivity to measure accurately concentrations of mercaptopurine normally encountered at therapeutic levels.

EXPERIMENTAL

Reagents—All compounds used in the assay were used as received.



I II III

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