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The Effects of Ethambutol on the Inactivation of Isoniazid

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Isoniazid(INH)는 體內에서 迅速히 吸收되어 一部는 治療効果가 있는 遊離 INH로 變하고 一部는 治療効果가 없는 acetylisoniazid, isonicotinic acid, isonicotinuric acid等으로 代謝되어 不活性化된다.

家兎의 尿 및 血漿中에서 總 INH에 對한 遊離 INH의 比를 呈色的으로 定量 算出함으로써 INH의 不活性化에 미치는 Ethambutol(EMB)의 影響을 實驗하였다.

家兎에 INH를 經口投與한 結果 그 代謝에 依한 不活性化比는 같은 家兎에서는 比較的 一定하고 個體間의 差異는 현저하였다.

INH에 EMB를 配合投與하거나 分子化合物을 投與했을 경우 EMB에 依해 INH의 代謝가 抑制되어 INH 單獨投與時보다 遊離 INH가 尿 및 血漿中에서 增加하였다.

EMB에 依한 遊離 INH의 上昇은 INH單獨投與보다 平均 1.5倍이며 EMB分子化合物이 EMB配合物보다 血漿中에서 1.3倍 높게 나타났다.

Isoniazid(INH) has been known to be a highly active antituberculous agent. In 1945, Chorine[1] reported that nicotinamide possesses tuberculostatic action. Examination of the compounds related to nicotinamide related that many pyridine derivatives possess tuberculostatic activity. INH was synthesized with the thiosemicarbazons and isonicotinic acid in favour of synergistic effect[2].

INH is both tuberculostatic and tuberculocidal; the minimal tuberculostatic concentration is 0.025 to 0.05μg/ml.

In human, INH is readily absorbed when administered orally and peak plasma concentration develops 1 to 2 hours after oral ingestion.

INH is converted to pyruvic acid isonicotinoylhydrazone, acetyl-INH, isonicotinic acid, isonicotinuric acid, acetylhydrazine and 1,2-diacetylhydrazine etc. which have no or only a very slight therapeutic effect[3].

Matilla et al.[4][5][7] have reported that the rate of INH inactivation to which INH is

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broken down to the above mentioned compounds in the human body varies from one individual to another or race, but it seems to be quite constant over a certain length of time for one and the same person, not influenced by sex or age. Many investigators reported that the INH inactivation, by INH metabolism, has been inhibited on adding other drugs[8-13]. Lauener et al.[13] also reported that a significant increase of therapeutic effects has been demonstrated in human when PAS is administered simultanecusly with INH, Bell et al.[12] have observed that the simultaneous administration of PAS, PBA, sulfanilamide and sulfadiazine produces higher concentrations of free active INH in the blood, Johnson et al.[9] have identified that a large number of amino, amido and hydrazino compounds inhibit the acetylation of INH by means of pigeon liver extract test.

A relationship between the INH metabolism and the success of INH therapy has been postulated by Mitchell et al.[14] Hence, the simultaneous administration of other antituberculous agent has been used more useful than INH alone.

INH-Ethambutol(EMB) therapy has been used since EMB had been developed.

In human several investigators have reported the clinical effects of INH-EMB combined therapy, but none of investigations on INH inactivation has not yet been demonstrated when EMB is administered simultaneously with INH.

In these studies, the authors have found something about the effects of EMB on the inhibition of INH inactivation(metabolism).

EXPERIMENTAL

Instruments and Reagents——Spectrophotometer(Shimadzu MPS 5000): ammonium sulfate(GR); borax(GR); methanol(GR); $KMnO_4$(GR); ceric sulfate(GR); trichloracetic acid(GR); ascorbic acid(GR); isoamyl alcohol-ether(GR); 1-Chloro-2,4-dinitrobenzene(1.25 per cent in absolute ethanol, always freshly prepared); ammonia buffer solution(dissolve 87gm of K_2HPO_4 and 107gm of NH_4Cl in about 400ml of water and add 6.7ml of concentrated ammonia(28%) to make 500ml)

Materials——Rabbit(2.5-2.6kg); INH(USP); EMB(USP); Isoetam(INH: EMB=1:1, molecular compound, Ferrer, Spain)

The Constancy of the Degree of Individual Inactivation——To examine the constancy of the degree of individual inactivation, single oral dose of 30mg of INH was given to 6 rabbits. In the twenty four-hours urine both free INH and total INH(INH+INH metabolites) were estimated. The test was repeated three times at intervals of three days.

The Effect of EMB on INH Inactivation——Single dose of 30mg of INH alone were given to 12 rabbits. Blood was withdrawn three hours after the administration of 30mg of INH, both free INH and total INH were estimated, and the free INH in per cent of the total INH was calculated. The per cent of free INH in urine of 0-4hrs

4-10hrs, 10-24hrs were determined as above.

One week later, the same 6 rabbits were given single dose of 80mg of Isoetam, as well as the same other 6 rabbits were given single dose of 30mg of INH plus 80mg EMB mixtures. The per cent of free INH in plasma and in urine ware calculated as above.

Determination of Free INH——The free INH in plasma was extracted with isoamyl alcohol-ether according to the method of Kelly et al.[10] and estimated in comparison with the method of Scott[21] with aid of the color reaction with 1-chloro-2, 4-dinitrobenzene. Thus, for establishing a standard curve, the standard INH dilutions in plasma must be extracted exactly according to the same method.

Determination of free INH in plama. In a glass-stoppered shaking bottle, 3.2gm of ammonium sulfate were weighed, and 5ml of plasma plus 0.5ml of NaOH 30ml isoamyl alcohol-ether were added. The mixture was shaken for 30 minutes using shaker and was then centrifuged. 20ml of ether solution were withdrawn and then shaken for three minutes with 1.3ml of 0.1N HCl. 1ml of the aqueous solution was transferred into a test tube and 1ml of 0.1N alcoholic KOH plus 100mg of borax plus 8ml of 1-chloro-2.4-dinitrobenzene solution were added. The tubes were placed in a boiling water bath for 20 minutes which resulted in complete evaporation of the ethanol. The tubes were then cooled and 3ml of methanol were added to the residue and the mixtures were shaken and filtered. The solutions were determined at 530nm. The blank solution was prepared using plasma obtained prior to the administration of INH.

Determination of free INH in urine. 1ml of diluted urine(1:10 to 1:20) was mixed, without extraction, with borax and chlorodinitrobenzene solution according to the procedure described above.

Determination of Total INH——By oxidation with $KMnO_4$ or ceric sulfate[22] INH, acetyl-INH, and isonicotinic acid are converted to isonicotinic acid, and then estimated using BrCN reagent by the method of Rubin et al[23].

Determination of total INH in plasma. 2ml of plasma plus 2ml of distilled water plus 2ml of trichloracetic acid(10%) were mixed in a centrifuge tube and the mixture was vigrously centrifuged. The clear supernatant fluid was poured into a glass stoppered test tube, 8ml of ether were added, and the mixture was shaken for 5 minutes. After seperation of the phases, the ether solution was completely sucked off and 4ml of the extracted aqueous solution were introduced into a graduated test tube, 1ml of 20% sulfuric acid was added, and the tubes were placed in a boiling water bath for 20 minutes. To this solution, aqueous ceric sulfate solution(15% in 0.5N H_2SO_4) was added dropwise until a yellowish color appeared. The excess ceric sulfate was reduced by means of ascorbic acid(1%). To the colorless solution, phenolphtalein and 4N NaOH were added until a pink color appeared. Ammonia buffer was added up to 10ml and the resulting precipitate was collected on a filter. 5ml of the clear

colorless filtrate were mixed with 1ml of aqueous BrCN solution(10%), and the extinction of the resulting yellow dye stuff was estimated after 3 minutes at 437nm. (blank solution; with plasma obtained prior to the administration of INH)

Determination of total INH in the urine. 2ml of urine diluted with water(1:10) were introduced into a glass-stoppered test tube, 2ml of 0.1N HCl plus 8ml of ether were added, and the mixture was shaken for 5 minutes. The ether was completely sucked off, and 2ml of the aqueous solution were introduced into a graduated test tube. To this were added 2ml of distilled water plus 1ml of H_2SO_4(20%), and the mixture was heated for 20 minutes in a boiling water bath. The above procedure was then followed as for the plasma.

RESULTS and DISCUSSION

The amounts of excreted free INH and of the total INH after administration of 30mg of INH by mouth are shown in Table I.

Three estimations, at intervals of 3 days, showed the percentage of free INH in urine to be relatively constant. In view of the constancy of the individual degree of inactivation, the effect of adding EMB to a constant INH dose can be examined.

It may be seen in Table I-V that, or adding EMB, a significant increase of the percentage content of free INH in plasma and in urine resulted. On the average, about 1.5 time more free INH was found on giving combined drugs than on administering INH alone. According to the plasma values, a little more free INH seemed to be marked on INH-EMB molecular compounds than on INH-EMB mixtures.

In the 10-24 urine fractions the effect of EMB was greater because the proportion of free INH, without adminstration of EMB, was usually very small. Calculated with reference to 24 hour excretion, this is less important, however, because most of the INH is excreted within 10 hours.

Examination of Table I shows that the excretion of the total INH reached 100% of the administered dose in only one case, and that in 2 cases it amounted 60% or less. This fact is attributed to incomplete absorption. According to findings reported by Iwainsky et al.[24] in urine of normal subjects, recovered 45 to 96% of the orally administered INH dose in the form of metabolites, but they also found considerable amounts(up to 50%) in the feces. From the variability of the absorbed amounts it appears that it is preferable to use the relative rather than the absolute concentrations of free INH for the calculations.

Likewise, the fraction in percetage of free INH does not remain entirely constant during the three tests days, e.g., rabbits a.b.f. Thus, the increase in free INH concentration after administration of INH-EMB mixture or molecular compound must be considerably greater than the chronologic variations.

Table I-IV indicate that some rabbits excrete small amounts of free INH. while

Table I—Urinary Excretion of Free and Total INH in 24 hrs After Oral Administration[a)]

Rabbit	INH, 30mg Dose		Free INH / Total INH %
	Free(mg)	Total(mg)	
a	5.93	27.53	21.83
	6.85	30.00	22.83
	7.51	25.81	29.09
b	5.98	25.33	23.61
	4.01	26.37	15.21
	3.32	20.01	16.60
c	3.47	21.05	16.48
	4.12	24.21	17.02
	3.83	23.11	16.56
d	7.90	27.71	28.51
	5.18	17.77	29.82
	7.82	26.71	29.28
e	6.44	23.77	27.10
	7.40	25.35	29.20
	6.33	21.67	29.22
f	5.49	27.01	20.33
	6.87	25.15	27.32
	3.30	17.31	19.06

a) Experiment were performed in triplicate at intervals of 3 days.

others excrete rather large amounts. Evidently, the first group will benefit from the administration of EMB. Johonson et al.[8)] have shown a further reason why only some rabbits might benefit from PAS with respect to the inactivation of INH is that the possible effects of EMB as judged by studies in the rabbit are concerned only with the acetylation of INH. In many human subjects, however, INH is also split to a large extent into isonicotinic acid. Lauener et al.[13)] have established that on the administration of PAS the breakdown of INH to isonicotinic acid is not influenced. It is therefore readily seen that PAS is effective only in persons in whom acetylation predominates in the process of inactivation.

Likewise, it is possible that the effects of administering EMB are also concerned with the inhibition of the acetylation of INH. Therefore, the effects of EMB may be due to the acetylation of INH only in subjects in which acetylation predominates in the processes of inactivation.

Peters[24)] has found that the addition of PAS failed to increase the free INH. Likewise, it is assumed that Peters might have worked with the subjects in which preferentially acetylation does not predominate in the processes of inactivation.

Bonicke et al.[25)] found no connection between the degree of the excreted free INH

and the therapeutic success of the medication, but Mitchell et al.[14] have reported that in patients with high plasma concentration of biologically active INH, tubercle becilli disappear more rapidly. In an investigation involving 100 cases of tuberculosis, Viven et al.[26] have observed that, in patients with a plasma concentration of at least 0.5 μg/ml of free INH, quick recoveries occur more frequently than in patients with low plasma concentrations of free INH.

These findings that the more percentage contents of free INH in plasma and urine of rabbits was found on giving INH-EMB mixture or INH-EMB molecular compound than on administering INH alone show that an increase of therapeutic effects according to the report of Mitchell et al.[14]

On the average, about 1.5 times more free INH was found on giving EMB daily in association with INH than on administering INH alone, it is similar to that of PAS, but is not established in view of chemical strutcure.

Table Ⅱ—Percentage of the Free to Total INH in Plasma and Urine after Oral Administration of 30mg INH

Rabbit	Plasma			Urine											
	3hrs			0~4hrs			4~10hrs			10~24hrs			0~24hrs		
	Free (μg)	Total (μg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free/Total %		
A	9.25	39.36	23.50	2.25	12.51	17.99	2.65	12.35	21.46	0.28	3.91	7.16	18.00		
B	10.78	29.27	36.83	2.17	11.35	19.14	2.34	15.98	14.64	0.12	2.15	5.58	15.70		
C	13.33	13.95	95.56	2.95	6.25	47.20	2.78	10.26	27.29	0.85	2.86	29.72	35.01		
D	14.21	39.12	36.32	3.75	12.37	30.31	2.71	13.15	20.60	0.21	2.25	9.33	24.01		
E	14.55	35.51	40.97	2.35	11.57	20.31	2.62	13.35	19.66	0.47	3.17	12.61	19.37		
F	13.97	33.75	41.39	3.31	6.21	53.30	3.57	6.35	56.22	0.53	3.05	17.38	47.47		
Mean			35.80			31.38			26.65			10.41	22.41		
G	14.21	39.13	36.31	2.57	10.55	24.36	2.18	14.71	14.82	0.49	4.61	10.62	17.54		
H	13.36	37.75	35.39	3.65	12.67	28.80	2.55	14.98	17.02	0.17	2.11	8.06	21.40		
I	12.65	25.55	49.51	3.86	10.95	35.25	3.77	14.27	26.42	0.35	1.33	26.31	30.05		
J	10.77	35.22	30.57	4.38	8.55	51.22	3.47	16.51	21.01	0.15	1.51	9.93	30.10		
K	13.35	38.95	34.27	2.67	13.21	20.21	2.88	12.84	22.43	0.28	1.93	14.50	20.11		
L	12.12	35.78	33.87	3.26	14.99	21.75	2.33	13.51	17.24	0.38	1.51	25.16	19.90		
Mean			36.65			30.27			19.82			10.78	23.18		

Table Ⅲ—Percentage of the Free to Total INH in Plasma and Urine After Oral Administration of 80mg Isoetam

Rabbit	Plarma			Urine										
	3hrs			0~4hrs			4~10hrs			10~24hrs			0~24hrs	
	Free (μg)	Total (μg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free/Total %	
A	33.13	35.36	93.17	3.25	13.15	24.71	3.18	12.21	26.05	0.57	3.88	14.69	23.94	
B	12.95	20.15	64.27	7.11	11.21	63.40	3.94	14.35	27.49	0.25	2.05	12.20	40.92	
C	10.66	20.25	52.64	4.34	7.95	54.59	5.49	11.31	48.52	1.21	2.68	45.15	50.32	
D	20.05	29.01	69.11	5.07	12.57	40.36	4.56	13.46	33.90	2.05	2.35	87.23	41.16	
E	21.35	30.15	70.81	4.22	11.97	35.24	3.88	13.38	29.00	0.88	3.66	24.04	30.95	
F	19.93	30.55	65.30	5.50	8.31	66.17	5.41	7.95	68.11	2.15	3.17	67.82	67.22	
Mean			64.42			43.89			32.99			32.78	37.46	

Table Ⅳ—Percentage of the Free to Total INH in Plasma and Urine after Oral Administration of INH-EMB Mixture(INH 30mg; EMB 80mg)

Rabbit	Plasma			Urine										
	3hrs			0~4hrs			4~10hrs			10~24hrs			0~24hrs	
	Free (μg)	Total (μg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free (mg)	Total (mg)	Free/Total %	Free/Total %	
G	13.15	28.95	45.42	10.91	11.31	96.46	12.31	14.51	84.84	2.15	4.51	47.67	83.60	
H	14.38	27.45	52.37	4.57	12.37	36.94	2.95	14.28	20.66	0.35	2.32	15.9	27.17	
I	13.27	20.35	65.23	3.15	10.58	29.77	4.11	14.53	28.29	0.23	1.43	16.08	28.22	
J	7.92	18.78	42.18	5.34	8.12	55.76	3.75	15.91	24.83	0.23	1.61	14.29	36.35	
K	16.16	29.85	54.15	5.59	13.22	42.27	3.23	12.13	26.63	0.71	1.82	39.01	35.08	
L	13.65	28.67	47.60	4.14	14.91	27.76	2.95	13.39	22.03	0.95	1.75	54.28	26.92	
Mean			51.66			34.26			24.49			31.21	30.75	

Table V—Ratio of Free INH in Plasma and Urine Following the Administration of an INH-EMB Combination and INH Alone[a)]

INH alone	INH with EMB or Isoetam	Ratio of the amounts of Free INH on Administration of an INH-EMB Combination to the Amounts on Administration of INH Alone				
		Plasma	Excretion			
		3hrs	0~4hrs	4~10hrs	10~24hrs	0~24hrs
30mg	80mg of of Isoetam(Equivalent to 30mg of INH)	1.80	1.40	1.24	3.15	1.67
30mg	30mg of INH+80mg of EMB	1.41	1.35	1.24	2.90	1.33

a) $\text{Ratio} = \frac{\text{Free INH(combined therapy)}}{\text{Free INH(single therapy)}}$

CONCLUSION

1) The percentage of free INH to total INH of individual in INH inactivation is relatively constant, while the variations from one to another were very marked.
2) About 1.4 times more free INH in plasma and about 1.3 times more free INH in urine were found on giving INH-EMB mixtures than on administering INH alone.
3) About 1.8 times more free INH in plasma and about 1.7 times more free INH in urine were found on giving INH-EMB molecular compounds than on administering INH alone.
4) About 1.3 times more free INH in plasma and in urine were established on giving INH-EMB molecular compounds than on administering INH-EMB mixtures.

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