# Dual marker one day pancreolauryl test

M R Green, S Austin, L T Weaver

## Abstract
**There is a pressing need for a simple non-invasive test of exocrine pancreatic function for use in children. The pancreolauryl test has been modified by the addition of a second marker (mannitol) to achieve a single day test without the need for two timed urine collections. Six healthy subjects and nine patients with cystic fibrosis were studied. Fluorescein, fluorescein dilaurate, and mannitol were taken by mouth, alone or in combinations, followed by 10 hour urine collections in two hourly aliquots to study the comparative pharmacokinetics of these markers. Urinary fluorescein was determined spectrophotometrically and urinary mannitol enzymatically. When fluorescein dilaurate and mannitol were taken together and the results expressed as ratios of percentage fluorescein to percentage mannitol recovery (F:M ratio) (mean (SD)) there was clear discrimination between healthy subjects and those with cystic fibrosis regardless of enzyme replacement treatment (57·3 (18·2) $v$ 3·4 (1·4) $v$ 3·2 (1·6) respectively). The differences in F:M ratios reached statistical significance in urinary aliquots collected between two and eight hours after marker ingestion. This single day tubeless test will greatly simplify the investigation of the child with suspected exocrine pancreatic dysfunction.**
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There are currently few reliable tests of exocrine pancreatic function for use in children which do not require upper intestinal intubation and stimulation of pancreatic secretion. The PABA test[1 2] and the measurement of faecal chymotrypsin[2 3] have proved useful, and isotope labelled breath tests[4] have attracted attention. We present here a modified and simplified version of the pancreolauryl test, which is ideal for repeated use in childhood.

In adult studies the pancreolauryl test has proved to be a sensitive and specific measure of exocrine pancreatic function.[5–10] In its standard, unmodified form fluorescein dilaurate is taken by mouth, it is cleaved by pancreatic esterases, and the recovery of the free fluorescein absorbed and excreted in a 10 hour urine collection is measured. The test is repeated on a second day with an equimolar dose of free fluorescein. The result is expressed as a ratio of fluorescein recovered from fluorescein dilaurate on the test day to free fluorescein recovered on the control day. Studies of adults[5–10] and four reports in children[11–14] show the test to be safe and reliable.

The difficulties of obtaining two 10 hour urine collections on separate days, owing to irregular, incomplete, and unpredictable bladder evacuation, limit the use of the test in infancy and childhood. This problem may be overcome by the development of a dual marker system which involves the inclusion of a second marker with absorption, distribution, and excretion characteristics similar to that of free fluorescein, to eliminate the second day of the test and to shorten the duration of the collection of urine. Mannitol, which has been used extensively for the measurement of intestinal permeability in infancy and childhood,[15–17] has been chosen as such a marker.

The aims of this study were to compare fluorescein and mannitol as markers and to establish the suitability of the latter for combination with fluorescein dilaurate in a simplified one day test of exocrine pancreatic function. We have used the new test to measure exocrine pancreatic function in healthy controls and in those with depressed function due to cystic fibrosis.

## Subjects and methods
### SUBJECTS
Six healthy adults (four men, two women, aged 29–40 years) were studied, and nine subjects with cystic fibrosis (six male, three female, aged 6–25 years). All the patients with cystic fibrosis had had the diagnosis confirmed by sweat test and were judged clinically to require pancreatic enzyme supplementation.

### TEST PROCEDURE
On the day of each test the subject fasted overnight and, after the collection of a pretest urine specimen, ate a breakfast of toast and butter. During breakfast the markers were ingested according to the following regimen: test 1, fluorescein 0·5 mmol; test 2, mannitol 25·0 mmol; test 3, fluorescein 0·5 mmol and mannitol 25·0 mmol; test 4, fluorescein 0·25 mmol and mannitol 12·5 mmol; test 5, fluorescein dilaurate 0·5 mmol; and test 6 fluorescein dilaurate 0·5 mmol and mannitol 25·0 mmol.

Fluorescein and fluorescein dilaurate were taken as capsules (pancreolauryl test, Charwell Pharmaceuticals) and mannitol as an aqueous solution (25 mmol in 200 ml). A high fluid intake was encouraged during the test and urine was collected in aliquots every two hours for 10 hours after ingestion of the markers. Six patients with cystic fibrosis did not take their enzyme supplements for the 24 hours preceding the test (CFWO). These six, and three further subjects with cystic fibrosis, were also studied while taking their pancreatic enzyme supplements (CFW). The subjects with cystic fibrosis performed test 6 only.

### BIOCHEMICAL MEASUREMENTS
After measurement of volume, a 20 ml aliquot of each urine specimen was frozen at $-20^\circ$C.

**MRC Dunn Nutrition Unit, Cambridge**
M R Green
S Austin
L T Weaver

Correspondence to:
Dr M R Green, Academic Unit of Paediatrics and Child Health, Clinical Sciences Building, St James's University Hospital, Leeds LS9 7TF.

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Table 1 *Percentage recovery (mean (SD)) of fluorescein in urine of healthy controls*

| Test | Substrate* | 0–2 Hours | 2–4 Hours | 4–6 Hours | 6–8 Hours | 8–10 Hours | Total |
|---|---|---|---|---|---|---|---|
| 1 | F | 13·3 (4·3) | 11·8 (6·1) | 4·8 (1·2) | 2·2 (0·7) | 1·3 (0·5) | 33·3 (6·8) |
| 2 | M | – | – | – | – | – | – |
| 3 | F+M | 17·0 (6·8) | 13·2 (4·3) | 5·7 (1·1) | 2·5 (1·0) | 1·5 (0·8) | 39·7 (8·9) |
| 4 | 1/2 F+M | 26·5 (14·7) | 17·5 (8·9) | 8·3 (4·8) | 4·0 (1·4) | 2·2 (1·1) | 57·3 (11·0) |
| 5 | FDL | 1·3 (2·1) | 7·5 (3·2) | 6·0 (3·5) | 3·0 (1·2) | 1·0 (0·0) | 19·2 (5·5) |
| 6 | FDL+M | 0·8 (0·7) | 7·6 (2·2) | 6·0 (2·5) | 2·9 (0·8) | 1·3 (0·5) | 18·7 (3·8) |

*F=fluorescein; M=mannitol; FDL=fluorescein dilaurate.

Table 2 *Percentage recovery (mean (SD)) of mannitol in urine of healthy controls*

| Test | Substrate* | 0–2 Hours | 2–4 Hours | 4–6 Hours | 6–8 Hours | 8–10 Hours | Total |
|---|---|---|---|---|---|---|---|
| 1 | F | – | – | – | – | – | – |
| 2 | M | 16·0 (4·7) | 11·2 (3·1) | 5·2 (1·5) | 3·0 (0·6) | 1·5 (0·8) | 36·5 (4·5) |
| 3 | F+M | 16·2 (3·2) | 12·0 (2·4) | 5·7 (1·1) | 3·5 (1·0) | 2·2 (1·1) | 40·0 (6·6) |
| 4 | 1/2 F+M | 15·8 (2·9) | 14·0 (4·2) | 5·5 (2·0) | 4·0 (1·7) | 2·0 (0·9) | 41·0 (8·8) |
| 5 | FDL | – | – | – | – | – | – |
| 6 | FDL+M | 14·4 (3·6) | 11·7 (5·1) | 3·8 (0·7) | 3·5 (2·0) | 1·7 (0·9) | 35·0 (11·1) |

*F=fluorescein; M=mannitol; FDL=fluorescein dilaurate.

Fluorescein was determined spectrophotometrically. A 0·5 ml volume of urine was added to 4·5 ml 0·1 M sodium hydroxide and incubated at 70°C for 10 minutes to hydrolyse any fluorescein glucuronide, which is colourless, to free fluorescein. After centrifugation, the absorbance (A) of the supernatant was measured at 492 nm against water. Fluorescein recovery, expressed as a percentage of the dose administered, was calculated from the equation: $(A_{492} \times \text{urine volume (ml)})/35$. The denominator was determined from the dose administered, the dilution and the absorption coefficient (pancreolauryl test: technical usage leaflet, Charwell Pharmaceuticals). When a half dose of fluorescein was used (test 4) the denominator was halved to 17·5.

Mannitol was determined by a spectrophotometric method using a Cobas-Bio centrifugal analyser (Roche). A 20 µl volume of urine and 10 µl water w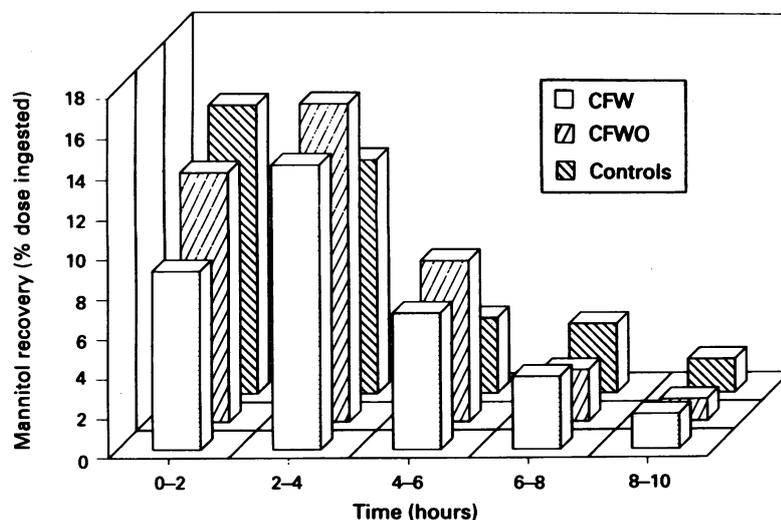ere added to 40 µl glycine buffer-NAD reagent and the absorbance measured at 340 nm. A 15 µl volume of mannitol dehydrogenase was added and the increase in absorbance followed for five minutes. The change in absorbance was a measure of the amount of NAD converted to NADH and therefore directly proportional to the mannitol concentration of the sample.[18]

The recovery of each marker was expressed as a percentage of the dose administered, and the test result was expressed as a ratio of fluorescein recovery to mannitol recovery (F:M ratio). In accordance with other studies[5-10] a ratio of 30 was chosen as the cut off between normal and decreased exocrine pancreatic function. The significance of the differences between marker recoveries and F:M ratios was assessed using $t$ tests. All studies were carried out with informed consent and approval of the local ethical committee.

## Results

### HEALTHY SUBJECTS

In healthy adults there was no significant difference between total fluorescein recovery whether given alone (test 1), with mannitol (test 3), or in a half dose with mannitol (test 4) (table 1). There was no significant difference in total mannitol recovery whether given alone (test 2), with fluorescein (test 3), in half dose with fluorescein (test 4), or with fluorescein dilaurate (test 6) (table 2). Fluorescein recovery from fluorescein dilaurate (test 5) was not modified by the presence of mannitol (test 6) (table 1). The percentage fluorescein recovery for each two hourly urine aliquot is shown in table 1 and for mannitol in table 2.

### CYSTIC FIBROSIS

When fluorescein dilaurate and mannitol were given together (the modified test, test 6) it was possible to distinguish clearly between patients with cystic fibrosis and healthy controls (whether or not the patients were receiving enzymes) due to a highly significant reduction in urinary fluorescein recovery. Mean (SD) 10 hour urinary volumes were similar for healthy controls, for those with cystic fibrosis not taking their enzyme treatment (CFWO), and for those with cystic fibrosis who were receiving enzyme replacement (CFW) (1475 (278) $v$ 1008 (443) $v$ 1129 (348) ml). Ten hour mannitol recovery was not significantly different from that of healthy adults (mean (SD) percentage dose administered: 35·8 (10·6)% in patients with cystic fibrosis receiving enzymes and 40·1 (18·1)% in those not taking enzymes). Neither was there a significant difference in urine mannitol recoveries found every two hours (fig 1).

There was a highly significant (p<0·001) difference in total mean (SD) percentage fluorescein recoveries, however (18·7 (3·8)% (controls); 1·2 (0·5)% (CFWO); 1·1 (0·7)% (CFW)) (fig 2), resulting in a highly significant difference in total 10 hourly fluorescein to mannitol (F:M) ratios (57·3 (18·2) $v$ 3·4 (1·4) $v$ 3·2 (1·6)) (controls $v$ CFWO and CFW; p<0·001) (fig 3).

When the results were expressed as F:M ratios



Figure 1 *Mean urinary recovery of mannitol in two hourly aliquots after ingestion of 25 mmol mannitol and 0·5 mmol fluorescein dilaurate. CFW=patients with cystic fibrosis taking enzyme supplements; CFWO=those not taking pancreatic enzymes.*
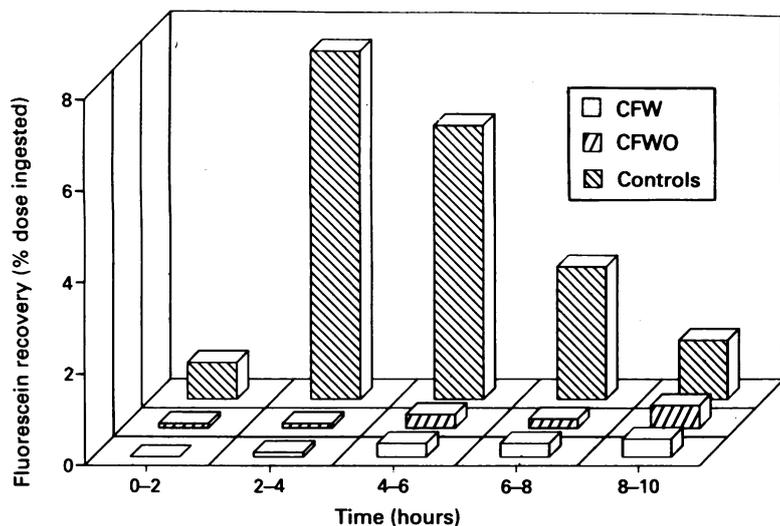
Figure 2  Mean urinary recovery of fluorescein in two hourly aliquots following ingestion of 25 mmol mannitol and 0·5 mmol fluorescein dilaurate. CFW=patients with cystic fibrosis taking enzyme supplements; CFWO=those not taking pancreatic enzymes.



Figure 4  Fluorescein:mannitol (F:M) ratios calculated for aliquots of urine between two and eight hours. Open circles represent patients with cystic fibrosis who were not taking enzyme supplements.

for each urine aliquot the differences between healthy controls and patients with cystic fibrosis, receiving or not receiving enzymes, were highly significant in aliquots collected between two and eight hours after marker ingestion (figs 3 and 4) (p<0·001).

## Discussion

Fluorescein dilaurate is a colourless, poorly water soluble synthetic ester which when given by mouth is hydrolysed by aryl esterases of the pancreatic secretions to lauric acid and water soluble fluorescein.[19] The latter is absorbed in the small intestine, partly conjugated in the liver, and excreted in the urine where it may be detected spectrophotometrically. In the absence of pancreatic digestion fluorescein dilaurate remains colourless because the opening of the lactone ring, which stabilises the double bonds of one of the phenolic groups responsible for fluorescence, does not occur. Urinary free

fluorescein recovery is therefore an indirect measure of exocrine pancreatic function. Fluorescein resists metabolism in the gastrointestinal tract and after uptake. Its pharmacokinetics after administration by mouth and intravenously have been studied in adults[20] and are broadly comparable with those of mannitol.

Mannitol is a hexitol which resists metabolism within the gastrointestinal tract and systemically. It has no affinity for active transport systems,[21-23] its distribution is restricted to the extracellular space,[24] and it is wholly and solely excreted in the urine with a clearance rate similar to that of creatinine.[23] It is present in some infant feeds[25] and has proved to be a useful and safe marker to measure changes in the passive intestinal permeability of infants in a variety of physiological and pathological disorders.[15-17] A dose of 25 mmol was chosen because this had been established as a suitable, safe, and detectable dose in these earlier studies.

By combining fluorescein dilaurate and mannitol in the same test the factors which affect absorption, distribution, and excretion of free fluorescein and mannitol apply equally to both, and changes in the urinary recovery of the former compared with the latter (F:M ratio) will be an indirect measure of pancreatic digestion.

The fluorescein dilaurate-mannitol test was well tolerated by all subjects. In contrast with other studies[26] we did not find the mean mannitol recovery to be significantly higher in patients with cystic fibrosis. Most of the fluorescein and mannitol was recovered within eight hours of ingestion. When fluorescein dilaurate and mannitol were given together and the results were expressed as a fluorescein to mannitol ratio there was clear discrimination between healthy adults and patients with cystic fibrosis, whether they were receiving pancreatic enzyme supplements or not. This indicates that exogenous enzyme preparations do not contain the esterases capable of cleaving fluorescein dilaurate.

When F:M ratios were calculated for each two hourly urinary aliquot it was clear that from two to eight hours there was a significant difference between healthy subjects and patients with cystic fibrosis (fig 3). The period before two hours probably represents the time taken for the lauric acid residues to be cleaved from fluorescein
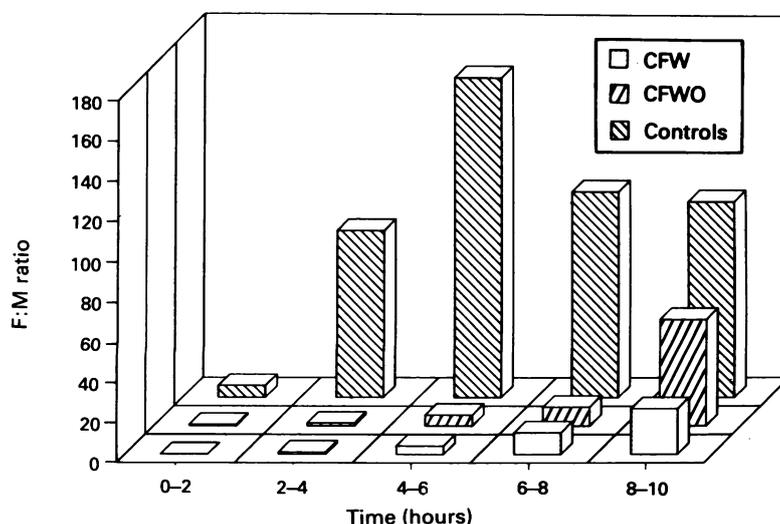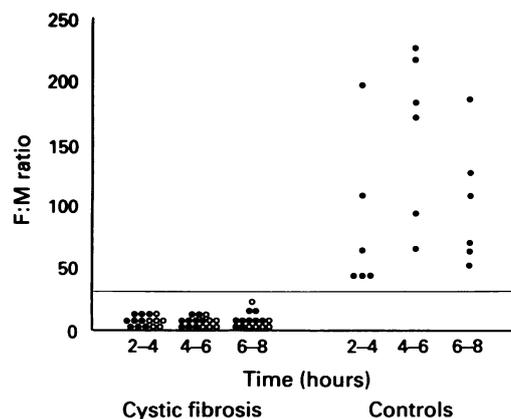


Figure 3  Mean fluorescein:mannitol (F:M) ratios calculated from two hourly aliquots of urine. CFW=patients with cystic fibrosis taking enzyme supplements; CFWO=those not taking pancreatic enzymes.

dilaurate and for the fluorescein released to be absorbed and excreted in the urine. In patients and healthy controls 95% of mannitol excreted in the urine was recovered within eight hours of ingestion. In the healthy subjects 93% of fluorescein excreted in the urine was recovered within eight hours of ingestion. After this time the recovery of the two markers was so small in patients and controls (figs 1 and 2) that expression of the result as an F:M ratio was no longer reliable (fig 3).

Using a combination of fluorescein dilaurate and mannitol we have shown that the second test day is no longer required and that the test may be performed using a shorter urine collection time. A reliable result may be obtained by collecting a single urine specimen at any time between two and eight hours (fig 4). This is of major importance if the test is to be adapted further for use in infancy. We believe that this new tubeless one day test of exocrine pancreatic function should be used as follows. After an overnight fast, the subject should empty his or her bladder and obtain a pretest urine sample. Fluorescein dilaurate 0·5 mmol and mannitol 25 mmol should then be taken by mouth followed by a light breakfast which includes at least 500 ml of clear fluid. Two hours later the bladder should be emptied and the urine collected discarded. The next urine specimen passed thereafter (between two and eight hours) should be used to measure the F:M ratio. It is expected that a test kit will become commercially available. Studies aimed at further modification of the test for use in infancy are underway.

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