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Gel permeation chromatography used to determine the stability of FITC-Dextrans in human saliva and porcine small intestinal mucus

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Summary

Fluorescein isothiocyante dextrans (FITC-Dextrans) have been used extensively as high molecular weight markers. The stability of FITC-Dextrans has been demonstrated in many situations, but there are instances where dextrans have not been stable. A gel permeation chromatography (GPC) method has been established that can be used to monitor molecular weight, concentration, polydispersity and free label in FITC-Dextrans. This has been applied to stability studies of FITC-Dextrans in human saliva, porcine small intestinal mucus and with human α-amylase. The molecular weight of FITC-Dextran was reduced in human saliva with a half-life of 5 h. The reduction in molecular weight was less rapid in porcine small intestinal mucus and non-existent with human α-amylase; sodium azide helped reduce degradation but did not eliminate it. The degradation of FITC-Dextrans is thought to be mediated by dextranase-producing micro-organisms. The successful use of FITC-Dextrans as high molecular weight markers must be accompanied by careful quality control of FITC-Dextrans, particularly when incubation times are long and microbiological spoilage is a possibility.

Introduction

Dextrans are glucose polymers that can be produced to a defined molecular weight. This has led to them being used clinically as plasma expanders where high molecular weight dextrans remain osmotically active in blood for extended periods.

Dextrans have also been used in research as high molecular weight markers. Dextrans themselves are difficult to assay, so they have been labelled in a variety of ways. Labelling with fluorescein isothiocyanate (FITC; De Belder and Granath, 1973) has been used extensively. FITC-Dextrans have been used to study microcirculation (Stock et al., 1989), the uptake of macromolecules by different cell types (Oliver et al., 1984; Eissenberg and Goldman, 1988), and the permeability of a variety of membranes (Bekker et al., 1988; Hulbert et al., 1989; Nakagomi et al., 1989).

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FITC-Dextrans have been shown to be stable for extended periods of time (Kurtzhals et al., 1989), with no release of free label or change in molecular weight, but there are instances where dextrans appear not to be stable; for example, in human saliva (Lukie, 1983) and faeces (Åberg, 1953), in the small intestine (Bloom and Wilhelmi, 1952) and together with certain micro-organisms (Hehre and Sery, 1952). The stability of FITC-Dextrans under these conditions remains to be established.

Preston et al. (1987) showed that apparent endocytosis of FITC-Dextrans into yeast cells reflected the uptake of low molecular weight impurities and not high molecular weight FITC-Dextrans. Consequently, they suggested careful quality control should be employed before using commercially available FITC-Dextrans as high molecular weight markers.

Successful use of FITC-Dextrans as high molecular weight markers requires stability of label and molecular weight if erroneous results are to be avoided. Molecular weight stability and purity of marker at the start and end of experiments must be established.

Many methods have been used to quantify the molecular weights of dextrans. Gel permeation chromatography (GPC) has been used widely as the method of choice (Alsop and Vlachogiannis, 1982; Cullen et al., 1985, Van Dijk et al., 1987). This method allows mean molecular weights and molecular weight distributions to be determined.

In the present work, a GPC method has been established and validated to determine the stability of FITC-Dextrans in human saliva and porcine small intestinal mucus. This method allows molecular weight to be followed and demonstrates release of free label.

Materials and Methods

Materials

The GPC apparatus consisted of an HPLC pump (HPLC Technology, U.K.), TSK G2000SW column, 7.5 × 600 mm, fitted with TSK SWP guard column, 7.5 × 75 mm (LKB, U.K.) and fluorescence detector (LKB, U.K.) with chart recorder (J.J. Instruments, U.K.).

Sodium chloride, sodium azide, disodium hydrogen phosphate and sodium dihydrogen phosphate were of Analar grade and fluorescein sodium and calcium chloride of GPR grade (BDH, U.K.). Fluorescein isothiocyanate labelled dextrans (FITC-Dextran) with different molecular weight and labelling efficiency and human salivary α-amylase (type IX-A) were obtained commercially (Sigma, U.K.).

Methods

GPC methods and conditions

A mobile phase consisting of 0.9% w/v sodium chloride + 0.05% w/v sodium azide was pumped through a TSK G2000SW, 0.75 × 60 cm column fitted with TSK SWP guard column, 0.75 × 7.5 cm at a flow rate between 0.1 and 0.5 ml/min. Samples were injected via a Rheodyne injection port fitted with a 20 μl loop. The eluent was monitored with a fluorescence detector fitted with a 450 nm narrow-band excitation filter and wide-band (> 490 nm) emission filter. The chromatographic profiles were recorded on a chart recorder.

FITC-Dextrans with different molecular weights and labelling efficiency (Table 1) and free fluorescein were dissolved at different concentrations in phosphate-buffered saline (PBS, pH 7.4). These standards were used to calibrate the system with respect to molecular weight and concentration. Reproduciblity was tested by repeated injec-

TABLE 1

Sigma FITC-Dextran molecular weight markers used in calibrating the TSK G2000SW column

Molecular weight	Labelling efficiency (mol FITC/mol glucose)	Experimentally measured molecular weight at 95% peak height
3860	0.04	–
4400	0.06	1006–13269
9400	0.003	1584–35325
18900	0.004	9055–34491
35600	0.011	5616–57082
40500	0.03	–
71200	0.003	–

tions of samples from a single batch over a period of 5 days.

Collection of saliva and small intestinal mucus

The stability of FITC-Dextran was tested in human saliva and porcine small intestinal mucus.

Human saliva was collected by the following protocol (Lukie, 1983). The mouth was rinsed out with 100 ml of distilled water; after 5 min saliva production was encouraged by the subject chewing on paraffin wax sealing tape (Parafilm). Saliva was collected for 5 min and used immediately.

1-m lengths of duodenal small intestine were collected from freshly slaughtered pigs and stored on ice. These were cut open and the mucus layer removed by gently scraping with a glass slide. Mucus scrapings from six animals were pooled and diluted 1 : 1 with ice cooled PBS and stirred for 30 min at 4°C. The mucus suspension was centrifuged for 2 h at $40\,000 \times g$ and 4°C; the supernatant was removed and stored at 4°C before use.

Stability measurements

150 µl FITC-Dexran solution (0.5 mg/ml in PBS, MW 35 600) was mixed with 150 µl of saliva or PBS as a control and incubated at 37°C. Samples were taken at predefined time intervals and immediately frozen. Before assaying, the samples were thawed and centrifuged at $13\,225 \times g$ to remove any insoluble material.

Stability measurements with small intestinal mucus were conducted in a similar manner but FITC-Dextan of MW 18 900 was used.

The effect of adding the preservative sodium azide on stability was tested. 10 µl of sodium azide (to a final concentration of 0.05% w/v) or PBS as a control were added to the samples before incubation.

Present in saliva are amylase enzymes. These break down polysaccharides and could have an action on FITC-Dextrans. Amylases work by cleaving specific α-1,4-glucose bonds in polysaccharides. The glucose units in dextrans are linked predominantly by α-1,6 bonds (Virnik et al., 1975), with only a few α-1,4 bonds used for branching.

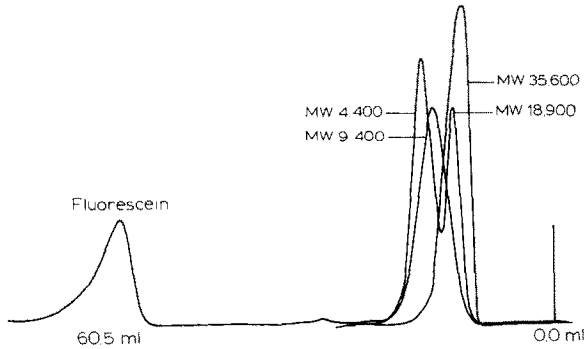

Fig. 1. GPC chromatograms of FITC-Dextran molecular weight markers using TSK G2000SW (60 cm) column.

The effect of human salivary α-amylase on FITC-Dextrans was studied. α-Amylase was dissolved in 1.0 mM calcium chloride to a concentration of 1 U/ml (1 U will liberate 1.0 mg maltose from starch in 3 min at pH 6.9 and 20°C; Bernfeld, 1955). 100 µl of amylase solution was mixed with 100 µl FITC-Dextran (MW 18 900) and incubated at 37°C. Samples were taken at set time intervals and processed as before.

Results and Discussion

GPC chromatography

The different molecular weight FITC-Dextrans were eluted as symmetrical peaks (Fig. 1). Molecular weights between 3855 and 35 600 could be fitted to a linear log MW/retention volume calibration line (Fig. 2, $r = -0.9934$, $n = 5$). Above a molecular weight of 35 600 all FITC-dextrans were

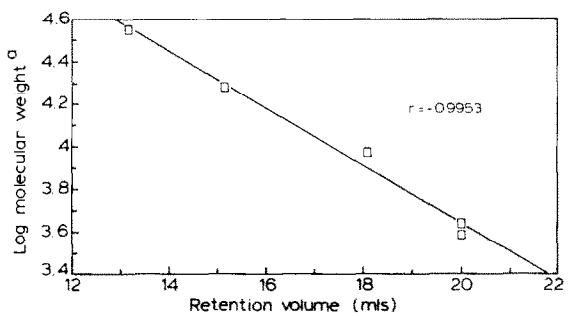

Fig. 2. Molecular weight calibration line for TSK G2000SW (60 cm) column using FITC-Dextrans. [a] Molecular weight is expressed as modal molecular weight of chromatograms.

excluded from the column and had similar retention volumes. The samples were very polydisperse; the peak widths were measured at 95% peak height to give a measure of polydispersity. Table 1 shows the molecular weight ranges measured. The FITC-Dextrans of 9400 and 35 400 molecular weight were particularly polydisperse when compared to FITC-Dextran 18 900.

Free fluorescein could be detected with a very long retention volume (65 ml). Since the total volume of the columns was 30.5 ml, it is likely that in the low ionic strength mobile phase, fluorescein was interacting with the polar silica bed (Barth, 1986).

The system was calibrated with respect to concentration with FITC-Dextran MW 4400 and 18 900 (Fig. 3). These proved to be linear down to 1.0 μg/ml ($r = 0.999$, $n = 6$) for both samples. The calibration lines are superimposable, since detection is based on the same fluorescent marker: the lines are simply shifted up or down depending on labelling efficiency.

Reproduciblity of the system was very good with coefficients of variation of 0.57% over the 5 day period with respect to retention volume (Table 2).

In the method developed, without an extraction step and with fluorescence detection, only one small peak attributable to the mucus sample was observed (Fig. 4). This had a long retention time and did not affect the assay. Non-specific methods of detection (i.e. refractive index) require complex sample preparation if dextrans are to be assayed

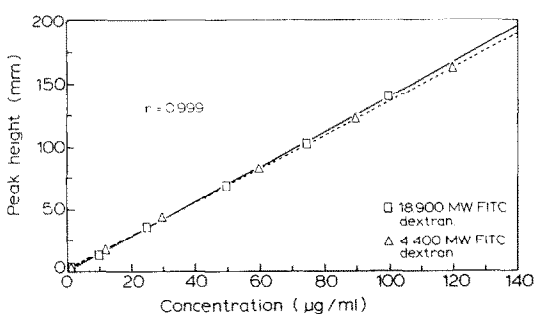

Fig. 3. Concentration calibration line for TSK G2000SW (60 cm) column using using 4400 and 18 900 molecular weight FITC-Dextrans.

TABLE 2

Reproduciblity of injecting FITC-Dextran (MW 4400) over a period of 6 days

Time	Retention volume (ml)	Percent original peak height
Day 1		
a.m.	21.76	100.0
	21.60	101.8
	21.60	104.3
	21.68	100.0
	21.60	101.4
	21.76	
p.m.	21.84	
Day 2		
p.m.	21.52	
Day 3		
a.m.	21.52	
p.m.	21.84	
Day 4		
Day 5		
Day 6		
a.m.	21.68	
p.m.	21.48	
	21.84	
Coefficient of variation	0.57%	1.6%

from body fluids (Cullen et al., 1985). The use of FITC-Dextrans and fluorescence detection has overcome this problem.

In previously published methods the efficiency of labelling affects retention volumes (Kurtzhals et al., 1989), thus the column must be calibrated with respect to molecular weight and labelling efficiency. This limitation has not been found in the present method where only molecular weight determines the retention time.

The TSK G2000SW column resolved FITC-Dextrans over a narrow molecular weight band (MW 35 600 to MW 4000). The mobile phase was at a pH of 7.0 to maximise the fluorescence of FITC-Dextrans; below pH 7.0 fluorescence decreases markedly as the FITC label becomes unionised. At alkaline pH, stability of silica-based columns must be considered (Kurtzhals et al., 1989) but over a 2 month period the performance of the column used did not deteriorate noticeably. pH 7.0 has been used routinely with this type of column before with no reported deterioration

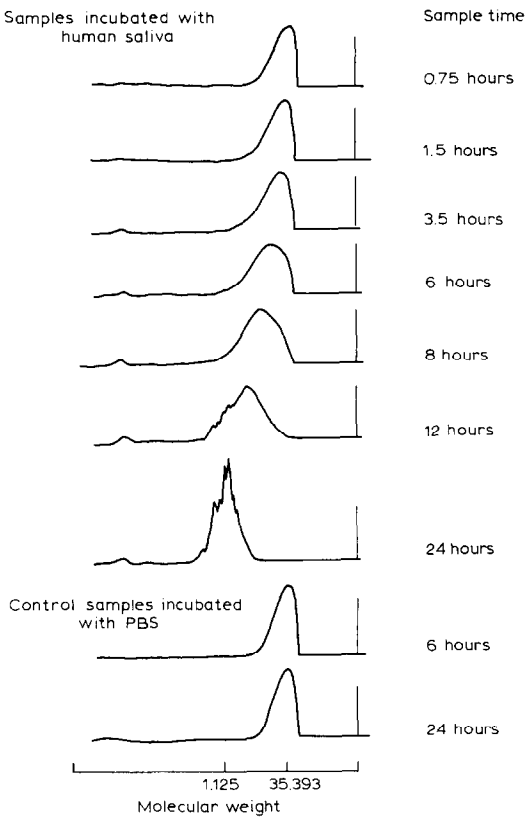

Fig. 4. GPC traces for FITC-Dextrans after incubation with human saliva at 37 °C.

(Kato et al., 1980), but long-term stability must be considered.

Stability of FITC-Dextrans in human saliva and pig small intestinal mucus

The stability of FITC-Dextran in human saliva was examined. Fig. 4 shows the results obtained for one subject (one sample per time interval). The peak shifts to a longer retention volume (lower molecular weight) and broadens. After 12 h (and seen fully at 24 h) low molecular weight material is seen as a collection of small peaks. By extrapolating the calibration line below the smallest standard (MW 3860) the molecular weight of the peaks is estimated to range from 100 to 4000. These represent the fully degraded FITC-Dextran and are probably short-chain oligosaccharides. However, no standards exist to allow identification.

The chromatograms allow the molecular weight to be followed with time (Fig. 5). After a short lag time the molecular weight falls rapidly with a half-life of approx. 5 h in this sample. A blank, without saliva present, shows FITC-Dextrans to be stable at 37°C. Under no conditions was free fluorescein detected and the label appears to be firmly attached to the dextran chains. This observation is supported by the previous work of De Belder and Granath (1973).

FITC-Dextran stability in porcine small intestinal mucus was tested with FITC-Dextran MW 18900. Fig. 5 shows the mean molecular weight ($n = 3$) changing with time. Stability was higher than in saliva with only small changes in molecular weight occurring in 24 h. It has been suggested that dextrans are not stable in the small intestine (Bloom and Wilhelmi, 1952) and that gut microflora could be involved in their metabolism (Hehre and Sery, 1952).

The effect of preservation of both mucus samples on the stability of FITC-Dextrans was studied. Table 3 shows the mean ($n = 3$) percentage reduction from the original molecular weight (18900) after 12 and 24 h. As seen previously, the unpreserved sample in human saliva degraded rapidly with the molecular weight being reduced to 17.0% of the original value in 12 h. When preserved with sodium azide the degradation was reduced: at 12 h the sample had a molecular weight of 45.7% of the original molecular weight, but at 24 h degradation was complete.

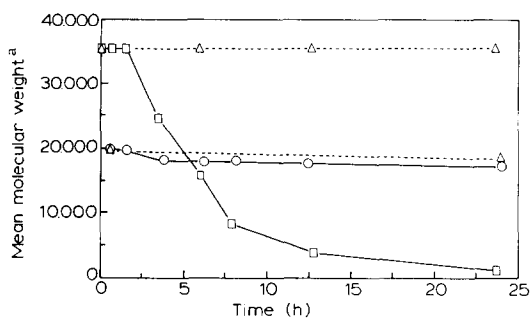

Fig. 5. Molecular weight changes in FITC-Dextran when incubated with human saliva and porcine small intestinal mucus at 37 °C. [a] Molecular weight is expressed as modal molecular weight of chromatograms. —□—, Incubated with saliva at 37 °C; ····△····, incubated with PBS at 37 °C; —○—, incubated with PSI mucus at 37 °C.

TABLE 3

The effect of preservation on the stability of FITC-Dextran (MW 18900) in human saliva and pig small intestinal mucus (mean of three experiments)

Mucus sample	Percent reduction from original molecular weight	
	12 h	24 h
Human saliva preserved with sodium azide	45.7 [a]	5.6 [b]
Human saliva unpreserved	17.0 [a]	5.1 [b]
Pig small intestinal mucus preserved with sodium azide	89.0 [c]	80.1 [d]
Pig small intestinal mucus unpreserved	82.0 [c]	76.6 [d]

[a] Significantly different ($p = 0.05$, paired t-test).
[b,c,d] Not significantly different ($p = 0.05$, paired t-test).

Degradation of FITC-Dextran in small intestinal mucus was not reduced by sodium azide ($p = 0.05$, t-test).

Human saliva has a large microbial population (Gibbons and Van Houte, 1975) with many different types of micro-organism present. Different micro-organisms possessing dextranase enzymes have been isolated from the mouth (Staat et al., 1973). Thus, microbial contamination could cause the FITC-Dextrans to be broken down to lower molecular weight species. Sodium azide kills aerobic organisms by interfering with cellular respiration. Conversely, anaerobic organisms would not thrive in the oxygenated sample. This action of sodium azide in reducing microbial contamination in the samples could explain the reduced degradation. However, saliva degraded the sample totally in 24 h despite the inclusion of sodium azide. Cell lysis and subsequent enzyme release or enzyme release prior to cell death could account for dextranase activity. With a very large initial microbial population significant amounts of enzymes could be released. This may explain why degradation is seen despite the presence of preservative. Dextranases would be expected to remain active under the conditions in the study (Janson and Porath, 1966).

From Fig. 4 it can be seen that the molecular weight population gradually decreases to a multicomponent collection. This method of degradation implies that dextranase activity in the mouth is of an endohydrolytic type (breaking the dextran chains in the middle). *Bifidobacterium* isolated from the mouth has been shown to release enzymes that operate by this mechanism (Kaster and Brown, 1983).

Degradation in small intestinal mucus is less rapid than in saliva, and is further reduced by preservatives. FITC stability in porcine small intestinal mucus may be explained by removal of microbial contamination Since some time is required to prepare the mucus, most operations have to be completed at 4°C, and at this temperature the number of viable organisms could be reduced (Drašar et al., 1969). The human small intestine has a much lower microbiological population than the mouth (Drašar and Hill, 1974); this may also be true for the pig small intestine. Smith (1965) showed that many micro-organisms could be isolated from the pig small intestine, but in much lower numbers than in the stomach and large intestine. These two factors together make microbiological spoilage of FITC-Dextran less likely in small intestinal mucus than in saliva.

The stability of FITC-Dextrans was tested with large amounts of human salivary α-amylase. After 24 h of incubation no change in molecular weight was seen. Thus, under the experimental conditions used, amylase did not appear to be able to cleave the α-1,6 bond found in dextran.

Conclusion

A GPC method has been established with fluorescence detection for the analysis of FITC-Dextrans; quantitative data of concentration, molecular weight, polydispersity and stability of FITC label on dextran chains can be obtained. Samples require little preparation and analysis times of less than 30 min can be obtained.

FITC-Dextrans are unstable in human saliva due to microbiological spoilage. The microflora of the mouth possess dextranases that act in an endohydrolytic manner to reduce the molecular weight

of dextrans. No free FITC was released during degradation. Preservation of the samples with sodium azide assisted in retarding degradation but did not eliminate it.

Porcine small intestinal mucus degraded FITC-Dextrans to a limited extent; preservation reduced this further. The method of preparation and possible low initial microbial population help reduce degradation of FITC-Dextrans.

Human α-amylase did not affect the stability of FITC-Dextrans under the conditions of the experiment.

Experiments using FITC-Dextrans as molecular weight markers must take into account their stability. Under normal conditions they are stable, but if incubation times are long and microbiological contamination is possible then stability must be ascertained.

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