

# Binding and diffusion characteristics of $^{14}\text{C}$ EDTA and $^{99\text{m}}\text{Tc}$ DTPA in respiratory tract mucus glycoprotein from patients with chronic bronchitis

M S CHEEMA, S GROTH, C MARRIOTT

*From the Department of Pharmacy, Brighton Polytechnic, Brighton, Sussex; and the Department of Clinical Physiology and Nuclear Medicine, Finsen Institute and Rigshospitalet, Copenhagen, Denmark*

**ABSTRACT** Measurement of pulmonary clearance of an inhaled aerosol of technetium-99m labelled diethylenetriaminepenta-acetate (DTPA) by external detection methods has been used widely as an index of permeability across alveolar epithelium and bronchial mucosa. To determine the applicability of the tracer to measurement of permeability in the airways the diffusion and binding characteristics of  $^{99\text{m}}\text{Tc}$  DTPA and the chemically related ethylenediaminetetra-acetic acid labelled with carbon-14 ( $^{14}\text{C}$  EDTA) was studied in purified respiratory tract mucus glycoprotein from patients with chronic bronchitis. The diffusion coefficients for  $^{99\text{m}}\text{Tc}$  DTPA and  $^{14}\text{C}$  EDTA through mucus gels were significantly lower than those for tritiated water. Both molecules bound to the mucus gels with high affinity at two independent low capacity sites. Appreciable amounts of  $^{99\text{m}}\text{Tc}$  DTPA or  $^{14}\text{C}$  EDTA are therefore unlikely to cross mucus layers of physiological thickness over periods of four or five hours. This suggests that when pulmonary clearance is determined by the  $^{99\text{m}}\text{Tc}$  DTPA method the tracer retained in mucus lined airways will provide background activity. This study supports the assumption that pulmonary clearance measurements are mainly measuring alveolar epithelial permeability and should not be used to study bronchial epithelial permeability.

## Introduction

Rinderknecht *et al*<sup>1</sup> suggested the use of the pulmonary clearance rate of inhaled aerosolised  $^{99\text{m}}\text{Tc}$  labelled diethylenetriaminepenta-acetate ( $^{99\text{m}}\text{Tc}$  DTPA) as an index of pulmonary epithelial permeability. This  $\gamma$  emitting radiopharmaceutical is detected efficiently by external, non-invasive methods—for example, by the gamma camera. The pulmonary clearance is increased in most patients with diffuse interstitial fibrosis and sarcoidosis and in smokers.<sup>1-7</sup> The rate of pulmonary clearance is usually defined by the initial slope (the first 7-15 minutes) of the detected time-activity curve or as the time taken for half the tracer to be cleared from the lungs, which is usually less than 60 minutes.<sup>1-7</sup>

When administered as small aerosol particles, most of the  $^{99\text{m}}\text{Tc}$  DTPA will be deposited in the alveoli. As it is a relatively small hydrophilic molecule it is thought to cross biological membranes by paracellular

passage only. Pulmonary clearance measurements are therefore thought to yield information mainly on the integrity of the alveolar epithelium. Recently, to obtain information about the permeability of the bronchial mucosa, aerosolised  $^{99\text{m}}\text{Tc}$  DTPA has been administered by an inhalation manoeuvre designed to enhance deposition of  $^{99\text{m}}\text{Tc}$  DTPA in the airways.<sup>8-10</sup> Regardless of the aerosol administration procedure some  $^{99\text{m}}\text{Tc}$  DTPA will inevitably be deposited in the airways as well as in alveoli. Unless bronchial mucosal permeability and alveolar epithelial permeability are identical, the time-activity curve as determined by the gamma camera will be the sum of different components. The  $^{99\text{m}}\text{Tc}$  DTPA deposited on mucus lined airways will have to diffuse through mucus before it can be transported across the bronchial mucosa. If the diffusion of  $^{99\text{m}}\text{Tc}$  DTPA in mucus is slow, this may have a greater influence on the bronchial component of an externally derived time-activity curve than the permeability of the bronchial mucosa itself.

The aim of this study was to evaluate the impact of deposition of inhaled  $^{99\text{m}}\text{Tc}$  DTPA in airways on measurements of pulmonary clearance by determining the binding and diffusion characteristics of

Address for reprint requests: Professor C Marriott, Pharmaceutical Sciences Research Group, Cockcroft Building, Moulsecoomb, Brighton BN2 4GJ.

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$^{99m}\text{Tc}$  DTPA in purified respiratory tract mucus glycoprotein from patients with chronic bronchitis. Similar measures were determined for  $^{14}\text{C}$  EDTA as the physiological behaviour of this tracer in man is very similar to that of  $^{99m}\text{Tc}$  DTPA.

### Methods

Sputum was obtained from patients with chronic bronchitis as "normal" bronchial mucus from undiseased subjects was impossible to obtain in adequate quantities. It was collected, pooled, and stored at  $-20^\circ\text{C}$ . Purulent or blood stained samples were discarded.

### PURIFICATION

Samples were homogenised in an equal volume of a protease inhibiting solution before filtration through glass wool. The composition of the solution was: EDTA  $10^{-3}$  mol/l, phenylmethylsulphonyl fluoride  $10^{-3}$  mol/l, sodium chloride 154 mmol/l, and sodium azide ( $\text{NaN}_3$ ) 3 mmol/l. Samples were applied to a Sepharose CL4B gel exclusion column at  $4^\circ\text{C}$  and the excluded fraction was collected. This fraction was exhaustively dialysed against water and ultrafiltrated through an Amicon PM 10 membrane at a pressure of  $5\text{ kg/cm}^2$ . Ultrafiltration was carried out until gels were produced. These were exhaustively dialysed against 0.05 M tris hydrogen chloride buffer at pH 7.40. Gels were stored at  $-20^\circ\text{C}$  with no apparent deleterious effects.<sup>11</sup> They contained no residual EDTA from the protease inhibiting solution.

### DIFFUSION CHARACTERISTICS

Apparent diffusion coefficients of  $^{14}\text{C}$  EDTA and  $^{99m}\text{Tc}$  DTPA were determined by a capillary desorption method<sup>12</sup> and the results compared with those for tritiated water ( $^3\text{H}_2\text{O}$ ). The desorption of radioisotope from a geometrically defined slab<sup>13</sup> of purified mucus gel was monitored at  $4^\circ\text{C}$ . Desorption was performed in 2.0 cm long, flat ended, silica glass capillary tubes. The tubes were thoroughly washed, rinsed, and dried in an oven at  $80^\circ\text{C}$ . They were placed in silanising fluid for 15 minutes and stored in the oven at  $80^\circ\text{C}$  until required.

Mucus gels of known dry weight were prepared and placed in small sacs of dialysis tubing so that each sac contained about 1.0 ml of gel. The gels were dialysed against 10 ml buffer (pH 7.40) at  $4^\circ\text{C}$  for at least 72 hours. The buffer also contained the radiolabelled diffusant at a concentration of about 10 000 counts  $\text{min}^{-1}\text{ ml}^{-1}$ . After dialysis the radioactivity of 200  $\mu\text{g}$  aliquots of mucus gel was measured.

The remaining radioactive mucus gel was gently centrifuged to remove air bubbles. Ten capillary tubes were then filled by carefully drawing gel into each tube

by suction with a syringe and thick walled silicone rubber tubing. Care was taken not to introduce air bubbles. The capillary tubes were then sealed and carefully inspected for air bubbles, particularly at the gel-dialysis membrane interface. Any tubes containing air bubbles were discarded.

Each capillary was placed in a test tube containing 3.0 ml of buffer maintained at a temperature of  $4^\circ\text{C}$  by a large volume, refrigerated, constant temperature bath. The sink solution was agitated by a slow stream of bubbles of nitrogen gas or by compressed air that had been passed through concentrated sulphuric acid and a molecular sieve to remove moisture, which would otherwise condense in the test tubes. When compressed air was used the pH of the sink solution remained at 7.40, indicating that the production of carbonic acid by the dissolution of carbon dioxide from the air supply was adequately buffered. After 16–24 hours the tubes were removed, dried with absorbent tissue, and the dialysis membranes removed. The gels were recovered and weighed. Scintillant (4.17 ml) was added in the case of  $^{14}\text{C}$  EDTA and the total activity measured after vigorous shaking. The diffusion period was measured to the nearest second, and was chosen so that the ratio of total activities within the gel at the end of the experiment to that at the beginning was about 0.5.

The fraction of diffusant desorbed was used to calculate diffusion according to the equation suggested by Newman<sup>14</sup>:

$$\frac{N}{N_0} = 1 - \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp \frac{-D(2n+1)^2 \pi^2 t}{4L^2},$$

where  $N$  = activity at time  $t$ ,  $N_0$  = activity at time zero,  $t$  = time elapsed in seconds,  $L$  = half capillary length in centimeters, and  $D$  = apparent diffusion coefficient.

The calculated diffusion coefficient does not include a correction for the presence of a polyelectrolyte gradient, which would hinder desorption or reverse osmotic flux. The results were calculated as the mean of the 10 replicates.

### BINDING CHARACTERISTICS

The presence of binding sites for  $^{14}\text{C}$  EDTA and  $^{99m}\text{Tc}$  DTPA on mucus was determined by means of an ultrafiltration method at  $20^\circ\text{C}$ .<sup>15</sup> A Scatchard analysis<sup>16</sup> was used and the results are presented as the mean of 12 replicates.

### RADIOACTIVITY

$^{14}\text{C}$  EDTA and  $^3\text{H}_2\text{O}$  isotopic content was determined by liquid scintillation counting (Beckman model LS3133, Beckman, Purley, Surrey). The scintillant consisted of diphenyloxazole 5 g/l, 1,4-bis [5-phenyl-2-oxazolyl]-benzene 1.5 g/l, Triton X-100 322 ml/l in

Table 1 Diffusion characteristics for  $^{99\text{m}}\text{Tc}$  DTPA,  $^{14}\text{C}$  EDTA, and tritiated water in mucus

Diffusant	$D (\times 10^{-6}\text{cm}^2\cdot\text{s}^{-1})$	$T(N/N_0 = 0.5)$ (hours)
$^{99\text{m}}\text{Tc}$ DTPA	9.65 (SD 0.44)	14.40
$^{14}\text{C}$ EDTA	9.08 (SD 0.47)	15.30
Tritiated water	23.30 (SD 0.51)	5.96

D, diffusion coefficients;  $T(N/N_0 = 0.5)$ , time until half of the diffusant had been desorbed.

toluene. All materials were of scintillation counting grade (Scintran, BDH Chemicals, Poole, Dorset).  $^{99\text{m}}\text{Tc}$  DTPA was counted with a sodium iodide well counter and a multichannel analyser (Canberra series 80, Meriden, Connecticut) at a standard counting error of 1%.

## Results

The diffusion coefficients of  $^{99\text{m}}\text{Tc}$  DTPA and  $^{14}\text{C}$  EDTA through the mucus gels were significantly less than those seen with tritiated water ( $p < 0.005$ ; table 1). The time required for half the diffusant to

Table 2 Binding site capacity ( $n_1$ , the number of molecules of bound molecule or ligand per molecule of macromolecule) and the association constant ( $K_n$ , binding affinity) for each ligand

Ligand	$n_1$	$n_2$	$k_1$	$k_2$
			(litres/mol)	
$^{99\text{m}}\text{Tc}$ DTPA	5.33	12.20	3.38	0.645
$^{14}\text{C}$ EDTA	5.65	12.83	2.84	0.581

leave the mucus was in the region of 15 hours at the concentrations studied.

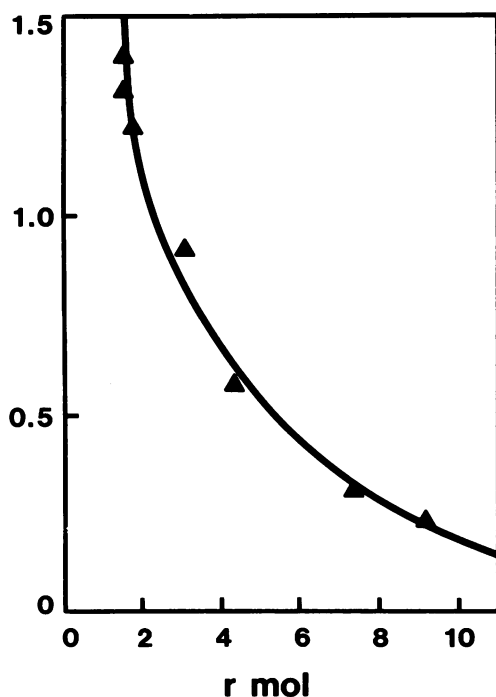
The Scatchard analysis indicated the presence of two independent binding sites (figure). The same sites appeared to bind both tracers, as evidenced by the similarity of the binding site capacity—that is, the number of molecules of bound molecule or ligand per molecule of macromolecule (table 2). The association constant ( $k$ ) for each ligand indicated that both sites had a high affinity for the ligands.

## Discussion

A major reason why  $^{99\text{m}}\text{Tc}$  DTPA is well suited for the measurement of pulmonary permeability, it has been suggested,<sup>6</sup> is that it is a non-polar agent and that the intercellular space (pores) between the alveolar cells is about 0.9 nm but the size of the  $^{99\text{m}}\text{Tc}$  DTPA is only 0.5 nm. When administered as an aerosol, regardless of the administration procedure, some of the  $^{99\text{m}}\text{Tc}$  DTPA will inevitably be retained on the airways.<sup>17</sup> Bronchial airway epithelium differs from alveolar epithelium in many respects. Alveoli are lined predominantly by surfactant and airway epithelium by mucus. It is likely that an externally derived time-activity curve for the clearance of  $^{99\text{m}}\text{Tc}$  DTPA will be made up of several components, reflecting the presence of different types of epithelium. The results of this study indicate one reason why there may be a difference between bronchial and alveolar clearance of  $^{99\text{m}}\text{Tc}$  DTPA. Even if the duration of the lung detection of radioactivity was extended to more than four or five hours, an insignificant part of inhaled  $^{99\text{m}}\text{Tc}$  DTPA aerosol retained on a mucus layer of physiological thickness ( $> 1 \mu\text{m}$ ) would have diffused through it.

This avid binding of  $^{99\text{m}}\text{Tc}$  DTPA to mucus means that measurements of pulmonary clearance will provide limited information about bronchial mucosa permeability. Most transpulmonary transport is likely to occur across non-ciliated terminal airways and alveoli. Airway  $^{99\text{m}}\text{Tc}$  DTPA will provide a background count on the time-activity curve. As, however, mucus is subject to mucociliary clearance, the mucus bound  $^{99\text{m}}\text{Tc}$  DTPA will not remain in the same position in relation to the detection equipment and the

$r/c \times 10^{-4}, 1$



Scatchard plot for the binding of  $^{99\text{m}}\text{Tc}$  DTPA to mucus, from a patient with chronic bronchitis, purified by an ultrafiltration method.

contribution to the background counts will not therefore be constant. Mucociliary clearance<sup>18</sup> is considerably slower than the pulmonary clearance rates usually reported.<sup>2-7</sup> In subjects without airflow limitation about 15% of slowly inhaled small particles (<2  $\mu\text{m}$ ) are deposited on the airways and 85% in the alveoli.<sup>6</sup> In patients with obstructive lung disease, however, there is increased airway deposition. The more <sup>99m</sup>Tc DTPA is deposited on the airways, the more pulmonary clearance measurements will underestimate true pulmonary epithelial permeability.

One approach to solving the problem of airway deposition of <sup>99m</sup>Tc DTPA would be to resolve the time-activity curve into several components. For this purpose it would have to be assumed that the relative amounts of tracer deposited on mucus lined airways and in the periphery can be estimated exactly or that a final slope of the time-activity curve can be defined. There is no external detection method capable of discriminating between the amount of aerosol retained in the airways and the amount retained in alveoli. The detection period could be prolonged until a final slope could be safely defined, but such a long procedure would be unsatisfactory in practice. Furthermore, the slow component cannot at present be identified with certainty as the bronchial component.

Elwood *et al*<sup>8</sup> suggested an approach to the determination of pulmonary clearance of aerosolised <sup>99m</sup>Tc DTPA that to some extent avoids the problems of airway deposition of the <sup>99m</sup>Tc DTPA. They estimated the transport of <sup>99m</sup>Tc DTPA from lung to blood from the count rate in plasma samples drawn at fixed times after the termination of the inhalation. The method, however, assumes that the plasma count rate is normalised with respect to an initial externally monitored count rate. With this method the <sup>99m</sup>Tc DTPA deposited on the airways may still influence the results. The method does, however, have the advantage that the results are independent of the magnitude of the mucociliary clearance. This was of particular importance in the study<sup>8</sup> where the approach was introduced, as it attempted to estimate the permeability of the bronchial mucosa in patients with asthma. The <sup>99m</sup>Tc DTPA was administered in a manner that should result in deposition of most of the aerosol on the airways. The lack of difference in permeability of the airway mucosal between asthmatic and normal subjects is probably due to the fact that permeability of the bronchial mucosa was never measured as most of the <sup>99m</sup>Tc DTPA is likely to have been bound to the mucus glycoprotein. Probably various solutes, including therapeutic agents, are administered to the airways without measurement or even consideration of the agent's binding to the mucus or diffusion through it.

Recently Groth *et al*<sup>19</sup> derived a method for cal-

culating mean transit time ( $\bar{t}$ ) for the transport of <sup>99m</sup>Tc DTPA from lungs to blood ( $\bar{t}(L)$ ) from the plasma *time-activity* curve without external detection. The method makes use of the fact that the  $\bar{t}$  of an inert tracer through compartments in series is the sum of the mean transit time through each compartment. After inhalation of the <sup>99m</sup>Tc DTPA the plasma *time-activity* curve is defined and used to calculate  $\bar{t}$  for the transport of <sup>99m</sup>Tc DTPA across the pulmonary epithelial membrane through the extracellular volume (ECV) until eliminated by the kidneys,  $\bar{t}(L + \text{ECV})$ . Subsequently, <sup>99m</sup>Tc DTPA is injected intravenously and the new *time-activity* curve used to calculate  $\bar{t}$  for the transport of <sup>99m</sup>Tc DTPA from ECV to the kidneys,  $\bar{t}(\text{ECV})$ .  $\bar{t}(L)$  can then be calculated as  $\bar{t}(L) = \bar{t}(L + \text{ECV}) - \bar{t}(\text{ECV})$ . In this way geometrical detection problems can be almost completely eliminated and <sup>14</sup>C EDTA, or rather chromium-51 (<sup>51</sup>Cr)EDTA can be used as a tracer; the latter is unsuitable for external detection because of unfavourable radiation energies. The method is, however, very time consuming.

Inevitably some inhaled <sup>99m</sup>Tc DTPA will be swallowed after being cleared by the mucociliary escalator, as will the tracer originally deposited in the mouth. The absorption of <sup>99m</sup>Tc DTPA from the intestinal tract, however, is very poor and slow, probably owing to firm binding of <sup>99m</sup>Tc DTPA to gastric and intestinal mucus. The poor absorption of <sup>99m</sup>Tc DTPA from the gastrointestinal tract has been exploited in clinical studies of gastric emptying using meals containing <sup>99m</sup>Tc DTPA.<sup>20-24</sup> Recirculation of gastrointestinal <sup>99m</sup>Tc DTPA will not therefore substantially influence measurements of pulmonary clearance.

The results of the present study do not provide evidence against the assumption that pulmonary clearance measurements are mainly measuring alveolar epithelial permeability. They suggest that, to improve interpretation of such measurements, the mucus binding of the tracer may need to be taken into consideration. Inhaled aerosolised <sup>99m</sup>Tc DTPA should not be used to study bronchial epithelial permeability.

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