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Studies on the Stability of Drugs in Biological Media. (1). Stability of Furylmethylketone Isonicotinoylhydrazone in Culture Media

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With furylmethylketone isonicotinoylhydrazone (FKI) used as an example, factors affecting drug stability in various culture media and its implications on the *in vitro* activity tests have been studied. In buffer solution (pH 2—10), FKI was stable in the neutral pH and unstable in acidic and alkaline pH regions. It became evident that in culture media the degradative reaction was greatly influenced by medium components such as serum and amino acids. The rate of degradation was four to ten times more rapid than in buffer solutions and considerable amounts of isoniazid were released long before one could expect growth of test organisms in culture. Among the medium components tested, amino acids such as asparagine and glutamic acid exhibited a dominant effect and the data seemed to indicate non-protonated amino group as catalytic species. Suggestions are made relative to the significance of these observations for the *in vitro* evaluation of anti-bacterial agents.

Although the stability of drugs in aqueous pharmaceutical preparations has been well studied from the physicochemical standpoint, their stability in biological media such as nutrient broth and body fluid has received relatively little attention. There are certain drugs which undergo at least partial degradation during the test incubation period. In these cases reading the end point after the standard incubation period might give misleading impression of activity of the compound.

The importance of the drug stability in culture media was recognized by Kanai and his co-workers.[2] Works along similar lines have been reported by Wick,[3] Colwell and his co-workers[4] in their studies on cephalothin and glucuronolactone isonicotinoylhydrazone respectively. Their studies, however, are largely of qualitative nature.

Furylmethylketone isonicotinoylhydrazone (FKI) was recently introduced as a new anti-tubercular agent. Previous work from this laboratory had shown that FKI was better absorbed from the rat intestine than any other isoniazid derivatives currently on market.[5] Its *in vitro* activity data, however, vary considerably ranging from almost equal to hundred times as potent as parent isoniazid and the question appears to be whether the salutary effects of FKI are merely due to the release of isoniazid contained in its molecule or it acts as a separate drug.[6-8]

The purpose of this work is, therefore, to investigate stability of this drug in various types of culture media as a function of catalytic components. It is anticipated that such physicochemical studies on the nature of the interaction of drugs with normal constituents of the media will significantly contribute to a rational approach to the evaluation of *in vitro* activity of investigational drugs.

1) Location: *Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto.*
2) K. Kanai, S. Okamoto, and T. Murohashi, *Kekkaku*, 38, 512 (1966).
3) W.E. Wick, *J. Bacteriol.*, 87, 1162 (1964).
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Experimental

Materials——FKI (Daiichi Seiyaku Co., Ltd.) and isoniazid (Takeda Chemical Industries Ltd.) were used as received. All other chemicals used were of reagent grade.

Assay——Samples were analyzed by ultraviolet absorption using a Shimadzu model QV-50 spectrophotometer. The absorbance at 310 mμ of aqueous or chloroform solution was read against a similarly prepared blank.

Procedure for Kinetic Studies in Buffer Solutions——Composition of buffer solutions is given in Table I.

Table I. Buffer Compositions

pH	Buffer
pH 2.0	HCl
pH 3.0— 5.0	CH_3COOH–CH_3COONa
pH 6.0— 8.0	KH_2PO_4–K_2HPO_4
pH 9.0—10.0	H_3BO_3–$Na_2B_4O_7 \cdot 10H_2O$

FKI was dissolved in the appropriate buffer solution containing a sufficient amount of potassium chloride to give the FKI solution an ionic strength $\mu=0.1$. The hydrolysis was carried out in a 50 ml volumetric flask which was immersed in a constant temperature bath regulated at 38°. Aliquots (1 ml) were withdrawn periodically and an appropriate amount of pH 6.0 phosphate buffer solution (0.1 M) was added. To the quenched solution, 5 ml of chloroform was added, shaken vigorously, and the chloroform solution being analyzed.

Procedure for Kinetic Studies in Culture Media——Media and components are listed in Table II. A series

Table II. Compositions of Culture Media

Component \ Media	Kirchner	Sauton	Long	Glycerol-broth	Glycerol agar	Ogawa
KH_2PO_4	4 g					1 g
$Na_2HPO_4 \cdot 12H_2O$	3 g					
Na-Citrate	2.5 g					
Asparagine	5 g	4 g	5 g			
Na-Glutamate	(or 10 g)		(or 10 g)			1 g
$MgSO_4 \cdot 7H_2O$	0.6 g	0.5 g	1 g			
Glycerin	20 ml	60 g	50 ml	50 g	50 g	6 ml
(Serum)[a]	(100 ml)					
Distd. water	1000 ml	1000 ml	1000 ml	1000 ml	1000 ml	100 ml
Ferric amm. citr.		0.05 g	0.05 g			
Amm. citr., dibasic			5 g			
K_2HPO_4		0.5 g	3 g			
Citric Acid		2 g				
NH_4OH		qs pH 7.4				
Egg solution						200 ml
Peptone				10 g	10 g	
Beef extract				10 g	10 g	
Agar					20 g	
NaCl				2 g	2 g	
2% malachite green						6 ml

a) additional component of 10% serum Kirchner-medium

of tubes of media containing FKI solution were allowed to incubate at 38°. Aliquots were withdrawn at given time intervals daily and analyzed. To an aliquot of the liquid sample, usually 1 or 2 ml, 10 ml of chloroform was added and shaken vigorously, then the chloroform solution was analyzed. In the case of solid media, whole media were transfered into a mortar with the aid of a small amount of pH 6.0 phosphate buffer solution (0.1 M), ground well, and centrifuged. To an aliquot of the supernatant (10 ml) from Ogawa medium 0.5 ml of 1 M sodium nitrite solution was added to effect clear solution. Chloroform (20 ml) was added to the clear solution and shaken vigorously. The chloroform solution was then separated, dried with

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2.5 g of anhydrous sodium sulfate, and analyzed. In the case of Glycerol Agar medium, 10 ml of chloroform was added to 3 ml of the supernatant and treated similarly.

Results and Discussion

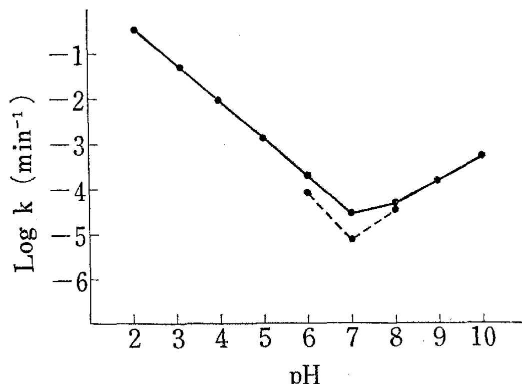

Fig. 1. The pH–Rate Profile of the Degradation of Furylmethylketone Isonicotinoylhydrazone in Buffer Solutions at 38°

——— μ=0.1 ……… μ=0

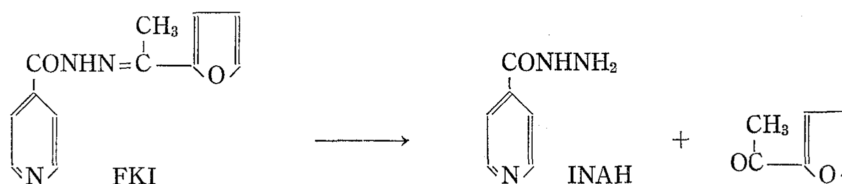

Chart 1. Hydrolysis of FKI

Stability in Culture Media

Despite the complexity of the reaction media, the degradation of FKI in culture media was found to obey first-order kinetics as that in buffer solutions. The route of degradation in culture media would be much the same as that in buffer solution since no degradation product other than isoniazid was observed. In all cases there was a linear relationship between time and logarithm of residual FKI concentration in media as shown in Fig. 2 and 3, where C indi-

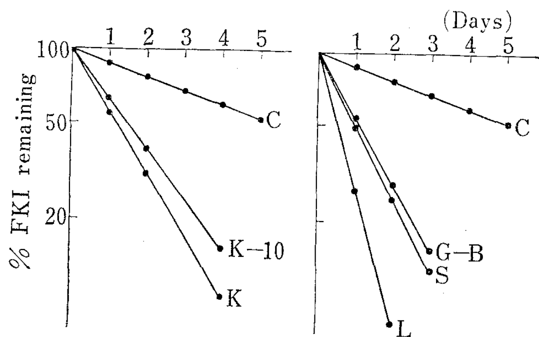

Fig. 2. Stability of Furylmethylketone Isonicotinoylhydrazone in Culture Media at 38°

C: control
K–10: 10% serum Kirchner
K: Kirchner
G–B: glycerol-broth
S: sautone
L: long

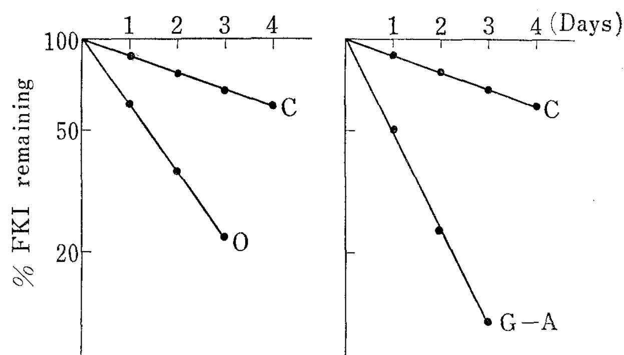

Fig. 3. Stability of Furylmethylketone Isonicotinoylhydrazone in the Culture Media at 38°

C: control
O: Ogawa
G–A: glycerol agar

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cates the stability of FKI in distilled water (pH 6.0). The apparent first-order rate constants k (in min^{-1}) and the half-lives (in day) are listed in Table III.

It is interesting to note that in culture media FKI was hydrolyzed four to ten times as rapidly as in plain buffer solutions. It would be expected that considerable amounts of free isoniazid are released from FKI long before one could expect growth of *Myco. tuberculosis* in culture. A slight stabilization by serum was observed in 10% bovine serum containing Kirchner medium. Less stability of FKI in Long medium would be due to the low pH of the medium Thus it could be postulated that medium components may be responsible for the net increase in the apparent degradation rate constant in culture media.

TABLE III. Stability of FKI in Culture Media at 38°

Media	Half-life (day)	k (min^{-1})	pH
Kirchner	1.20	4.01×10^{-4}	6.35
10% Serum Kirchner	1.45	3.32×10^{-4}	6.35
Sautone	0.95	5.06×10^{-4}	7.35
Long	0.55	8.75×10^{-4}	5.10
Glycerol-Broth	1.15	4.18×10^{-4}	7.10
Glycerol agar	1.00	4.81×10^{-4}	7.10[a]
Ogawa	1.40	3.44×10^{-4}	6.60[a]
Control (in water)	5.50	8.76×10^{-5}	6.00

a) pH measured before solidification

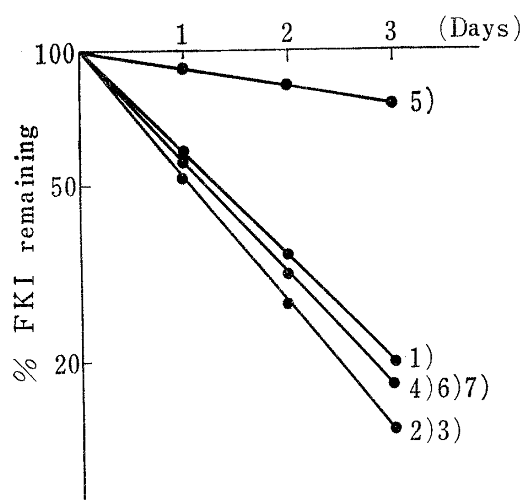

Fig. 4. Effect of Medium Components on the Stability of Furylmethylketone Isonicotinoylhydrazone in Kirchner Medium at 38°

Excluded Component	half-life (day)	pH
1) Control	1.30	6.35
2) KH_2PO_4	1.10	7.10
3) $Na_2HPO_4\cdot12H_2O$	1.10	6.05
4) Na-Citrate	1.25	6.25
5) Asparagine	7.50	6.35
6) Glycerin	1.25	6.35
7) $MgSO_4\cdot7H_2O$	1.25	6.35

Effect of Medium Components on the Stability

With the above in mind, it became desirable to study the effects of medium components on the rate of degradation of FKI in order to understand the enhancement observed in culture media. The medium selected for this phase of the study was Kirchner's. Effect of medium components on the stability of FKI is summarized in Fig. 4, where control stands for complete Kirchner medium. Exclusion of monobasic potassium phosphate and dibasic sodium phosphate resulted in slight change in pH. In other case, this change was negligible. It is obvious from Fig. 4 that asparagine has a pronounced effect on the degradation of FKI. This effect was also noted when asparagine was replaced by sodium glutamate.

Effect of Amino Acids on the Stability

Since amino acids displayed such pronounced catalytic effects on the degradation of FKI, detailed study of their effect on FKI stability was felt needed. Wenzel showed that coupling products were formed when isoniazid was treated with amino acids such as glycine, asparagine,

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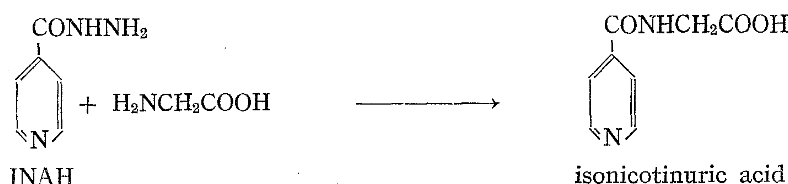

Chart 2. Formation of Isonicotinuric Acid

and glutamic acid in neutral pH region.[9–11] In the case of isoniazid with glycine, for example, isonicotinuric acid is produced as shown in Chart 2. Since isoniazid is the degradative product of FKI it became desirable to determine the effect of amino acid coupling reaction on the overall loss of FKI. This possibility was, however, ruled out by the experiment which employed FKI and glycine as a model coupling agent. From material balance and thin–layer chromatographic data obtained by the modification of the experiment by Diller and his co–workers,[12] it became obvious that no coupling product was formed as given in Fig. 5.

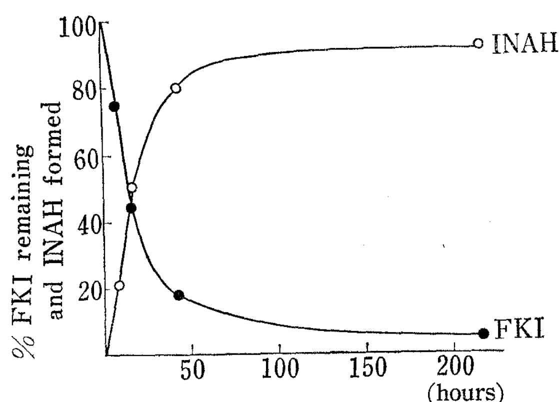

Fig. 5. Relationship between the Degradation of FKI and the Formation of INAH in 0.05M Phosphate Buffer Solution (pH 6.0) with an Addition of Glycine at 37°

The catalytic effect of glycine, asparagine, and sodium glutamate was investigated over the pH range 5.0 through 8.0. At a single pH the observed first–order rate constants were found to be directly proportional to amino acid concentration as shown in Fig. 6. The dependence of the apparent first–order rate constants on the catalytic species is defined for amino acid catalysis by:

$$k_{app}=k_0+C(A)$$

where k_{app} is the apparent first–order rate constant at a given concentration of amino acid, (A), and k_0 is the apparent first–order rate constant for hydrolysis in the absence of amino acid. These values are summarized in Table IV. The values of catalytic constant showed a tendency to increase with pH. This tendency, however, was not so significant between pH 5 and 6. Glycine was the most effective amino acid followed by asparagine and sodium glutamate. Effect of N–substitution was tested with N–methylglycine (sarcosine) and N–acetylglycine (aceturic acid) in the similar manner at pH 7.0. These compounds, as expected, did hardly show such catalytic effect as glycine. On the other hand, glycylglycine showed nearly fourty–five times as greater catalytic effect as glycine. Catalytic constant for aspartic acid was about one–half of asparagine, while glutamine, having two amino groups, was twice as active as sodium glutamate which in turn has only one amino group in a molecule. Accordingly it may be concluded that free amino group of amino acids or peptides is an catalytic species in the degradative reaction of FKI. The ratio of non–protonated to protonated amino group increases with pH thereby causing more pronounced effect in higher pH region as shown in Table IV. This result is consistent with the choice of non–protonated amino group as the primary catalytic species.

9) M. Wenzel, *Naturwiss.*, **42**, 370, 424 (1955).
10) M. Wenzel, *Arzneim.–Forsch.*, **6**, 58 (1956).
11) M. Wenzel, *Arzneim.–Forsch.*, **7**, 662 (1957).
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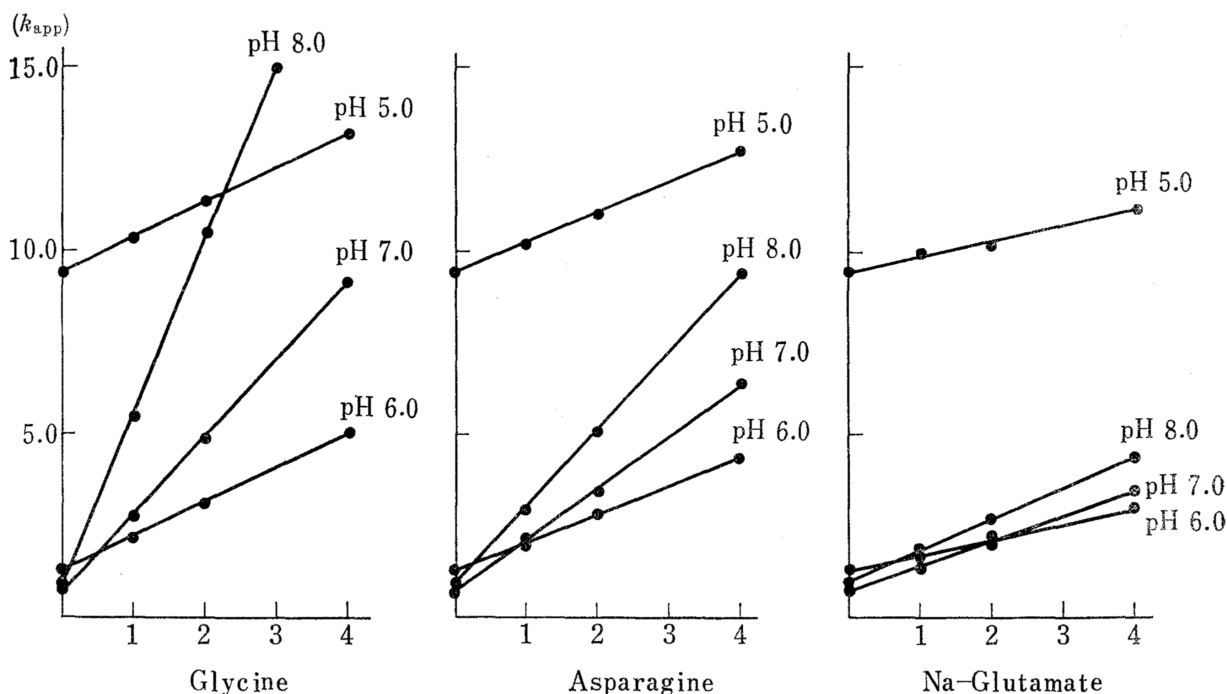

Fig. 6. Effect of Amino Acids on the Stability of FKI in 0.05M Phosphate Buffer Solution at 37°, Apparent Rate Constant k_{app} (10^{-4} min^{-1}) Plotted against Amino Acid Concentration(10^{-2}M/liter)

Table IV. Catalytic Constants for the Degradation of FKI in 0.05 M Phosphate Buffer Solution at 37°

pH	Glycine (min^{-1} M^{-1})	Asparagine (min^{-1} M^{-1})	Na–Glutamate (min^{-1} M^{-1})
5.0	9.3	8.3	4.4
6.0	9.4	8.4	4.6
7.0	20.4	13.4	6.9
8.0	47.8	21.0	8.6

It was described earlier that FKI was stabilized slightly in 10% serum containing Kirchner medium. Equilibrium dialysis experiments ruled out the possibility of stabilization by protein binding of FKI thus reducing the thermodynamic activity of the compound. Stabilizing effect of serum would be better explained by the decrease of free asparagine due to its binding to serum protein.

Since amino acids like asparagine and glutamic acid are indispensable as nitrogen source in most of the culture media for the growth of *Myco. tuberculosis* and other testing microorganisms and since drugs under investigation are usually kept in such media for a considerable period of time in a very dilute concentration, this pronounced catalytic effect of the medium components should be taken into account seriously in the analysis of *in vitro* activity data of anti-bacterial agents. Unless such stability problem or interactions are taken into consideration, *in vitro* results will bear little relationship to intrinsic activities of tested compounds and the test does not predict the potential efficacy of the drug, but rather may relate their stability in culture media.

Studies are currently in progress, to evaluate quantitatively the relationship between the stability in culture media of various anti-tubercular agents and their activity *per se* on *Bacillus Calmette-Guérin* (*BCG*).

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