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Development and Validation of Spectrophotometric Methods for Simultaneous Estimation of Lactose and FITC-Dextran in Ordered Mixtures

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Abstract

Mixtures of micronized fluorescein isothiocyanate (FITC)-Dextran (model drug) and sieved lactose (75-106 μm) in ratios ranging from 1:100 to 1:12 respectively were blended to produce ordered mixtures. Simple and sensitive spectrophotometric and flourometric methods were developed for the determination of lactose in the presence of FITC-Dextran in the mixtures. Calibration curves were prepared for each compound at the wavelength for peak absorption (490 nm) by a spectrophotometer. FITC-Dextran was separately analyzed by fluorimeter at excitation and emission wavelengths of 490 nm and 515 nm respectively. While lactose didn't interfere with the analysis of FITC-Dextran using the fluorimeter at the specified excitation and emission wavelengths, the latter showed proportional relationship between its concentration and emission. This allowed the calculation of the FITC-Dextran contribution to the absorbance of lactose in the mixtures and therefore omitting the interference in order to quantify lactose. The method of calculation was verified and found successful, therefore can provide meaningful way to assess segregation or redistribution of the formulation components resulting from vibration or fluidization.

Key Words: Lactose, analysis, FITC-Dextran, ordered mixture; spectrophotometer; fluorimeter.

Introduction

Three types of powder mixtures are recognized which include ordered, adhesive interactive (pseudo-random) and random mixtures (Sundell-Bredenberg and Nyström, 2001). Ordered mixtures have found important pharmaceutical applications, primarily for direct compression of tablets and dosing of the micronized active ingredient in dry powder inhalers (Zatloukal, 2004). The mixtures are normally consisting of fine (micronized) drug adsorbed on to the active sites on the surface of a larger carrier particle, sufficiently tenacious to resist being dislodged (Zeng et al., 1998). Therefore, as opposed to randomized mixtures, interactive mixtures provide more stable preparations that resist segregation, if the formulations are carefully designed. However, there are several factors that may lead to the segregation of ordered mixtures (Swaminathan and Kildsig, 2000) with three types recognized and these are the ordered unit, displacement and saturation segregations (Twitchell, 2002). The first occur when there are differences in the size of carrier particles themselves, while displacement segregation occurs in the ternary component

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systems when sufficiently fine particles of one component compete and displaces another component on the active sites of carrier particles (Zeng et al., 2000; Swaminathan and Kildsig, 2002). Saturation segregation occurs because of the saturation of the active sites of the carrier particles and this normally occurs at relatively low concentration of fine drug particles (Sundell-Bredenberg and Nyström, 2001). The remainders of fine particles are either free particles or aggregates that may segregate from the mixture, especially with the application of segregation forces such as vibration or fluidization.

While obtaining physically stable mixtures is important in both direct compression and inhalation formulations, the latter requires that upon aerosolization, fine particles are dispersed freely unadhered to the larger carrier particles to allow deeper penetration into the alveolar region of the lungs (Clark et al., 2007). Several researches have been carried out toward this end (Al-Tabakha and Arida, 2007; Al-Tabakha and Arida, 2007_a), however, such attempts increase the likelihood of segregation whether displacement or saturation types. In direct compression method to produce tablets, vibration is used to assess formulation flow through hopper into the dies of the tabletting machine. This also increases the likelihood of segregation with the resulting maldistribution of the drug in the tablets (Johnson, 1979; Thiel et al., 1986).

Lactose is one of the most popular excipients used in different solid pharmaceutical preparations and is the only one approved for general use in the United States (Cryan, 2005). It is inert, non-toxic and free flowing powder. FITC-Dextran is a model drug that can be obtained in different molecular weight extending from few thousands to millions (Xu and Groves, 2001) therefore, representing fast growing technology of compounds produced using recombinant DNA. FITC-Dextran can be detected fluorimeterically in minute concentration of less than 1 µg/ml. This makes FITC-Dextran an ideal candidate to substitute potent drugs in preliminary experiments (in't Veen et al., 2005). The effect of FITC-dextran molecular weight on its release from tablets has been studied before (Xu and Groves, 2001). Mixtures of micronized FITC-Dextran and sieved lactose formulations were also studied for aerosolization performance *in vitro* using the Andersen cascade impactor (Al-Tabakha and Arida, 2007; Al-Tabakha and Arida, 2007_a). In such experiments lactose can be used as a carrier to protect the hygroscopic drug, improve flowability and aid in the dispersion of the drug when aerosolized (Iida et al. 2005).

In order to study segregation in mixtures and to identify the type and mechanism of segregation, it is essential to analyze the components making them which are in this case FITC Dextran and lactose. The simplest method to use is spectrophotometric analysis. However, this may be complicated by significant spectral overlap of the components involved. The key fact that permits analyzing such mixtures is that at each wavelength the absorbancy of a solution (containing species X, Y, Z ...) is the sum of the absorbencies of each species (Manahan, 1986 Henry et al., 1991). Spectrophotometric tests for reducing sugars and polysaccharides have been available for a long time and rely on the action of concentrated (or near concentrated) sulphuric acid causing hydrolysis of all glycosidic linkages and the subsequent dehydration of the monosacharides released to give derivatives of furfural. The dehydration products react with a number of compounds such as L-cysteine, phenol (Dubois et al., 1956), orcinol (The Tintometer Ltd., 1967) and anthrone to give coloured products (White and Kennedy, 1986 The method has been used for the analysis of dextran (Williams and Taylor, 1992).

The case presented here is the calculation of the absorbancy contribution of FITC-Dextran to the total absorbancy when lactose is present in the mixture of both. Drug to diluent ratio frequently encountered in dry powder formulations for inhalation and in direct compressic tablets were used.

Materials and Methods

Materials: Lactose was obtained from Borculo Whey Products, UK. FITC-Dextran M. Wt. 4,400 Daltons (lot 106H1180), phenol (redistilled, 99+ %) and sorbitan trioleate (Span 85) were purchased from Sigma, UK. Chloroform (batch no. 9806708059) and sulphuric acid (S.G. 1.84; conc. 98%; analytical reagent) was purchased from Fisher, UK.

Apparatus: The LS-5 luminescence spectrometer (Perkin Elmer, UK) was used for all fluorimetric measurements. Spectra scans and absorbencies were recorded using Shimadzu (UV-1201): UV-Visible spectrophotometer (Shimadzu Corporation, Japan). All measurements were made using the standard 10 mm path-length quartz cuvette. Fluid jet mill (Glen Creston Ltd., UK) was used for micronizing FITC-Dextran while mechanical tap sifter (Pascall Engineering, England) was used for the sieving of lactose. Particle size measurements were made using Malvern system 2600 (Malvern Instruments Ltd., UK).

Micronization of FITC-Dextran (Mol Wt 4,400 Daltons)

FITC-Dextran was fed into the milling chamber of the jet mill, where two air streams meet at high velocity. Micronization results from collision of particles and size reduction occur by the combined impaction and attrition forces. The differential pressure set of 70 psig in the feeder side and 100 psig in the opposing side was used to micronize FITC-Dextran and lactose separately.

Preparation of coarse lactose (75-106 μm)

Sieving was carried out using mechanical tap sifter as described in the US Pharmacopeia (2000) using two sieves, one with an aperture of 75 μm and the other of 106 μm (laboratory test sieves, Endecotts Ltd, England).

Particle size measurement by LASER diffraction method

Micronized FITC-Dextran and sieved lactose were measured separately for particle size using Malvern system 2600. The sizing method employs laser diffraction according to Fraunhofer phenomenon. The suspending vehicle used for the powder samples was chloroform in which 0.1% w/v sorbitan trioleate was added. The suspension was sonicated for 20 seconds to disperse any clumps or aggregates before carrying out size analysis. Lens 300 mm was used when sieved lactose (75-106 μm) was measured. On the other hand micronized FITC-Dextran was measured using the lens 63 mm. The lens choice is according to particle size distribution of the measured samples. Obscuration which is a measure of concentration was adjusted between 0.10 and 0.18. Each sample was measured at least in triplicate.

Fluorimetric analysis of FITC-Dextran

The scanned excitation wavelength (λ_{ex}) and emission wavelength (λ_{em}) by the LS-5 of the solution containing FITC-Dextran were found at 490 and 515 nm respectively. FITC-Dextran solutions were prepared using phosphate buffer (pH 8.0 ± 0.05) as medium. The buffer was prepared in accordance to British Pharmacopoeia (2004). A standard calibration curve of FITC-Dextran fluorescence was prepared from an average of triplicate preparations of concentration ranging from 0 to 0.36 μg/ml.

Spectrophotometric determination of sugars and related substances

To 2 ml of the sample solutions (made of sieved lactose or FITC-Dextran or a combination of both), one ml of the 5% w/v phenol solution was added, followed by addition of 5 ml of sulphuric acid (98%). The solution was allowed to stand for ten minutes and then shaken. Reactions were allowed to take place for 20 minutes in a water bath at 25° C before spectrophotometric determinations were made. Standard calibration curves and absorbencies measurements were made at the peak absorption obtained following scanning of the materials spectra. For calibration curves a series of dilutions in triplicate (concentrations ranging from 0 to 60 μg/ml) were prepared for lactose and FITC-Dextran and analyzed using the Shimadzu UV-Visible spectrophotometer. The stock solutions were prepared by dissolving 120 mg of FITC-Dextran or lactose in 100 ml phosphate buffer (pH 8.0 ± 0.05) as medium followed by performing appropriate dilutions to obtain the desired concentration range.

Preparation of the powder blends

To verify the ability of our method to quantify the amount of sieved lactose in a mixture with micronized FITC-Dextran, blends of both components were prepared in different ratios. These blends were in the ratios of 1:100, 1:51, 1:25 and 1:12 respectively and were prepared and mixed by tumbling in a rotating container. To do so, the fractions of powders were accurately weighed and transferred to a jar. The container was tumbled on a roller for 1 hr at a speed of 90 rpm. Ten samples from each blend were weighed so that each contained an equivalent to 1 mg of FITC-Dextran. The samples were then analyzed by the use of the fluorimeter and spectrophotometer at the proper wavelengths as described previously.

Results and Discussion

Particle size

Laser sizing of micronized FITC-Dextran revealed that the volume median diameter (VMD) was 5.21 μm with all particles below 20.70 μm (figure 1). Sieved lactose fraction (75-106 μm) had a VMD of 116.63 μm. The Malvern 2600 user manual (Diffraction reference, 1993) explains that when the particles are not spherical, the VMD may fall outside the designated sieve fraction as was shown by the sizing results. The particle size distribution showed in figure 2 indicates the presence of small proportion of fine particles. These results of sieving lactose support other works (Zeng *et al.*, 1998; Al-Tabakha and Arida, 2007$_a$). Zeng *et al.* (1998) have attempted to remove fine particles from a sieved lactose fraction (63-90 μm) by subjecting it to an airstream with a flow rate of 160 l/minute, but were not successful.

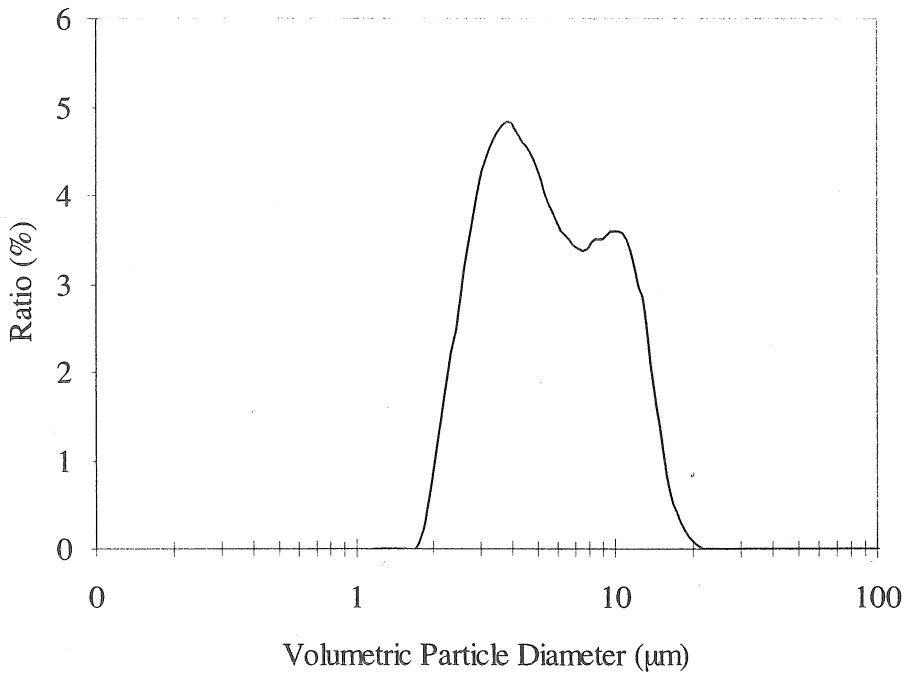

Figure 1. The particle size distribution of micronized FITC-Dextran.

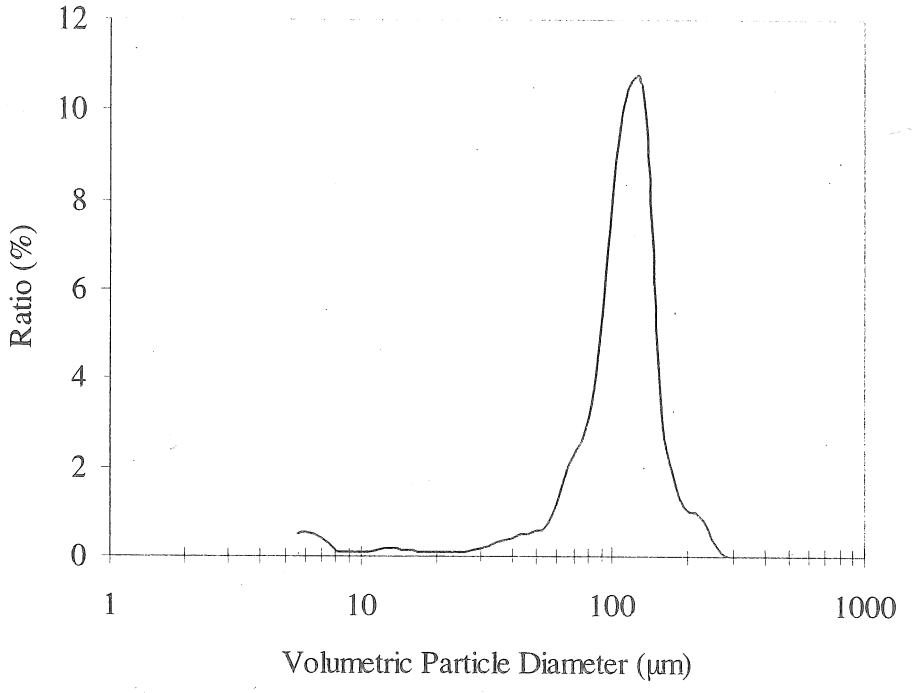

Figure 2. The particle size distribution of a sieved lactose fraction (75-106 μm) after sifting by mechanical tapping.

For ordered or interactive mixtures, many researches have used fine particles of drug with VMD approximately 5 μm, while the carrier particles which were frequently referred to as coarse passed through the sieve 106μm (Swaminathan and Kildsig, 2002; Beilmann *et al.*, 2007), however, the narrowing of the particle size distribution was important to prevent ordered unit segregation (Thiel and Nguyen, 1982). The concentration of the drug to carrier ratio varied with some studies recording the use of as low as 0.1% (Thiel and Nguyen, 1982) and as high as 32.5% (Zeng *et al.*, 2000) in the preparation of interactive mixtures. Therefore, the prepared micronized FITC-Dextran (VMD = 5.21 μm) and sieved lactose fraction (75-106 μm) blends in the ratios ranging from about 1% to about 7.7% are ideal in representing binary ordered mixtures or segregations that may occur for any of the reasons stated previously.

Colorimetric spectra overlap

The spectrophotometric analysis was used to carry out the individual scanning of the FITC-Dextran and sieved lactose over the wavelengths range from 200 nm to 800 nm. A base line was first made by the spectrophotometer for the same wavelength range using the prepared blank. The resultant spectra of FITC-Dextran and lactose proved to be very similar (Figure 3).

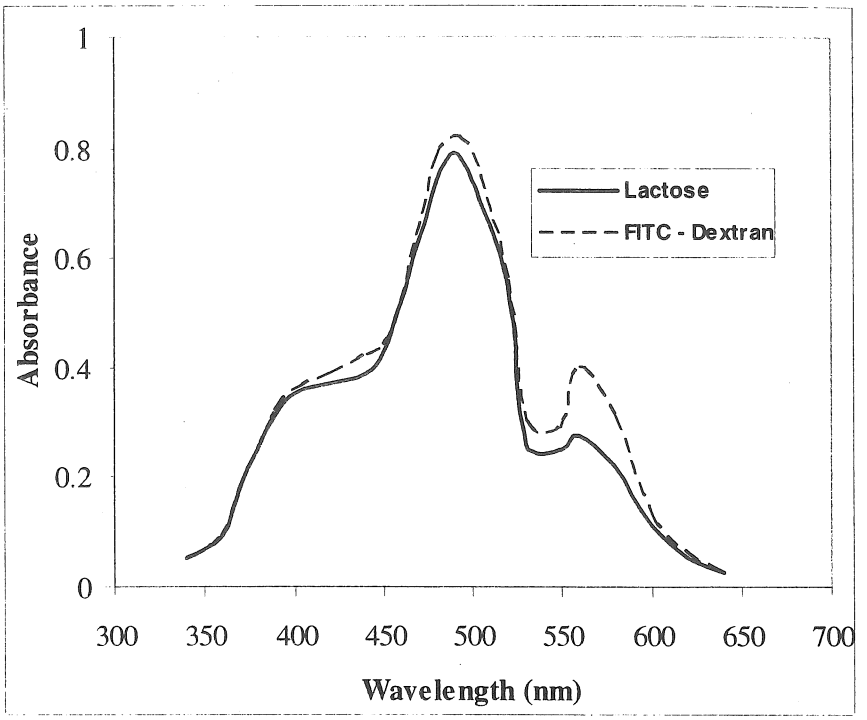

Figure 3. Absorption spectra of FITC-Dextran solution (60μg/ml) and lactose solution (60μg/ml).

The peak absorption wavelengths for FITC-Dextran from the scanned wavelength range 200-800 nm were found to be 490 nm (absorption = 0.822) and 565 nm (Absorption = 0.419). Similar peak wavelengths were found for lactose at wavelength 490 nm (absorbance=0.789) and 564 nm (absorbance= 0.277). Another peak wavelength was also found at 432 nm (absorbance 0.391).

Therefore the presence of lactose and FITC-Dextran together in solution would clearly result in an additive increase in the absorption of lactose because of the FITC-Dextran presence when measured at the peak wavelength 490 nm.

Standard calibration curves for FITC-Dextran and lactose

A good and reproducible linear relationship between concentration and fluorescence was obtained for FITC-Dextran. There was no difference in fluorescence of FITC-Dextran whether lactose was present or not. The linear regression equation representing the average of calibration curves obtained from the three data sets (n= 3) is y=1935.4x – 2.4528 with a relative standard deviation (RSD) of 0.5%. At the peak absorption wavelength 490 nm, spectrophotometric results showed good correlation between concentrations of FITC-Dextran and absorbencies. The equation obtained from the average of standard calibration curves was y= 0.0137x – 0.0005 (RSD of 0.04 %).

The analysis of different concentrations of lactose using the Shimadzu (UV-1201) spectrophotometer at the peak wavelength 490 nm showed good relationship between the concentrations used and the absorbencies obtained. The standard calibration curves average equation was y = 0.0133x + 0.0004 (RSD of 0.5 %) which is similar to that obtained from FITC-Dextran and absorbencies.

In order to develop equations whereby the problem of the interference of FITC-Dextran in the spectrophotometric analysis of lactose, it is suggested that for any mixture of compounds X and Y, two cases can be distinguished, one is that the absorption bands of pure X and pure Y overlap significantly everywhere and the other case is that the bands of X and Y have little overlap in some regions. In the case of FITC-Dextran and lactose this has shown significant overlapping of the two spectra as indicated previously (Figure 3).

Since the absorption spectra of FITC-Dextran and lactose are so similar, another analytical method is needed to quantify one component in the presence of the other. FITC-Dextran can be measured fluorimeterically without the interference of lactose and therefore can be used to omit its contribution in the spectrophotometric absorption provided standard calibration curves are available from the spectrophotometer for each of the pure species.

The equation of the linear fit resulting from the standard calibration graph of FITC-Dextran made fluorimeterically is:

$$A^{F}_{X_s} = 1935.4[X]_s - 2.4528 \qquad (1)$$

Where

$A^{F}_{X_s}$ Is the fluorescence reading of FITC- Dextran.

$[X]_s$ is the concentration of FITC-Dextran in µg/ml.

Similarly, the equation of the linear fit resulting from the standard calibration curve of FITC-Dextran made calorimetrically is:

$$A_{X_s} = 0.0137[X]_s - 0.0005 \qquad (2)$$

Where

A_{X_s} Is the absorbancy of FITC-Dextran.

$[X]_s$ is the concentration of FITC-Dextran in µg/ml.

The equation of the linear fit resulting from the standard calibration curve of lactose made calorimetrically is:

$$A_{Y_s} = 0.0133[Y]_s + 0.0004 \qquad (3)$$

Where

A_{Y_s} Is the absorbancy of lactose.

$[Y]_s$ is the concentration of lactose in µg/ml.

Now [X], the unknown concentration of FITC-Dextran sample can be calculated from equation 1 using the fluorimetric analysis method. The value of [X] can then be substituted in the equation 2 to calculate the absorbency contribution from FITC-Dextran. By solving equation 1 and 2, and provided the same unit is used for the concentration term, this can be calculated by the following equation 4:

$$A_{X_s} = 7.079\times10^{-6} A_{X_s}^{F} - 4.83\times10^{-4} \qquad (4)$$

When the spectrophotometric absorbancy of FITC-Dextran is calculated, it is subtracted from the total spectrophotometric absorbancy of the mixture; hence the absorbancy of lactose is obtained. From the calculated absorbancy of lactose, its concentration can then be calculated from equation 3.

The proposed method of calculation had to be verified by analyzing ten samples from the prepared blends by both spectrophotometer and fluorimeter. The relative proportions of FITC-Dextran and lactose in each of 10 samples for the four mixtures were then determined by the method described above. The results of the calculations are summarized in table 1. It is shown that not only the samples withdrawn agree with the formulated blend ratios, but also the relatively low RSD, a characteristic of an ordered mixture. According to the US Pharmacopeia (2000) for the content uniformity test, one of the pre-requisite is that the RSD does not exceed 6%.

Table 1. Ratios of FITC-Dextran to lactose based on a combination of fluorimetric and spectrophotometric analysis (n=10).

Prepared ratio	Ratio (1:100)	Ratio (1:51)	Ratio (1:25)	Ratio (1:12)
Calculated ratio from samples analysis (RSD*)	1:101.3 (1.29)	1:50.0 (0.54)	1:25.0 (0.71)	1:12.1 (0.67)

* RSD: Relative standard deviation is calculates based on the content of FITC-Dextran in the drawn samples.

Conclusion

The proposed method of calculation for determining the amount of lactose in a mixture with FITC-Dextran was verified experimentally and found to be acceptable. The mixing technique was also shown to be satisfactory otherwise the results would not agree with the values of ratios of the prepared blends. The method should also be suitable for applications into wide range of similar situations were two species interfere with each other analysis (without chemical interaction between them). The interference should be additive and there should be a method for quantifying one of the species. The current investigation provides valuable tools in assessing segregation or stratification that may occur in direct compression formulations. It should also be able to assess the redispersion of the dry powder particles upon aerosolization *in vitro*.

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