Autoradiographic Imaging of the Distribution of 5-Fluorouracil Through Cervical Tissue Following *in Vitro* Surface Application of a Bioadhesive Cervical Patch

A. David Woolfson,[1,3] Dermot F. McCafferty,[1] Paul A. McCarron,[1] and John H. Price[2]

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The distribution of 5-fluorouracil through cervical tissue has been assessed following the *in vitro* application of a bioadhesive patch to excised human cervix. The bioadhesive matrix contained a total of 20 mg of 5-fluorouracil spiked with 5-fluorouracil-6-^{3}H and was applied for fixed periods of either 4 or 24 hours. Tissue slices were sectioned perpendicular to the plane of the applied patch and the autoradiographic image developed by placing a frozen tissue slice on Hyperfilm with subsequent instant thawing and refreezing, the resulting bilayer being maintained at −18°C for 24 hours. The developed image was analysed by scanning densitometry and raster scans were visualised with three-dimensional contouring software. The autoradiograms showed darker areas surrounding tissue ducts, suggesting that 5-FU was spilling from the lumen into the surrounding stroma. Transport of 5FU via aqueous channels may thus make an important contribution to the rapid penetration of the drug through the cervical stroma. Three-dimensional autoradiographic images showed that, for a 4-hour patch application, there were areas of relatively low drug concentration within the upper 5 mm of tissue, where CIN lesions can exist in the glandular tissue or cervical crypts. However, extending the application time to 24 hours produced areas of high drug concentration extending throughout this region.

KEY WORDS: autoradiography; bioadhesive; cervical intraepithelial neoplasia; 5-fluorouracil.

INTRODUCTION

Drugs used to combat neoplasms interrupt the growth pattern of cancerous cells. Before cell death can be achieved, the concentration of drug presented to the abnormal cell must exceed a threshold, especially during particular stages of the mitotic cycle, otherwise, there is a risk of therapeutic failure and further cell invasion. Cervical intraepithelial neoplasia (CIN) begins in the epithelial layers and is initially of a non-invasive nature. Systemic delivery of drug to this epithelium relies on diffusion from underlying blood vessels, which do not penetrate this cellular layer. Therefore, the drug gradient acting into the epithelium requires high blood levels, leading to the possibility of unpleasant side-effects.

Drug delivery systems that incorporate bioadhesive polymers have been shown to be of use on various mucosal surfaces that have previously been unavailable to non-bioadhesive devices. Adhered devices are close to the epithelial surface and drug diffusion through this layer is expected to be good. Furthermore, therapeutic levels can be achieved in the proximity of the epithelium without the need for systemic dosing. In effect, the usual drug gradient becomes reversed and the underlying vasculature acts as a drug sink. This method of drug delivery is particularly suited for the treatment of epithelial carcinoma, such as CIN, since the external surface of the cervix is a readily accessible site. Thus, we have developed a novel bioadhesive cervical patch (1) that continuously delivers 5-fluorouracil (5-FU) from a drug-loaded bioadhesive matrix while remaining secured to the cervical epithelium for at least 24 hours (2). The advantages of such a system for both patients and clinicians have been previously identified (2).

Presently, treatment of non-regressing CIN lesions is by ablative or excisional surgical techniques (3). Such methods have a high success rate (4) that must be matched by any prospective local drug treatment. Of particular importance is the depth of drug penetration into the cervical stroma such that dysplastic lesions originating deep within ridges of the cervical canal will be exposed to a cytotoxic concentration of 5-FU. It is now recognised that, to ensure an effective therapeutic response, the externally applied cytotoxic drug must penetrate the cervical tissue to a depth of at least 4 mm in order to reach lesions residing in the glandular tissue or cervical crypts (5).

It has recently been demonstrated *in vitro* (2), using liquid scintillation spectrometry, that a bioadhesive cervical patch containing 20 mg of 5-FU produces a drug concentration approximately 2000 times in excess of the cytotoxic threshold at a depth up to 5.5 mm below the epithelial surface. This technique provides a quantitative 'snapshot' of drug concentration at a given sampling point in the tissue. However, it does not provide information on the overall distribution of drug throughout the cervix, an equally important factor for effective therapy. Such information is also of value in determining the method of drug penetration through the cervix, about which little is presently known. Radiolabelled 5-FU can be detected following its exposure to photographic emulsion (6). In the present study, therefore, cervical tissue sectioned perpendicularly to the tissue surface is used to obtain an autoradiographic image of the overall distribution of 5-FU through the cervical stroma following application of the bioadhesive cervical patch delivery system.

MATERIALS AND METHODS

5-Fluorouracil, 5-fluorouracil-6-^{3}H and 2,5-diphenyloxazole (PPO) were obtained from Sigma Chemical Co. (Poole, U.K.). Hyperfilm-^{3}H was a product of Amersham International plc (Aylesbury, U.K.). Hypam fixer and nuclear emulsion, grade K5, were obtained from Ilford Ltd. (Knutsford, U.K.). Universal Developer was supplied by Patterson Products (Dagenham, U.K.). Optimum Cutting Temperature Compound (OCTC) was purchased from Tissue Tek, Miles Inc., Elkhart). Carbopol 981 was supplied by B.F. Goodrich Ltd. (Hounslow U.K.). Medical grade polyvinyl

[1] School of Pharmacy, The Queen's University of Belfast,
[2] Department of Obstetrics and Gynaecology, Belfast City Hospital, Belfast, United Kingdom.
[3] To whom correspondence should be addressed.

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chloride (PVC) emulsion was obtained from Rusch Manufacturing (Craigavon, U.K.). Glycerin was British Pharmacopoeial grade. Water was Reagent Grade I obtained from a Milli-Q System (Millipore, Watford, U.K.). All other chemicals, used to prepare phosphate-buffered saline, were of analytical reagent grade.

Preparation of Bioadhesive Cervical Patches Containing 5-Fluorouracil-6-^{3}H

Cervical patches were manufactured as previously described (2) using cold 5-fluorouracil spiked with 5-fluorouracil-6-^{3}H (a non-labile form in which the radiolabelled hydrogen is bonded directly to a ring carbon atom, thereby eliminating the possibility of exchange) to give approximately 3 $\times$ 10^6 dpm in each square centimetre of patch. Patches were circular with a diameter of 26 mm and a 5-FU loading of 20 mg per patch. Sample patches were quality controlled by determining the total drug content per patch, bioadhesion to cervical tissue and the actual radioactivity, dpm cm^{-2}, as previously described (2).

In Vitro Drug Diffusion and Tissue Sectioning Procedures

Excised cervical tissue (from hysterectomies, donated with permission in all cases) was cut into slabs, 1 cm^2 across the epithelial face and approximately 10 mm deep. The tissue was clinically judged to be healthy, the donors being free of cervical disease, and was visually checked for surface damage before use. The tissue was supported on a stainless steel filter grid (Millipore Corp., Cambridge, Ma.) placed across the top of the reservoir of a Franz cell, as previously described (2). The reservoir was filled with 10.0 ml of sterile phosphate buffered saline (pH 7.2), sufficient to bring the fluid level up to the grid and expel traces of air.

A cervical patch, the bioadhesive matrix spiked with radiolabelled 5-fluorouracil and containing a total of 20 mg of 5-fluorouracil, was applied to the uppermost epithelial layer of the tissue slab such that the bioadhesive layer and tissue were in direct, intimate contact. Patches were applied for fixed time periods of either 4 or 24 hours. The patch and tissue were separated when the required penetration time had elapsed. The tissue was then flash-frozen by exposing it to a liquid nitrogen atmosphere, without immersion, for 3 minutes.

The tissue block was sectioned by positioning it on the microtome stage so that slices were taken perpendicular to the applied patch (Fig. 1). Slices were cut at a microtome setting of 50 μm and stored in a liquid nitrogen atmosphere until use.

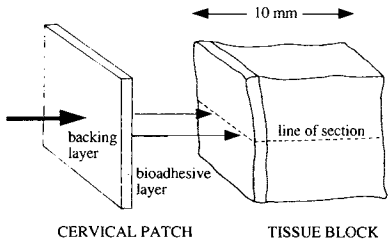

Fig. 1. Sectioning of cervical tissue prior to autoradiography. The cervical patch was applied to the epithelial surface and, following exposure, the tissue sectioned perpendicular to the plane of the applied patch.

Autoradiography

Slices were exposed as follows to either (i) Ilford K5 nuclear emulsion, using a dipping method or (ii) directly applied to Hyperfilm-^{3}H.

(i) Emulsion gel (Ilford K5) was melted at 45 °C and placed into a rectangular box, large enough to accommodate a glass slide. Slices of tissue were removed from cold storage, allowed to thaw onto glass slides for less than two seconds and immediately re-frozen. The tissue was freeze-dried for 24 hours. When dry, slides containing tissue specimens were dipped in emulsion and allowed to drain and dry. Slides were exposed for 24 hours at −20°C before development.

(ii) Frozen tissue slices were placed directly onto Hyperfilm-^{3}H, whereupon they instantly thawed and bonded onto it. This bilayer was immediately re-frozen in a liquid nitrogen atmosphere and stored at −18°C for 24 hours. The estimated length of time the tissue slices were unfrozen was less than two seconds.

Hyperfilm-^{3}H was developed and fixed under safelighting conditions (Kodak B6 filter). The photographic image was developed using Universal Developer at 20 °C for 2 minutes. The emulsion was removed, washed under water for 2 minutes and fixed in Ilford Hypam fixer for 2 minutes. The image was then washed in water for 10 minutes and allowed to dry. Emulsion-dipped slides were also developed for 2 minutes and fixed for twice the clearing time period, as recommended by the emulsion manufacturer. Dried images were then scanned using a scanning densitometer (Chromoscan, Joyce-Loebl, Gateshead, U.K.) and raster pictures visualised with three dimensional contouring computer software (UNIMAP, Uniras A/S, Soborg, Denmark).

RESULTS AND DISCUSSION

Autoradiographic procedures frequently involve dipping the prepared histological specimen into a bulk melted emulsion (6) or exposing the specimen to a flat plate coated with dry emulsion. The choice of procedure is dictated by the type and energy of the emitted radiation. Both methods were evaluated during this study. However, pre-prepared sheets coated with emulsion were found to be easier to manipulate. The grade of film chosen was Hyperfilm ^{3}H, which is a single-coated, clear plastic base sheet with no anti-scratch layer. Beta radiation from tritium is of such a low energy that an anti-scratch layer would prevent it from reaching the emulsion. The sensitivity of this film was rated such that 2 $\times$ 10^6 counts were required over a 5 mm slot during 24 hours to give a satisfactory image density (7).

Direct contact between frozen film and frozen tissue without an intermediate thawing stage did not produce satisfactory images, since the distance travelled by beta particles is extremely limited. In such cases, beta particles probably have insufficient energy to traverse the air gap between tissue and film. Indeed, the energy of beta particles emitted from tritium is so low that their range in photographic film is less than the average distance between the silver halide grains (8). In a solid medium, such as a thin layer chromatographic plate, it has been demonstrated (8) that only a small

percentage of beta particles can emerge from the surface of the plate. Thus, contact between frozen tissue and film, with the contact maintained by pressure alone, was insufficient to produce a resolved image.

To increase the visualisation of the image in the direct contact system fluorography was used in a similar procedure to the incorporation of a solid scintillator, such as PPO, into a thin layer chromatographic medium. In this design, a radionuclide-containing spot can be detected by exposing the thin layer plate to photographic film. Beta particles excite the scintillator, producing ultraviolet light. that, unlike the actual particles, is not absorbed and can escape to produce a photographic image. Therefore, in the present study freeze-dried tissue slices were flooded with 7% w/v PPO in ether and allowed to dry. Hyperfilm was maintained in close contact using pressure at −70 °C, a temperature previously shown (9) to give optimum emulsion sensitivity.

Fluorography is fundamentally different to autoradiography in that a photographic image is produced, the former being more sensitive by a factor of 10^3 or greater with the limits of sensitivity being, respectively, $>8 \times 10^6$ dpm cm^{-2} and 8000 dpm cm^{-2} (10). The increased sensitivity means that exposure time can be substantially reduced, often from over one month to about 24 hours (11), which in turn reduces aerial and chemical fogging. However, when the fluorographic images in this study were developed, there was no improvement in image quality. The major difficulty was physical damage caused by crystals of PPO to the photographic emulsion, the latter containing no anti-scratch layer.

The technique that produced the most satisfactory autoradiograms consisted of removing frozen tissue sections, thawing them onto Hyperfilm and immediately refreezing, all steps being performed under safe-lighting conditions. The total time the section remained thawed was less than 5 seconds, thereby ensuring that the drug gradient did not change significantly. This produced the required intimate contact sufficient to expose the silver halide to the beta particles and, consequently, the development time was reduced to only 24 hours. The presence of tissue was initially found to impede the action of the developer. However, pre-soaking the film in water for 1 minute to remove all traces of tissue greatly improved both the speed and resolution of the final image. Furthermore, the contrast between background grains and the image was improved.

Close studies of the autoradiograms showed that the outline of ducts within the tissue were darkly stained, as shown in Fig. 2, a representation of the actual autoradiogram obtained by exposing cervical tissue to a 20 mg 5 -FU patch for 24 hours.. This suggests that transport of 5FU via aqueous channels is an important contribution to the rapid penetration of the drug through the cervical stroma. Closer inspection of particular ducts indicated that 5-FU was spilling from the lumen into the surrounding stroma, as indicated by the darker image in these regions. Thus, the transport of 5FU through cervical tissue is, perhaps, not a simple case of diffusion through an homogeneous barrier, but a combination of diffusion through the cervical stroma and transport through aqueous pores. Interestingly, structures like Nabolian cysts were found not to retain radioactivity in their lumen even though they mostly consist of aqueous mucus, a medium 5-FU would be soluble in.

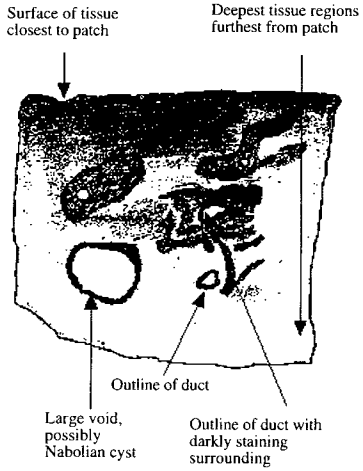

Fig. 2. Autoradiogram of a tissue slice from a cervical tissue sample taken following exposure *in vitro* to a 20 mg bioadhesive cervical patch for 24 hours.

To fully evaluate the images obtained by autoradiography, each was scanned using a scanning densitometer. The traces for typical tissue sections taken following 4 and 24 hour drug exposure experiments are shown, respectively, in Figs. 3 and 4. In Fig 3 it is clear that there is a linear concentration gradient across the tissue, indicating Fickian diffusion of the drug. In Fig. 4, by contrast, an increase in drug concentration is observed in the basal region of the tissue sample, suggesting an apparent departure from Fick's first law. However, it is significant that this increase in tissue concentration of 5-fluorouracil occurred in that part of the tissue sample closest to the receiving fluid. Some degradation of the tissue sample at its interface with the receiving fluid was observed at the end of the 24 hour contact period. As a result, it is likely that the receiving fluid containing 5-fluorouracil had penetrated into the basal layers of the tissue sample during the latter part of the experiment. This effect was not seen in Fig. 3, where little, if any, drug reached the receiving fluid and the shorter contact time allowed the integrity of the basal portion of the tissue sample to be maintained. The possibility of specific binding of the drug to the tissue can be discounted, since the effect is concentrated in only one region of the tissue and, in any case, incubation of tissue samples with 5-fluorouracil solution for 24 hours produced no reduction in the drug concentration in solution compared to controls. Thus, the apparently non-Fickian behaviour, despite the existence of sink conditions,

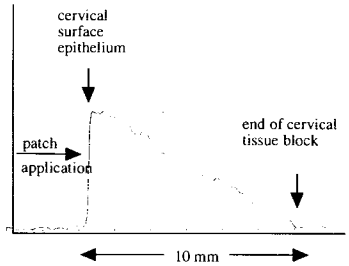

Fig. 3. Scanning densitometer trace of an autoradiogram of a tissue slice from a cervical tissue sample taken following exposure *in vitro* to a 20 mg bioadhesive cervical patch for 4 hours.

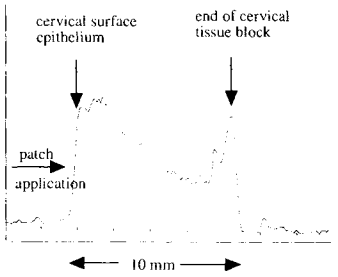

Fig. 4. Scanning densitometer trace of an autoradiogram of a tissue slice from a cervical tissue sample taken following exposure *in vitro* to a 20 mg bioadhesive cervical patch for 24 hours.

indicated in Fig. 4 is probably an unavoidable artifact relating to tissue degradation during prolonged contact with the receiving fluid.

The scanning densitometer had the facility to perform a raster scan, where the complete image could be represented by an alphanumeric grid-pattern. The autoradiogram was scanned in a pre-programmed series of parallel sweeps, thus converting the optical density of the image into an intensity grey scale. The greater optical densities corresponded to areas of film exposed to more intense radiation. In turn, such areas contained more 5-FU and were thus assigned a higher grey scale code. The raster scans from the 4 and 24 hour penetration autoradiograms were translated into spatial co-ordinates and visualised with three-dimensional contouring computer software. These contour maps are shown, respectively, in Figs. 5 and 6. Each alphanumeric character is translated into a value that forms the z co-ordinate on the contour map. The x co-ordinate is an arbitrary value and represents the distance along the plane perpendicular to the plane of the patch. If Fig. 6 is considered, the x axis of 60 marks the position where the patch met the tissue surface. Higher values than 60 represent space above the patch, whereas values from 60 to 20 denote the complete tissue specimen, a distance of 10 mm. The region below 20 represents the area of the receiving fluid in the reservoir. The y axis is a representation of distance across the tissue slab, but parallel to the plane of the applied patch. The plane of the image thus occupies the x-y co-ordinate plane and the grey intensity at every point is lifted into the z plane. In both Figs. 5 and 6 it can be seen that the drug gradient is acting away from the applied patch. Those areas of greatest drug concentration (radioactivity) are shown as areas of blue, decreasing sequentially through shades of green (dark to light), yellow, orange and red. Finally, the lowest drug concentrations appear as magenta and purple regions in the contour maps.

It is readily seen from Fig. 5, in which the tissue had been exposed to the 5-FU-loaded cervical patch for a relatively short time of 4 hours, that the most radioactive (green) regions are confined to an area immediately below the tissue surface, adjacent to the patch. Less radioactive (orange and red) regions extend over approximately 75% of the deepest region of the tissue, although a small yellow area at the base of the tissue does confirm that some drug had penetrated into the receiving fluid and was, in turn, in contact with the deep-

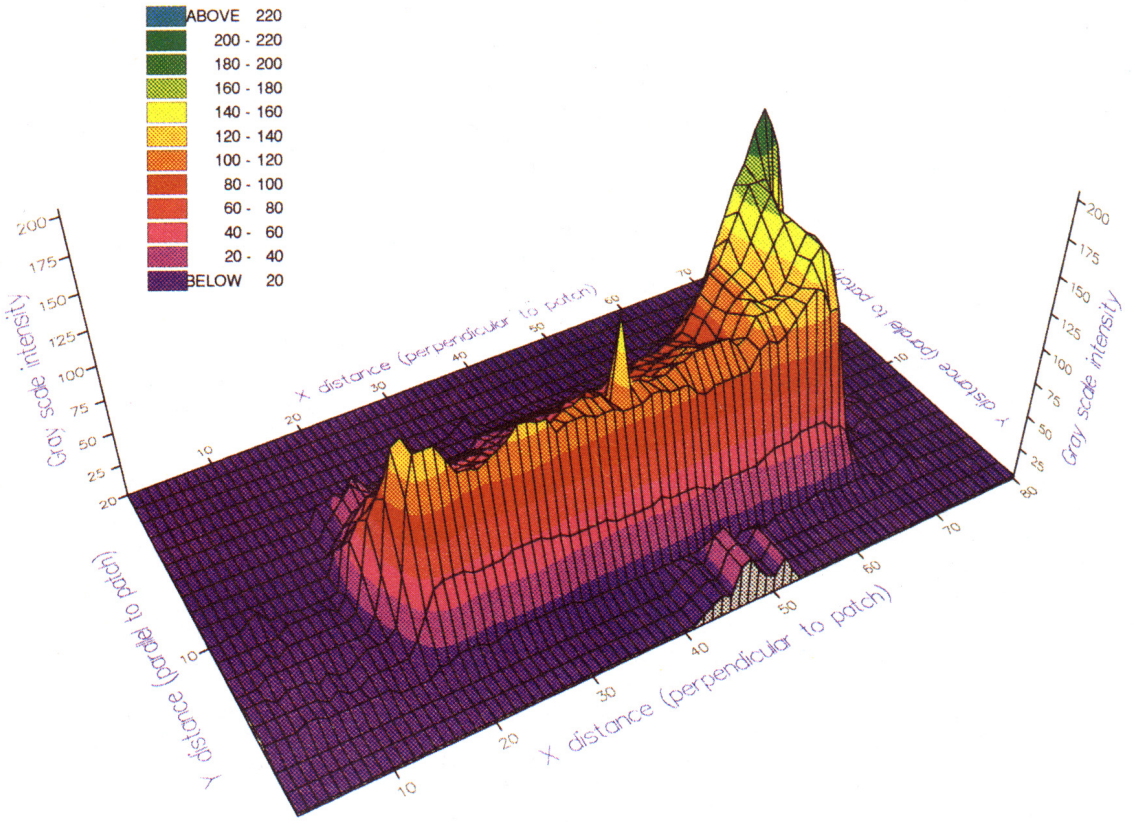

Fig. 5. Three dimensional contour imaging of an autoradiogram of a tissue slice from a cervical tissue sample taken following exposure *in vitro* to a 20 mg bioadhesive cervical patch for 4 hours.

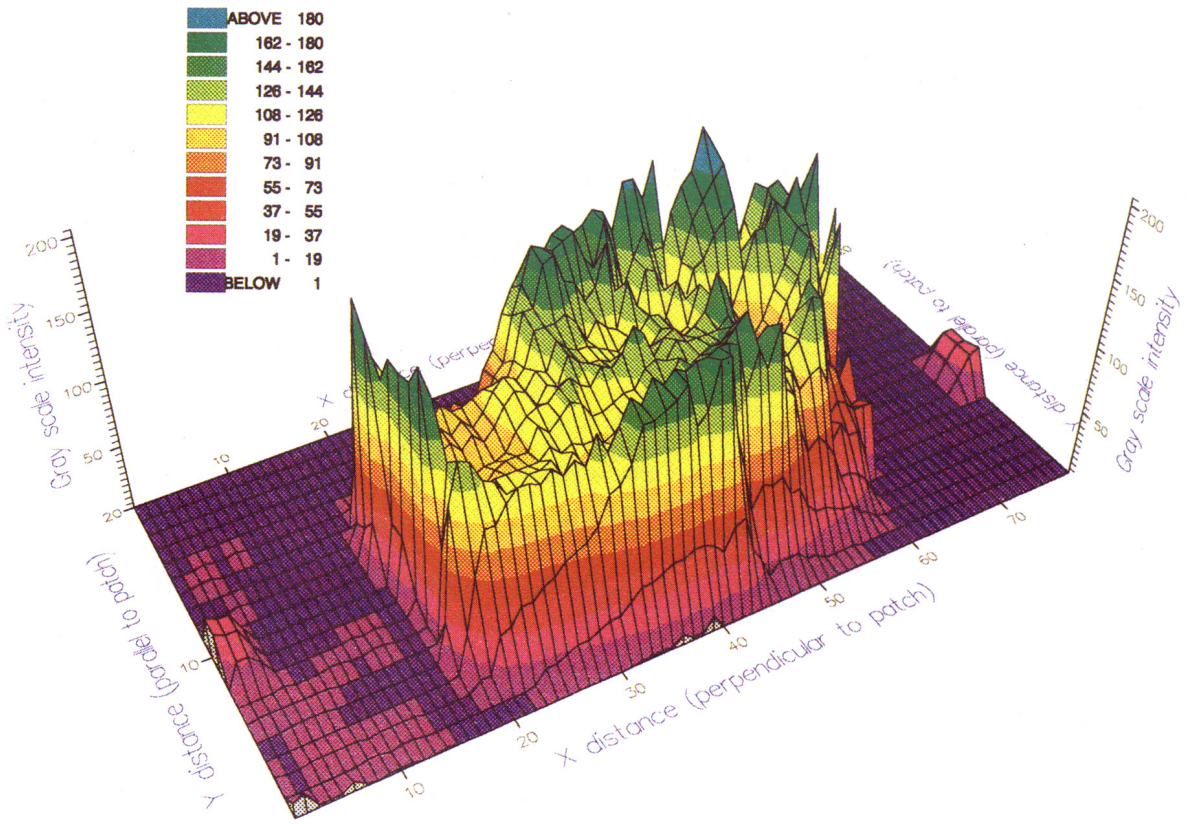

Fig. 6. Three dimensional contour imaging of an autoradiogram of a tissue slice from a cervical tissue sample taken following exposure *in vitro* to a 20 mg bioadhesive cervical patch for 24 hours.

est tissue, thereby producing a locally higher drug concentration. Quantitative determination of 5-FU by liquid scintillation spectrometry in an identical penetration experiment showed a similar pattern (2), thus confirming that a 4-hour application of the patch was insufficient to achieve a high drug concentration uniformly distributed throughout the upper half of the tissue, an essential requirement for effective clinical use of the patch.

Fig. 6, in which the patch was applied to the cervical tissue sample for 24 hours, shows blue and green (high drug concentration) areas extending deep into the tissue, down to a depth of approximately 5 mm. Interestingly, drug distribution is not completely uniform, some yellow areas, also an indication of relatively high drug concentration, also being present in this region and extending further into the tissue to a depth of approximately 7 mm. The fact that the drug distribution throughout the important upper 5 mm of tissue is not completely uniform may be a further indication that, for a comparatively water-soluble drug such as 5-FU, the route of drug penetration may be complex, involving both comparatively slow diffusion through tissue and rapid penetration through aqueous channels. Again, the deepest tissue region shows a localised green area of high radioactivity, where 5-FU that has penetrated through to the receiving fluid is in contact with the tissue. For both Figs. 5 and 6, it is also apparent that the drug concentration gradient does not tend to zero within the limits of the tissue, but stops abruptly at the point where the tissue and metal grid meet at the tissue/receiving fluid junction.

The results of this investigation, when combined with that of an earlier quantitative study (2) clearly show that drug distribution throughout the upper 5 cm of cervical tissue, following a 24-hour application of a 20 mg cervical patch, was sufficient to expose potential dysplastic lesions in these areas to a significant concentration of 5-fluorouracil. This information is essential before any consideration can be given to the clinical use of the cervical patch delivery system for the treatment of cervical intraepithelial neoplasia. In particular, autoradiographic imaging has shown that the drug distributions throughout the tissue samples were essentially linear and that tissue penetration was rapid. Examination of the autoradiographs suggests some evidence for locally high concentrations of drug, possibly in aqueous-filled pores known to be present in cervical tissue. It may be that, in the case of a relatively water-soluble drug such as 5-fluorouracil, shunt diffusion plays a significant role in the drug penetration process.

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