The Survival of Different Fungal Spores During Tabletting

Raghad A.Al-Shikli[*,1] , Alaa A.Abdul-Rasool[**] and Mustafa M. Al-Hiti[*]

[*] Department of Clinical Laboratory Science,College of Pharmacy, University of Baghdad, Baghdad, Iraq.
[**]Department of Pharmaceutics, College of Pharmacy, University of Baghdad, Baghdad, Iraq.

Abstract

The survival of dried spores of A.flavus, Penicillia Spp., and Cladosporia Spp.inoculated into multivitamins and folic acid tablets were examined at different compression pressures.Survival of fungal spores decreased with increasing compression pressure. The level of survival at particular pressures was shown to depend on the size of the contaminating fungal spores.The lethal effect of tabletting was attributed to shearing forces upon the contaminating spores generated by interparticulate movement. This hypothesis was supported by the dependence of survival upon spore size.

Key words: Fungal spores, microbial contamination.

الخلاصة

لقد تم تصنيع نوعين من الأقراص الدوائية الصيدلانية وهي حامض الفولك ومجموعة الفيتامينات وتم إدخال ثلاثة انواع من الفطريات وهي بنيسيلية-اسبار جلس كلادوسبورية وبتركيزين ١٠^٢, ١٠^٤ سبور/غرام وتم كبسها بطريقة الكبس المباشر تحت ضغوط مختلفة وقد تبين إن زيادة الضغط تؤدي الى نقصان في عدد السبورات وان معدل البقاء تحت ضغط معين يعتمد على حجم البوغ الملوث.التأثير القاتل لtabletting نُسِبَ إلى قَصّ القواتِ على بويغاتِ الثلويث ولَدَ بحركةِ interparticulate. هذه الفرضيةِ كانتْ مدعومة من قبل إعتمادِ البقاء على حجم البويغةِ.

Introduction

Microbiologically contaminated tablets may cause disease and under humid storage conditions lead to visible deterioration of the tablet.Various workers have examined the effect of compaction process upon natural contaminants present in the granulate.In all cases, compression of granules to significant reduction in the levels of microbial contamination. [1- 3]Tabletting machines exert some antimicrobial effect . The shearing forces and localized heat involved in pressing tablets is sufficient to destroy many mould spores and vegetative organisms, although Bacillus spores appear to survive. Reduction (67-93)% in total viable counts of dry blended product has been reported to be produced by tabletting [(1)]Increasing compression pressures and tabletting speed enhance the anti microbial effect[(4-8.)] .Fassihi and Parker[(9)]granulated lactose powder using a granulating fluid containing A. niger spores .They demonstrated a linear relationship between the log survival and the applied pressure (28-271 MN/m2). Such linearity was not observed by Yanagita et al. [(10)] for Rhodotorula glutinins, E.coil and bacillus subtilis contaminated crystalline cellulose.plumpton[(11)] found that the levels of survival within four formulations were similar for a given organism, yet patterns emerged according to the mechanism of compaction of the material.Survival following compaction in lactose and emdex , which compact by a process of fracture, was inversely related to pressure over the entire range tested , whilst for sta-Rx and potassium chloride such relationship was only exhibited up to compaction pressure of (194 MN/m2). At low compaction pressure (0-39 MN/m2)there was a little inactivation of the microorganisms in any of the four formulations. Also Plumpton[(11)] found that levels of survival were inversely related to the size of the organisms for any given pressure, survival being greatest for B.megaterium spores (cell dameter approximately 1.5µm), intermediary for A.niger spores (4µdiameter) and least for S.cerevisiae (mean cell diameter approximately 8 µm). Similar trends in survival according to cell size were suggested from the work of Yanagiitu et al. [(10).] These workers examined the survival during compaction of vegetative cells of E.coli and R.glutinis and spores of B.subtillis within an Avicel/skin milk formulation. B.subitillis spores were found to be highly resistant to tabletting. Where as vegetative cellsof R.glutinis were much more sensitive to tabletting than those of E.coil. Blair[(7)] found that extent of kill on tablet compression was great for the larger organism, E.cloacase, on studying separate batches of lactose monohydrate , maize starch and cellulose

[1] Corresponding author E- mail : raghadrazook@yahoo.com
Received : 28 / 12 / 2008
Accepted : 31 / 5 / 2009

microcrystalline contaminated with Staphylococus aureus or Enterbacter cloacae and tabletted at various compression force,indicating that size is important and cell rupture by shear,rather than heat,is the mechanism of kill.In this study,we examined the effect of tabletting on survival of dried spores of A.flavus,Penicillia Spp. and Cladosporia Spp. inoculated into the multi vitamines and folic acid tabletsduring compression with two levels (10^2 and 10^4)spore/gram at three compression pressure and one conpression pressure (10^2and10^4) spore/gram respectively.

Materials, instruments and methods

Chemicals

Acetonitrile ,Acetone, Ammonium Hydroxide ,Anhydrous Sodium Sultate , Benzene, Chloroform, Glacial Acetic Acid ,Hydrous Disodium Hydrogen Phosphate (Na2Hpo4.12H2o), , Methanol, Potassium Hydroxide, Potassium Chloride, Sulfuric Acid, Sodium Hydroxide, Sodum 1-Hexane Sulfonate, Sodium Perchlorate, Sodium Chloride, and Tween 80 (Polysorbate 80) Suppied by BDY England. Monobasic Potassium Phosphate From Fluka-Swizerland. Heexan supplied by Merch-W. Germany. Microcrystalline cellulose (Avicel pH 101, Avicel pH 301), Folic Acid, Maize Starch, Vitamin B1 (Thiamin mononitrite), Vitamin B2 (Riboflavin),Vitamin B6 (pyridoxine Hcl), Methionine, Talc and Magnesium Stearate supplied by FMC coproation and Kindly supplied from (Sammara Drug Industires SDI. Iraq).

Microorganisms

Aspergillus flavus , penicillia Spp. and Cladospria Cladosporoids were obtained from College of Agriculture ,University of Baghdad. Culatures were stored on Sabouared Ager slants following incubation at 25°C for 5 days. Fresh cultures were prepared every 4 weeks .

Culture media

Sabouraud Dextrose Agar.
RoseBengal Agar
MacConkey Broth

Solution used for dilutions and preparation of spore suspension

Relative Humidity of the prepared solutions .
Extraction Solvent of Aflatoxin.
Relative Humidity Containers .

Tablet formulations

Prepation of dried spore powder

0.1 ml aliquots of 7days cultures of A.flavus, Cladosporia and Penicillia were inoculated onto the surfaces of predied sabourad dextrose agar plates, incubated at 25°C for 5days. After this time spores were clearly visible on all the plates. Three ml of sterile water containing 0.1%tween 80 (as a dispersin agent) Were add to each plate . the spores were then dislodged by using glass spreader. The spore suspensions obtained were then stirred by using a vortex mixer for one minute. The spore suspension were then filtered through a sterile cotton wool in order to get rid of the hypha. The filtrates were then harvested by centrifugation at (10,000xy for10 min). The supernatant liquids were then decanted and the residues were resuspended in 20 ml of sterile distilled water and washing were repeated three times. The number of spores of the resultant sopre suspensions was determined by viable count technique, spore suspensions were adjusted to obtain $2.16x10^6$ spore/ml for A.flavus, $1.68x10^6$ spore/ml for Penicillia Spp. and $1.98x10^6$ spore/ml for C. cladsporoids. Multivitamin tablets and folic acid tablets were prepared using the formulas listed in tables (1) and (2), respectively . The following excipient were used Avicel pH 301 as a direct compression excipient, starch (5% w/w)as a disintegrant, magnesium stearate (0.5%)and stearic acid (2% w/w) as lubricants, were mixed with the active ingredients and compressed directly using a single punch tabletting machine with 7-mm flat –faced punches.

Table 1 : The formula of the prepared folic acid tablet

Ingredient	Amount / tab
Folic acid	1mg
Avicel pH 301	118.6 mg
Maize starch	6.5 mg
Mg.stearte	2.6 mg
Talc	1.3 mg
Total	130 mg

Table 2 : The formula of the prepared multivitamin tablet

Ingredient	Amount/tab
Thiamin Mononitrite	1.5 mg
Riboflavin U.S.P	20 mg
Pyridoxine Hcl U.S.P	2.0 mg
Methionine	2.0 mg
Avicel pH 301	105 mg
Maize Starch	6.5 mg
Talc U.S.P	6 mg
Stearic acid (powdered)	3.0 mg
Mg.Stearte (powdered)	2.0 mg
Total	130 mg

Preparation of contaminated tablets

For preparation of 500 contaminated tablets a(0.3 ml, 30µl) of A.flavus .(0.4 ml 40µl) of penicillia Spp and (0.3 ml, 30 µl) of C. cladosporiods were transferred to a sterile mortars and placed in the incubator until completely evaporation of water .Dried spores were scraped off and were included in direct compression formulation by dry mixing to 102 get spore / gram and 104 spore / gram for each of A.flavus, penicillia Spp. and C. cladosporids respectively. Ingredients including dried microorganisms spores weighed and lightly mixed in a glass morter by the method of geometric dilution technique for 20 min. Preliminary experiments had established that this method gives a uniform distribution of the microorganisms within the formulation , screen in the lubricant (magnesium stearte or stearic acid) and mixed for an additional 5 minutes. Quantities each of 130mg were accurately weighed and poured into 7-mm diameter die.

Determination of viable number of spores in the prepared tablets

Viable number of spores in prepared tablets was determined immediately after their production at different compression forces and after storage up to 8 weeks. Eight tablets (total wt.=1gm) were disintegrated in trypic soy broth (9ml) according to B.P 98 using a flask and suitable serial dilution in tryptic soy broth were prepared. One- ml sample of each dilution was poured in a sterile petridish and then 15 ml of molten dextrose agar was added to the plate. The sample and molten sabouraud dextrose agar were mixed together in forward and backward movement and swirled movement. The plates were allowed to solidify on a leveled surface. The plates were incubated at 35°C for 2-5 days. Survivals as colony forming units were estimated as the mean of triplicate determinations and expressed as a percentage relative to an uncompressed control sample of the contaminated formulations.

Physical properties of tablets

The result of physical properties of tablets are shown in table (3). Tablet weight and thickness, friability, hardness and disintegration time were measured.

Table 3 : Physical ,Chemical and Microbiological (Control) evaluation of folic acid and multivitamin tablet

Tablet evaluation	Multivitamin	Folic acid
Weight (7min)	**130 mg**	**130 mg**
C.pressure		148.3MN/m^3
Wt.Uniformity	0.9%	0.9%
Hardness (Kp)	6.6 ± 0.4	6.5 ± 0.6
Thickness (mm)	2.85	2.65
Friability %	0.2	0.2
Disintegration time	2.3 min	2 min
Assay		
B_6 Pyridoxine %	133.76%	
B_1 Thiamin %	148.2%	
Folic acid %		905
Microbiological Quality		
One day after preparation		Less than 10 *CFU/gm
After storage for 8 week at 35 °C 75 % RH		Less than 10 *CFU/gm
After storage for 8 week at 35 °C 85 % RH		Less than 10 *CFU/gm
After storage for 8 week at 35 °C 95 % RH		Less than 10 *CFU/gm

Interrelation of compression pressure and survival of A. flavus, penicillia and Cladosporia spores in multivitamin and folic acid tablets

Multivitamin formulation were contaminated with A.flavus spores or cladosporium spores or penicillia spores using 102 and 104 spore/gm. Tablets were prepared at a variety of compression pressure (137.9, 144.8, and 148.3) MN/m2 and the number of survival was determined immediately upon ejection of tablets from the die. The number of survival was plotted as logarithmic function of compression pressure.

Results and Discussion

Tablet evaluation

Folic acid and multivitamin tablets were prepared as previously mentioned (Tables 1 and 2, respectively). The prepared folic acid and multivitamin tablets were evaluated physically, chemically and microbiologically. The results areshown in table 3.

Interrelation of compaction pressure and survival of A. flavus, penicillia and cladosporia spores in multivitamin and folic acid tablets

The results of the effect of compression force upon percent survival are shown in figure(1) and table (4a)for a contamination level of 102 spore/gm using three compression pressures of (137.9, 144.8 and 148.3 MN/m2) to get tablets with optimum physical properties. The results (fig. 1) and (table 4a) in which a 10^2 was used indicate that a loss of

50.0, 49.0, and 94.0 percent in viability of the spores was obtained at pressure of 148.3 MN/m^2 for A.flavus spores , Penicillia Spp. Spores, and C.cladosporoids spores, respectively. The data also show that the survival at a contamination level of 102 spore/gm was inversely proportional to the compression pressure i.e, increasing pressure from (137.9 to 148.3)MN/m^2 caused a decrease in survival from 60 to 50.0 percent for A.flavus spores, from 50.0 to 41.0 percent for Penicillia Spp . spores and from 10.0 to 6.0 percent for C.cladospooids. That reduction is statistically significant (p=0.05). In general a higher reduction in viability was observed for Cladosporia than of Penicillia and A.flavus, size of the spores were in order of(3-6)µm in diameter, (4-6)µm in diameter, 3-7(-11)x2-4(5) µm in diameter)for A.flavus, Penicillia, and C.cladosporoids, respectively. On the other hand, Figure (2) and table (4b) show the effect of compression pressure upon percent of survival using 104 spore/gm contamination level and (148.3MN/m2) compression pressure to to obtain optimum physical properties of the tablet. The results indicate that the pressure applied caused a significant reduction in percent of survival (p=0.05)as 50.0 ,37.0 and 16.0 percent for A.flavus, penicillia, and C.cladosporoids ,as shown in figure (2). (4b).These results are well support the hypothesis that an inverse relationship is existed between spore size and compression pressure. Chesworth et al.[1] attributed the lethal effects of compression to a combination of two events, generation of heat and shearing between particles causing mechanical damage to the cells.Fassihi et al. [12]regarded localized heating within the formulation as being the critical parameter associated with inactivation of A.niger spores during compression of lactose granules .They proposed that during compression process, the pressures applied between the upper and lower punches were exerted only upon those particles directly in contact with the punch surface and that stresses on the remaining particles were through inter particulate contact . Such a phenomenon would result in very high pressures being exerted over small areas of inter particulate contact .This might cause the existence of localized (hot spots) within the tablets and bring about death of the microorganisms by heat alone. Such high temperatures have been shown to result in the melting of some materials during compression [13-18]. If such a mechanism were applicable in our systems one would expect the levels of survival for different organisms to reflect their heat sensitivity rather than cell size. This was not the case, results suggested therefore that shearing forces(size) are contributory to the observed lethal effects of compression rather than heat per sec.If pressure alone was responsible for the observed inactivation of microbial spores then once again no differences would have been expected between organism types. Conversely, larger sized microorganisms would be more likely to be subjected to shearing forces within a compact than smaller sized ones, supporting the hypothesis that inactivation is brought about by a direct physical trauma.

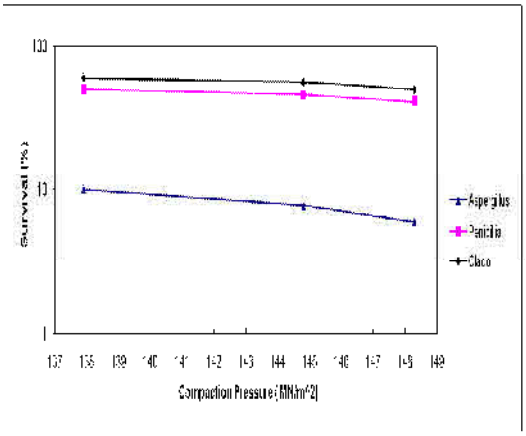

Figure 1 : effect of compression pressure upon the survival of (10^2 spore/gram) different fungal spores in Multivitamin tablet L.S.D = 3.7

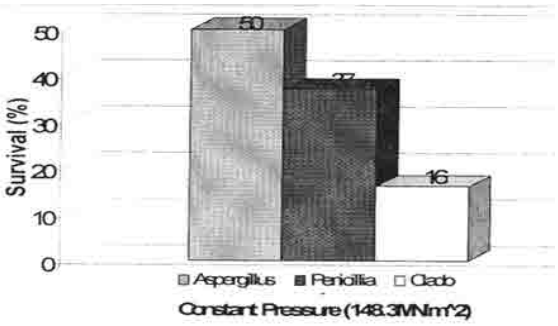

Figure 2 : survival of 10^4 ASP flavus penicillia and cladosporia spores at constant pressure in multivitamin tablet

Table 4: effect of pressure on survival of different fungal spores expressed as a % in multivitamin and folic acid tablets using 10^2 spore /gm and using 10^2 spore/gm contamination levels

4- a

Pressure (MN/m^2)	% Mean No. of spore / gm for 10^2 spore/gm contamination level		
	A.flavus	Penicillin Spp.	C. cladosporoids
137.9	60.0	50.0	10.0
144.8	56.0	45.7	7.7
148.3	50.0	41.0	6.0

4- b

Pressure (MN/m^2)	% Mean No. of spore / gm for 10^2 spore/gm contamination level		
	A.flavus	Penicillin Spp.	C. cladosporoids
148.3	50.0	37.0	16.0

Conclusion

The results emphasize on the existence of relationship between survival of fungal spores and the pressure used during tableting. Survival of fungal spores decreased with increasing compression pressure and level of survival at particular pressure depends upon the size of the spore. The lethal effect of tabletting was attributed to shearing forces upon the contaminating spores.

References

1. chesworth, K.A.c.; Sincletr, A.; atertten , R.J; and Hayer,W.p. Micro bios lettus 1977,4:41.
2. Morris,S.J- proceedings of thye Guild of Hospital oharmacists, hondon, ,1981 10:63.
3. Ayorinde, J.o. odeku, o.A. and Tida, o.A. the survival of B.subtilis spore in dicacium phosphate, hactose and starch and ther binary mixtures during tableting. Pharmaceutical technology, 2005, 29 (12):56-67-
4. plumpton E.J.;gilbert,p.; and fell, J.T the survival of microgranisms during tableting Int.J. pharm. 1986a ,30:241-246;
5. Plumpton E.J.;Jilbert,P.;and Fell J.T.Effect of special distribution on contaminant microorganisms with in tablet formulations on subsequent in in activation through compaction Int.J.Pharm.1986b,30:237-240;
6. Fassihi,A.R;and Parker,M.S.Inimicable effects of compaction speed on microorganisms in powder systems with dis-similar compaction mechanisms J.pharm.sci,1987, 76:466-470.
7. Blair T.C. ; Buckton ,G. ;and Bloomfield ,S.F; Baird, R;Leak R.E .;and Leech , R (ods). Microbial quality assurance in pharmaceuticals , cosmetics and Toiletries .Ellis.Horwood,Chichest :1991,pp:104-161.
8. Odeku,O.A,Awe,O.O.,Popoola,B.Odenigi, M.A.and Itida,O.A.Compreccssion and me mechanical properties of tablet formulations containing corn,sweet potato and cocoyam starches.Phar.Tech ,2005 29(4):82-90.
9. Fassihi,A.R.;and parker,M.S.Journal of applied acteriology,43meetings xvii;1977a
10. Yanagtta,T.;Miki,T.;Sakat,T.;and Itorkoshi ,chemical and pharmaceutical bulletin, 1987 ,26:185-190.
11. PlumptonE.J.Studies upon the survival of various microorganisms in solid dosage forms,ph.D.thesis, university of manchester;1982
12. Fassihi,A.R.;and parker, M.S.the influence of water activity and oxygen tension upon the survival of aspergillus and pencillia species and tablets .Int .Biodeterior . Bull , 1977,13 :75-80.
13. Bowden,F.P.and tabor , D . (Friction and lubrication of solids) , vol. 2 , Clarendon press,oxford;1964 .
14. Jayasinghe ,S.S;pllpei,N.and Harwood ,C.F.Materals science and Engineering ,5 ,287 ;1969,1970
15. Odeka,O.Aand Itiolao A.Evaluation of Khaya gum as abinder in paracetol tablet formulation on pharm.pharmacol commum, 1998, 4:183 -188.
16. Espinasse.V,Mperria cornet.J, Amartceat, Gervais. P.High pressure in activation of dried microorganisms. Drug developed and industrial pharmacy, 2002,81ssae: 329-337.
17. IF Eynwa F.Obuekwe and Florence Eichie , Actapolonae pharmaceutica-drug research , ;2006 ,6 (2) :121-125.
18. Obuekwae,I.F.,Ogbimi A . O. , Pakistan J. sc. Ind. Res. 2002,45:341.