Studies on the Interaction between Isonicotinic Acid Hydrazid (INH) with Serum Protein by Polarography

by

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Introduction

It has been found by Goldstein¹⁾ that many drugs react with plasma protein and, furthermore, the combination of sulfonamide and its derivatives with plasma protein was reported by Davis²⁾³⁾⁴⁾⁵⁾ and Sakai⁶⁾. Emi⁷⁾ described that the dosage of INH was decreased in case of adding INH in Kirchner protein culture. Gomi⁸⁾ and others studied on the combination of antituberculous agent with serum protein and they concluded that antituberculous agents combined with serum protein at some constant percentage.

The determination of the concentration of INH in blood after administration of INH orally or injectionally was carried out by many workers⁹⁾¹⁰⁾, but the obtained values were generally too low to the amount of administrated INH. From these facts, it may be conceivable that the cause of degradation of INH in serum is the interaction of INH with serum protein to form INH-protein complexes.

Thus, we studied on the interaction of INH with serum protein by dialysis technique and polarographic methon.

Experimental

INH was determined by the method reported in our previous paper¹¹⁾. Polarograms were recorded by Yanagimoto pen recording polarograph PB-105. The dropping mercury electrode had a capillary constant of $1.33 \text{ mg}^{\frac{1}{2}} \text{ sec}^{\frac{1}{2}}$ at zero potential *vs.* S.C.E. in thermostat at $25^{\circ}\text{C} \pm 0.1^{\circ}\text{C}$. The equilibrium point of salting-out was determined by analyzing, below the temperature of 5°C ., the contents of INH in the inside and outside of dialysis cellophane bag (No. 300). Phosphate buffer pH 8.4 were used throughout these experiments, whose pH was measured with a Beckman Model G pH meter. Protease made by Teikoku Chemical Industry Co., Ltd., and trypsin made by Mochida Pharmaceutical Manufacturing Co., Ltd. were used for the decomposition of INH-protein complexes. Phosphate buffer solution of pH 8.4, containing 5,000 P.U.N.* protease or 5,000 H.U.M.* trypsin per 1 ml was employed for the enzymatic decomposition. Human albumin and γ -globulin used were made by The Japan Blood, Ltd. Apparatus for freezing and drying of INH-protein complexes was Kyowa Ōyo Physical Laboratory Type RL 500.

* P.U.N. and H.U.M. are units of Enzyme.

Results and Discussion

1) Degradation of INH in rabbit serum, human serum and water (for control). 100 γ of INH is dissolved in 1 ml of rabbit serum (containing 6.2% protein), human serum (containing 7.4% protein) or water (for control). These reaction mixtures are kept at 15°C and the concentrations of INH are determined by the polarographic method at time intervals as shown in Table I. It is seen in the Table that the degradation of INH in serum is fairly higher than the control.

Table I. Degraded values of INH in rabbit serum, human serum and water (for control) at 15°C.

Time (hour)	Rabbit serum (6.2%)	Human serum (7.4%)	Water (control)
0.5	58.0	60.0	0
1.0	73.5	74.8	0
3.0	76.0	75.2	0
5.0	76.8	76.7	5.0

2) Percentage of INH bound with protein of rabbit serum or human serum. 40 γ –140 γ of INH is dissolved in 1 ml of rabbit serum (containing 6.2% protein) or human serum (containing 7.4% protein). These serums containing INH are put in dialysis bags using distilled water or pH 8.4 phosphate buffer as an outside solution. Dialysis is continued until the concentrations of INH of the inside and the outside solutions become equal and then percentage of INH bound with protein is calculated by the following formula. The results are shown in Table II. It is clear that percentage of INH bound with protein is approximately constant notwithstanding the change of amount of the added INH, when the concentration of protein is kept constant.

Calculation formula of percentage of INH bound with serum protein :

$$\frac{\text{INH added in serum } (\gamma/\text{ml}) - \left(\frac{\text{Concn. of INH found after dialysis } (\gamma/\text{ml}) \times \text{Sum of volumes (ml) of inside and outside solutions}}{\text{INH added in serum } (\gamma/\text{ml})} \right) \times 100 - 2.9 \text{ or } 2.6^*}$$

Table II. Percentage of INH bound with rabbit serum or human serum.

	Concn. of added INH γ/ml								
		40	60	80	90	100	120	140	Average
Rabbit serum (6.2%)	Outside solution								
	H ₂ O	—	—	75.4	72.4	73.0	72.0	73.2	73.2
	pH 8.4 Buffer	—	—	73.2	72.0	72.3	71.5	72.0	72.2
Human serum (7.4%)	H ₂ O	—	—	75.2	74.2	74.1	73.8	73.5	74.1

3) Percentage of INH bound with human albumin or γ -globulin. 60 γ –100 γ of INH is dissolved in 1 ml of human albumin solution (containing 4% or 5% protein) or γ -globulin solution (containing 100 mg/ml of protein) and then percentage of INH bound with protein is determined polarographically after the dialytic equilibrium was attained using water as an outside solution. The results are shown in Table III.

From Table III, it may be concluded that human albumin and γ -globulin interact with INH to form INH-protein complexes and that albumin combines with larger amount of

* These figures are average values of percentage of INH adsorbed onto cellophane No. 300 when water or pH 8.4 phosphate buffer were used as an outside solution.

Table III. Percentage of INH bound with human albumin or γ -globulin

Protein	Concn. of Protein	Added INH (γ /ml)	INH bound with albumin or γ -globulin	Average
Human albumin	4 %	60	40.4	34.4
		80	33.4	
		90	32.2	
		100	31.7	
	5 %	70	59.5	54.4
		80	58.7	
		90	51.1	
		100	53.5	
γ -globulin	100 mg/ml	60	52.5	52.1
		70	54.4	
		80	51.3	
		90	50.9	
		100	51.6	

INH than γ -globulin.

4) Enzymatic decomposition of INH-protein complexes and determination of INH bound with protein. 100 γ , 400 γ or 800 γ of INH is dissolved in 1 ml of rabbit serum (containing 6.0% protein), human albumin (containing 2% protein) or γ -globulin (containing 1% protein). In order to remove nonbinding INH perfectly from the test solution these reaction mixtures are put in dialysis bags and dialyzed below 5°C using distilled water as outside solution and then crystallized INH-protein complexes are obtained from these dialyzed solution by freezing at -30°C and drying in vacuum at 40°C for 4 hours.

These INH-protein complexes are dissolved in buffer solution together with enzymes (protease and trypsin), and they are incubated at 37°C for the hours as shown in Table IV. And thus isolated INH is determined by the previous method.¹¹⁾

Table IV. Decomposition and Determination of INH-protein complexes by protease and trypsin

Time of incubation (hour)	Protein	Rabbit serum (6 %)		Human albumin (2 %)	γ -Globulin (1 %)
	Added INH (γ /ml)	100	400	100	800
	Enzymes and units	Used INH-protein complexes (mg.)			
		60	60	20	10
1	Protease 5,000 P.U.N./ml	42.0	44.0	11.5	9.0
4		49.0	44.0	17.0	14.5
7		49.0	49.0	17.2	15.0
20		51.8	50.0	17.3	15.0
1	Trypsin 5,000 H.U.M./ml	0			
4		23.1			
7		25.0			
20		31.0			

5) Degradation of INH by protease and trypsin at 37°C . Protease of 5,000 P.U.N. and trypsin of 5,000 H.U.M. dissolved in phosphate buffer of pH 8.4 are added to INH solution (concentration of INH: 100 γ /ml; 40 γ /ml) and then incubated at 37°C for the hours as shown in Fig. 1. At each time, the concentration of INH is determined by polarogra-

phic method to get an information about the degradation of INH by these enzymes in phosphate buffer. From Fig. 1, it is concluded that the decrease of INH in high concentration is greater than that of INH in low concentration and the decrease of INH by protease is smaller than that by trypsin, therefore protease is suitable for the decomposition of INH-protein complexes.

Fig. 1 Degradation of INH by protease and trypsin in pH 8.4 phosphate buffer at 37°C.

6) Determination of INH bound with protein and free INH in serum after oral administration of INH to rabbit. Concentrations of INH bound with protein and free INH in serum are determined by polargraphic method together with enzymatic decomposition of INH-protein complexes which are obtained by the above-mentioned dialysis procedures. It is roughly estimated from the data in Table V that the ratio of INH bound with protein to free INH in serum is 3~5:1.

Table V. Concentrations of INH bound with protein and free INH in 1 ml of serum.

Hours after administration	1	2	3	6	8
Free INH (γ /ml)	17.2	23.1	15.5	13.7	10.4
INH bound with protein (γ /ml)	68.0	76.0	60.0	64.0	56.4
$\frac{\text{INH bound with protein } (\gamma/\text{ml})}{\text{Free INH } (\gamma/\text{ml})}$	3.9	3.3	3.8	4.6	5.4

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ポーラログラフ法によるイソニコチン酸ヒドラジド (INH) と血清蛋白との相互作用に関する研究

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薬剤の血漿蛋白との相互関係については Goldstein の綜説的報文があり, さらにサルファ剤と蛋白との結合性に関しては Davis, 酒井らの報文がある. 江見は蛋白加キルヒナー培地において INH の減少度の大きいことを光電比色による定量法で認めている. また抗結核剤と血清蛋白との結合性に関しては五味らによる報告がある.

我々は INH の血中濃度が投与量に比して一般に低いこと, また家兔血清に INH を添加したさい Table I にみられる様になりに大きく経時的に減少していることより INH と血清蛋白とが何等かの相互作用により複合体を形成しポーラログラフ法による定量法に陰性となるためと考え, セロファン膜による透析凍結乾燥による複合体の精製さらにこの複合体を酵素分解してえられる INH のポーラログラフ法による定量を行ないその結合性につき種々検討を行なった.

家兔血清, 人血清, 人アルブミン, γ -グロブリンに対する INH の結合率は Table II, III の如く蛋白濃度一定であれば INH の結合率はほぼ一定値を示し,

人アルブミンは γ -グロブリンより高い結合性を示した. またこれらのものに INH の一定濃度を添加し, 一定時間 incubate したのち透析凍結乾燥を行ない複合体を結晶となし, これを酵素分解してえられる INH を測定したところ Table IV の結果をえた. この結果よりして複合体を形成した INH は定量可能と考えられる. なお酵素分解に使用した酵素は Trypsin より Protease がよい結果をえた.

実際に INH を家兔に 50mg/kg 経口投与し血清中の遊離 INH と複合体との INH 量の比を測定したところ経過時間により異なるがほぼ 1 : 3 ~ 5 の割合であつた.

以上の実験には柳本ペン記録式ポーラログラフ PB-105 を使用し, 特性 $\text{mg}\% \text{sec}^{-1} = 1.33 (0v.)$ の滴下水銀電極で飽和甘汞電極を対極として用いた. 透析にはセロファン No. 300 で作成したセロファン裏を使用し, 複合体の凍結乾燥には協和応用物理研究所 RL500 を使用し -30°C で凍結し, 40°C にて 4 時間真空乾燥を行なった.