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A Review on In-vitro Bioequivalence Studies and its Methodologies

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Abstract: Nowadays choosing a perfect alternate for an original drug and maintaining its bioavailability plays important role in the pharmaceutical industry. Bioequivalence is conducted for the comparison of different formulations of a same drug. Dissolution test is a simple and an important tool for assessing bioequivalence. The main aim of this review is to study the *in-vitro* bioequivalence, its associated parameters and compile the research works carried out in this field.
Key words: bioequivalence, *in-vitro* and *in-vivo*, dissolution, fit factor, biowaiver.

Introduction:

Bioequivalence is the study of different brands of a same drug and its dosage forms. Two different formulations of a same drug are bioequivalent when their rate of dissolution and absorption is same. As there is an increase in production and consumption of generic drugs, the need for bioequivalence study is also rising. Nowadays drug's cost increases due to the expensive original drug. This cost can be reduced by substituting cheaper generic copies. For this, generic copy should be therapeutically equivalent to the original drug. In order to find this, bioequivalent studies are conducted. According to the Food and Drug Administration (FDA) [(1)] bioequivalence is defined as:

"the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study".

Two ways are followed widely to conduct a bioequivalence study, *in-vitro* and *in-vivo* bioequivalence study. *In-vivo* bioequivalence study is usually carried out in human and animal subjects by measuring the rate and extent of drug absorption in the blood stream after a drug has been administered. *In-vivo* study gives highly reliable information but there are many parameters which cannot be controlled. Moreover living organisms exhibit greater variability. So we need to go for a number of trials and cost also matters.

In-vitro bioequivalence study is carried out in dissolution apparatus. All the necessary biological conditions are provided and samples are collected and assayed periodically. By performing *in-vitro* studies we can have a control over the system. It also allows to mimic the biological conditions. *In-vitro* studies reduce the cost and number of trials. It also offers benefits in terms of ethical considerations and drug performance. Biowaiver is an exemption granted by the US FDA to conduct *in-vivo* bioequivalence studies. It implies that it is not necessary for generic products to conduct the *in-vivo* studies for product approval. Instead a dissolution test can be adopted. Biowaiver can be suggested only for solid, orally administered immediate release products (85% release in 30 min), containing highly soluble drugs over a pH range of 1 to 7.5. For a bioequivalence study

of a waiver, the test and reference product should exhibit similar dissolution profile (f2 >50). However, it is not applicable for buccal, sublingual, oral dispersion and modified release formulations. Biowaiver reduces the cost of bringing new products to the market. It has a major advantage in reducing the time for approval of a product.

Tests conducted to study *in-vitro* bioequivalence:

Uniformity of content:

In order to conduct bioequivalence study it is important to know if there are variations in % content of active ingredients. To check whether a tablet contains a proper amount of drug, % content of drug should be routinely measured. Analysis of drug potency in tablets indicates the presence of drug in dosage form and their stability. The content uniformity test has been included in the monographs for all dosage forms and the samples of tablets are selected and assayed individually. Maximum tablets must have assay content within ±15% of the declared potency and should not exceed ±25%. Uniformity of weight ensures consistency of dosage units during compression [(2)(3)].

Weight variation:

Factors that affect tablet weight includes tooling of the compression machine, head pressure, machine speed and flow properties of the powder. Weight variation is calculated by taking twenty tablets from each brand. An analytical weighing balance is usually used for weighing the tablets. From the mean value average weights for each brand and percentage deviation were calculated. According to pharmacopoeia, not more than two of the individual weights should deviate from the average weight [(2)(3)] .

Hardness:

Hardness test is important as it determines the resistance of the tablet to chipping, abrasion, or breakage during storage, transportation and handling before usage. Space between the upper and lower punches at the time of compression, weight of the material used and pressure applied during compression affect the hardness of the tablet. Different types of apparatus used for measuring hardness are as follows: Monsanto or Stokes hardness tester, Pfizer hardness tester, Strong cob hardness tester and Heberlain or Schleeniger hardness tester [(2)(3)].

Friability:

Friability is a phenomenon where surface of tablet is damaged or shows a site of damage due to mechanical shock [(2)(3)]. This test is performed to make sure that the edges of tablet do not break away. Apparatus used is Roche friabilator. Initial weight (W_1) of randomly chosen 20 tablets is calculated. After subjecting the tablets to the friabilator for 4 min at 25 rpm, the final weight (W_2) is calculated. % loss is determined using the formula

$$\% \text{ Friability} = ((W_1-W_2)/W_1) *100$$

Disintegration:

Disintegration study is important for the evaluation of drug release. To find time taken for the tablets or capsules to completely disintegrate, disintegration test is performed. Earlier in order to find the uniformity of compression characteristics, disintegration test is employed. Nowadays for the optimization of compression characteristics we prefer this test. If disintegration time is too high, the tablet is highly compressed or capsule shell gelatine is not of required quality. If disintegration time is not uniform it results in lack of batch uniformity and batch inconsistency. There are different types of disintegration apparatus for different drugs but principle and construction remains the same. The apparatus consist of basket with six tubes of equal diameter. A wire mesh is fixed to each of these tubes. Reciprocating motor is used for the movement of the basket. The entire assembly is kept immersed in a vessel containing the medium in which the test is carried out [(2)(3)(4)] .

Dissolution test:

The dosage effectiveness depends on the amount of drug dissolving in the body fluids and its absorption into the systemic circulation. Thus it is important to calculate the dissolution rate of a dosage form. In a dissolution apparatus biological conditions are maintained by providing appropriate dissolution media and

temperature with the help of thermostat. Samples are withdrawn at regular intervals. In order to maintain sink conditions an equal amount of media is added. Assays are carried out accordingly. For a successful dissolution test, selection of dissolution media, apparatus and agitation rate plays an important role[5]. Dissolution test is conducted for

- Optimization of therapeutic effectiveness during product development and stability assessment.
- Routine assessment of production quality to ensure uniformity between production lots.
- Assessment of 'bioequivalence'.
- Prediction of *in-vivo* availability, i.e. bioavailability (where applicable).

Different types of apparatus are employed for this purpose as per the Pharmacopeia for different dosage forms [2][3][4]. They are given below:

DISSOLUTION APPARATUS AND DETAIL AS PER USP[3]		
APPARATUS	NAME	DRUG PRODUCT
Apparatus I	Rotating basket	Tablets
Apparatus II	Paddle	Tablets. capsules modified drug products
Apparatus III	Reciprocating cylinder	Extended-release drug products.
Apparatus IV	Flow cell	Drug products containing low-water-soluble drug
Apparatus V	Paddle over disk	Transdermal drug products.
Apparatus VI	Cylinder	Transdermal drug products.
Apparatus VII	Reciprocating disk	Extended-release drug products

Analytical parameters:

According to the Pharmacopoeial limits, the amount of drug dissolution should be more than 80% of the labelled amount during the first 30 min. When the drug dissolution is more than 85% within 15 min, further mathematical evaluations are not necessary. For those that did not meet the criteria mathematical evaluations were used to demonstrate bioequivalence. The mathematical evaluation involves the calculation of fit factor (similarity and dissimilarity factors), dissolution efficiency, correlation coefficient, ANOVA test and Dunnett's test.

Fit factor:

Similarity factor f_2:

For comparison of *in-vitro* dissolution profiles, similarity and difference factors are emphasized by US FDA. As the name specifies, similarity factor (f_2) stresses on the comparison of closeness of two comparative formulations. The f_2 parameter is commonly used to establish similarity of two dissolution profiles .The FDA defines Similarity factor as " the logarithmic reciprocal square root transformation of one plus the mean squared (the average sum of squares) differences of drug percent dissolved between the test and the reference products". The formula to find similarity factor is as follows [6]:

$$f_2 = 50 + \log \{[1+ (1/n) \sum_{t=1} * n (R_t-T_t)^2]^{-0.5} \times 100\}$$

R_t and T_t are the cumulative percentage dissolved at each of the selected n time points of the reference and test product respectively. Two profiles are considered to be same when f_2=100. f_2 value results in 50 at an average difference of 10% for all measured time points. f_2 value between 50-100 indicates similarity between two dissolution profiles. In other words if difference at each sampling time is less than or equal to 10% f_2 value will be between 50 and 100. Therefore, a quick way to establish similarly of two profiles is to see if differences in dissolution results at each sampling time are less than 10%. The limit of 50-100 range for f_2 may be in conflict with the commonly accepted pharmacopoeia (e.g. USP) standards, where the acceptable deviation is significantly higher than 10%. f_2 is suggested for evaluating products with formulation and to find out whether there exists any manufacturing differences. f_2 is 100 when two comparative groups of reference and test are identical. F_2 becomes zero as the dissimilarity increases[7].

Dissimilarity factor (f_1):

Dissimilarity factor focuses on the difference in percent dissolved between reference and test at various time intervals. Therefore the factors directly compare the difference between percent drug dissolved per unit time for a test and a reference product. f_1 factor is used to calculate the approximate % error in drug release profile. f_1 should be between 0 and 15. Dissimilarity factor f_1 is given as [6]

$$f_1 = \{[\Sigma_{t=1}^{n} |R_t - T_t|] / [\Sigma_{t=1}^{n} R_t]\} \times 100.$$

The criteria stated by US FDA [1] for dissolution profile are as follows:

1. The dissolution profiles can be compared only when the number of dissolution unit used is equal to or greater than 12. f_2 should be computed from average mean dissolution data of 12 units.

2. For accurate calculation of similarity factor, statistical approach of establishment of confidence intervals to determine whether the reference and test are statistically significant or not may be used.

3. The dissolution conditions should be identical for both reference and test products like the strength of dosage form, test time intervals, temperature, rpm, total test time etc.

4. The literature also states to consider only one time after 85% dissolution of product, since f_2 values are sensitive to number of dissolution time points.

5. For rapid dissolving products, that may dissolve 85% in 15 minutes, comparison of dissolution profiles is not mandatory.

6. Similarity factor of 50-100 ensures sameness of two products.

7. Difference factor of 0-15 ensures minor difference between two products.

Therefore prior to *in-vivo* study, comparison of *in-vitro* dissolution profiles using fit factors may be the promising surrogate. Though calculating Similarity factor for comparison of dissolution profile is important, it didn't account the issue of variability in dissolution data[8].These methods are useful in formulation development also.

Dissolution efficiency:

Dissolution efficiency is the area under the dissolution curve within a time range ($t_1 - t_2$) expressed as a percentage of the dissolution curve at maximum dissolution Y_{100}, over the same time frame [9].

$$DE = \frac{\int_0^T Y\,dt}{Y100 \times T} \times 100\%$$

Research articles related to *in-vitro* bioequivalence studies:

In the paper published by Tanjinatus Shams *et al.*, [10] ten generic Atorvastatin tablets (10 mg tablets coded from A to J) from different manufacturers were used for the *in-vitro* bioequivalence study. *In-vitro* drug diffusion study was also carried out for brand A and D. For this study, Atorvastatin solution in methanol and Atorvastatin dispersed in water were compared. Brand C and E did not comply uniformity of weight test. From dissolution profile, both brand to brand and within a brand variations were observed. From the *in-vitro* diffusion study it was found that membrane permeability rate was proportional to the *in-vitro* dissolution rate. Brand D whose *in-vitro* dissolution rate was higher passed the membrane quickly. From the calculation of fit factor it was found that all brands are similar with brand D and can be used interchangeably. Brand D showed high dissolution efficiency. Dissolution efficiency of B, C, I and H are more or less similar to brand D and can be considered as interchangeable.

C.O. Esimonea *et al.*, published a paper on i*n-vitro* bioequivalence study of nine brands of Artesunate tablets marketed in Nigeria [11]. In this paper eight brands of Artesunate tablet was compared with reference drug (AT_1 to AT_8 and AT_9 is ref). The paper aims at studying the comparison of T_{50} (50% dissolution time), T_{90} (90% dissolution time) and similarity factor f_2. Three tests were conducted, crushing, disintegration and dissolution test. Both crushing and disintegration tests were analysed using t test. Dissolution media used was SIF. Four brands, AT_5, AT_6, AT_7 andAT_8 exhibited more than 90% dissolution in less than 10 min where as AT_1, AT_2, AT_3, AT_4 and the innovator drug, AT_9 dissolution rate was less than 85% so further mathematical evaluation was conducted for these five brands to demonstrate bioequivalence. The T_{50} and T_{90} values of AT_2, AT_3, AT_4

and AT_9 were similar. AT_1 had a T_{50} value of 30 min and for T_{90} it was greater than 120 min. this indicates poor dissolution, hence poor bio availability of AT_1. Based on the calculated similarity factor AT_2, AT_3 and AT_4 were bioequivalent with AT_9. AT_5, AT_6, AT_7 and AT_8 can be interchanged in clinical settings. The disintegration time of AT_3 was found to be low but it gave a high dissolution rate. Except AT_1 other brands showed good bioequivalence and hence good bio availability

H.N.Tense and M.A.Ibrahim studied *in-vitro* bio equivalence of different brands of Chloroquine phosphate syrup commercially available in Nigeria [(12)]. Five brands of immediate release Chloroquine phosphate syrup were studied. In this paper three tests were conducted. Chloroquine phosphate content was varied. % absorbed vs time profile was found to be more appropriate than concentrate or amount absorbed vs time. Brands 1 and 2 were found to follow first order absorption kinetics while brands 3 and 4 followed zero order kinetics. Brand 5 did not seem to follow either first or second order kinetics. It has been reported that the 5 brands of Chloroquine phosphate syrup are not bioequivalent. This result point out the importance of bioequivalence studies for different brands of the same drug for drug regulatory agencies.

Parvin Zakeri-Milani *et al.,* took eight brands of Metformin tablets for their study [(13)]. They were subjected to various tests to evaluate bioequivalence. Content uniformity, average weight, SD, RSD and acceptance value for each brand were calculated. The requirement for dosage uniformity was met and all drugs were in acceptable value range i.e less than 15%. Dissolution apparatus with paddle type was used as per USP. Media was 900ml phosphate buffer. After 100 fold dilution samples were assayed using UV. The concentration was determined from the calibration curve. All brands except C released 80% of the drug in 30 min.

N. C. Ngwuluka *et al.,* published a paper [(14)] on the *in-vitro* bioequivalence of Ciprofloxain. Six different brands of Ciprofloxacin each 500mg, available in Nigeria were taken for the study. Pure Ciprofloxacin HCl powder was considered as reference. Various tests were conducted to study bioequivalence of Ciprofloxacin. All brands were in accordance with USP specification for assay while brands C, D and F did not meet the BP standard. As per BP uncoated should disintegrate within 15 min and film coated within 30 min. As per USP both uncoated and film should disintegrate within 30 min. Brands A, C, D and E were uncoated while B and F were film coated. Dissolution test, as per USP basket method was employed. B released 92% of drug at 15 min. The amount released by other brands is less than 85%. The percentage dissolved was calculated using one-way analysis of variance (ANOVA) and Dunnett's test. Pair wise comparison of A, C, D and E against B were carried out. Dissolution efficiency was also performed. The dissolution profiles of A and E were found to be similar and most probably bioequivalent to B and can be used interchangeably.

In this paper *in-vitro* bioequivalence of Sulfisoxazole tablets were published by V.P.Pandey and J.K.Pandit [(15)]. Four fabricated tablet dosage forms (2 chewable type c1 & c2 and two swallow type s1 & s2) of Sulfisoxazole were compared with one commercial tablet (M). Hardness, friability, disintegration, weight variation and dissolution tests were carried out. Though S1 hardness was lower than reference, integration time was almost equal for both, which indicated that hardness was not a control factor. S2 with a slightly higher hardness resulted in an increased disintegration time. S2 was comparable with reference tablet in dissolution characteristic. S1 showed much lower disintegration time than S2.

Ramu.A and Srinivasa Babu published a paper on *in-vitro* evaluation of Glimepride tablets [(16)]. Four brands of Glimepride GM1-GM4 (each of 2mg) were used for this study and 1 pure Glimepride for reference. Various test like weight variation, hardness test, friability test, content of active ingredients, disintegration test and dissolution test were conducted. Dissolution test was performed in a paddle type dissolution apparatus at 50 rpm. This paper made use of three types of dissolution media: 0.1NHCl, pH 7.4 phosphate buffer, 0.5% sodium lauryl sulphate with distilled water. Friability value was found to be less than 1% in all cases. GM2 had greater release rate then other (order: GM2, GM3, GM4, GM1). Drug release was faster in 0.5% SLS with distilled water media. All the dissolution parameters of Glimepride brands were higher than pure drug.

Anagha Joshi *et al.,* published a paper on the effect of alcoholic beverages on sustained release formulations [(17)]. They studied how alcoholic consumption affects the sustained and instant release of Metformin and Diclofenac. Four alcoholic beverages namely Kingfisher strong (500ml) and mild beer (500ml), rum (60ml), 40% alcohol (15ml) were used for this study. Dissolution study was conducted for both drugs. For Metformin, the volume of alcoholic beverage affected the instant release where as sustained release was affected by strength of the alcoholic beverage. Sustained release form of Diclofenac showed a similar release profile. The release of instant release formulation was quicker in Kingfisher strong beer, mild beer, Rum and 40% alcohol then in water. For sustained release formulation, release was faster in 40% alcohol and in rum than in

water. This study proved that the consumption of alcoholic beverages during treatment with sustained release formulations affects the dissolution profile and leads to some side effects like dose dumping.

Ashraful Islam *et al.,* published a paper on the bioequivalence study of Aceclofenac tablets in two different dissolution media [(18)]. In this paper five brands of Aceclofenac drug each of 100mg is taken and formulated for dissolution study. They were analysed by using UV spectroscopy and HPLC. Assays were performed from which the potency is calculated of the peak area appeared. For assaying 20 tablets were taken and weighed powdered and dissolved. The buffer used to dissolve is phosphate buffer of 100ml at pH6.8.It was shaken for 10min and sonicate d for 5mins.Then the solution was diluted upto 100ml and again diluted to 12mcg/ml. After sonication dissolution test was done using paddle type dissolution tester. Phosphate buffer and sodium lauryl sulphate were used as the dissolution media. HPLC shows positive as it gives similar peak for both standard and sample and it is highly selective. It was observed that the use of 900ml of pH6.8 phosphate buffer at 37+/-0.5 degrees with a paddle speed of 50 rpm for 1hr gives the best result for the formulation of Aceclofenac.

Nifedipine is an important drug used for the treatment of cardiovascular disorder. R. Panchagnula *et al.*, studied the bioequivalence of nine brands of Nifedipine available in Indian market [(19)]. They were designated as N1 to N9. Tests conducted include weight variation, friability, dissolution, assay and hardness. Dissolution study was conducted using paddle type dissolution apparatus. Media used was 900ml phosphate buffer (pH 6.8) which contained 1% SLS. The influence of pH was also studied using different pH levels of pH 2.0, 5.0 and 7.4. Dissolution profile was studied using fit factors and compared with the dissolution profile of NIPER formulation. Results from dissolution studies showed that N1 had a lag of 2hr for initiation of drug release. N6 and N8 showed almost 80% of drug release in 1 hr while N3, N4, N5, N7 and N9 had a sustained release. Only N1 and N7 were subjected to further study. From the dissolution data N1 followed both zero and first order kinetics while N7 followed first order kinetics. Finally it was concluded that N1 and NIPER were superior to other marketed drugs.

Olubukola O. *et al.*, conducted the *in-vitro* equivalence studies of generic Metformin hydrochloride tablets and Propranolol hydrochloride tablets under biowaiver conditions in Lagos State, Nigeria [(20)]. The drug samples taken were all conventional, immediate-release, solid oral dosage forms. The innovator products, Inderal for Propranolol hydrochloride 40-mg tablets and Glucophage for Metformin hydrochloride 500-mg tablets were used. Two generic versions of Propranolol hydrochloride 40-mg tablets and four of Metformin hydrochloride 500-mg tablets were used. Propranolol hydrochloride and Metformin hydrochloride were the reference standards. Active contents of generic and innovator brands were assessed as per BP. For the dissolution test the following media was employed: 0.01 N HCl acid (pH 2), phosphate buffer (pH 4.5), and phosphate buffer (pH 6.8). The assay result for samples of Propranolol hydrochloride tablets were within *BP* limits. Propranolol hydrochloride tablets of both the generic and innovator products were rapidly dissolving releasing more than 85% of the labelled amount within 30 min. None of the four brands of Metformin tested met the condition of dissolution of 85% or more within 15 min. But the generic form had over 85% dissolution within 15 min in the three media. Two generic products of Propranolol HCl were compared with the innovator product. For comparison of the dissolution profiles, similarity factor (f_2) was used. From the f_2 values it was found that all generic samples were not similar to the innovator product. The study concluded by saying that the generic products assessed did not qualify for biowaiver. Therefore *in-vitro* dissolution studies for bioequivalence should be performed for regulatory purposes so that the manufacturers can consider the factors that affect solubility and permeability of their products while formulating them.

Patrico JP *et al.,* published a paper on *in-vitro* dissolution profile of two commercially available iron preparations [(21)]. The paper presented the *in-vitro* dissolution study of two similar commercially available formulations of iron- and folic acid-containing supplements, Folifer® (Bialport – Produtos Farmacêuticos, S.A., Portugal) and Ferroliver® (SM Pharma c.a., Venezuela). These two were selected because they had similar amounts of elemental iron. Three dissolution media replicating intestinal juices were used. pH range was from 1.5 to 6.9. The release of iron from each tablet was evaluated over a 4-hour period. Titrations of samples were carried out by treatment with cerium ammonium sulphate in order to determine the rate of iron released under different pH conditions. % of dissolved iron was calculated as a cumulative frequency. Dissolution similarity was evaluated using the f_2 statistic formula. The paper concludes by saying that Folifer® releases more iron than Ferroliver® and they were not equivalent.

Pharmaceutical evaluation of different brands of Levofloxacin tablets (250 mg) available in local market of Karachi (Pakistan) was studied by Raheela ban *et al.,*[(22)] For this study, six brands of Levofloxacin

designated as Levo 1 to Levo 6 were used. It was observed that all the six tablets were in accordance with USP and BP. Levo 6 was found to be thicker than all other tablets under study. The results of the assay of chemical content of Levofloxacin tablets showed that the active content in all formulations were between 95% and 105% of the labelled amount. From the statistical evaluation (ANOVA) it was observed that there was no significant variation in content of active moiety. From this paper it was evident that the variation in the weight and thickness of the tablets play a key role in the compliance of patients. At the same time they have no meaning when the thickness of the tablets was adjusted accordingly. The amount of Levofloxacin in different brands was between 95 to 105% which indicate that the amount of active ingredient in each brand was within the pharmacopoeia limits.

A paper was published by A.R. Chandrasekaran *et al.*,[23] based on the *in-vitro* bioequivalence evaluation of Paracetamol tablets. Six brands of Paracetamol 500 mg tablets were taken for this study. Weight variation, hardness, friability, disintegration time was carried out according to USP and BP limits. Dissolution test was demonstrated using USP apparatus I with dissolution media phosphate buffer of pH 5.8. Brands B, C, D, E and F were readily available in solution. All the dissolution characteristics of six brands appear to be similar and there was no significant difference from various manufactures.

Bioequivalence of twelve brands of Sulphadoxine Pyrimethamine in Nigera was studied by A. Ochekpe1*et al*,.[24] The study was conducted using three different dissolution media. 0.1 N HCl and phosphate buffers at pH 4.5 and 6.8 were the dissolution media used. Dissolution apparatus used for this study was dissolution test apparatus II. According to the USP specification, in pH 6.8 phosphate buffer the drug release in the media after 30 min should be more than 60%. For brands 2, 4, 5, 7, 8, 9, and 10 the release of both active ingredients were more than 60% while for Brand 1 drug release was less than 50% and Brand 11 showed less than 20% after 60 min. From the *f*1 and *f*2 values brands 2, 5, 7, 8, and 10 were similar to the reference product.

Conclusion:

In the current industrial practice, to compare with the multi brand generic molecules and to provide enough therapeutic activity of the dosage form, *in-vitro* bioequivalence plays an important role. In this review article we have compiled the information on *in-vitro* bioequivalence studies along with its importance, various methods involved in bioequivalence studies which is suitable for both institutional and industrial practice as per the Pharmacopeia limits and requirements. The post marketing *in-vitro* bioequivalence studies of different brands provides the significant information regarding the quality of the different brands. It is confirmed by the result published by various authors with different generic molecules and compared with different brands. With this we would conclude that *in-vitro* bioequivalence studies are needed and can make an impact on the dosage form manufacturing companies.

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