Liquid Scintillation Spectrometry of 5-Fluorouracil in Cervical Tissue Following *in Vitro* Surface Application of a Bioadhesive Cervical Patch

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The potential use of bioadhesive technology for the treatment of cervical intraepithelial neoplasia was investigated. A cervical patch was designed containing 5-fluorouracil in a bioadhesive matrix and polyvinyl chloride as the backing layer. The concentration of 5-fluorouracil at specified tissue depths from the cervical surface was determined *in vitro* in relation to the ability of the drug to reach precancerous foci in cervical crypts up to 4 mm below the tissue surface. Thus, tissue was exposed to drug-loaded patches spiked with 5-fluorouracil-6-^{3}H and subsequently sectioned to obtain tissue slices at different depths. The concentration of 5-fluorouracil was determined by liquid scintillation spectrometry. Drug penetration into cervical tissue exceeded a depth of 5.5 mm. Furthermore, the concentration in the tissue depended on the drug loading in the patch. Patches containing 10 and 20 mg of 5-fluorouracil produced a linear drug gradient that was established after a 4 hour application of the patch and persisted over 24 hours. However, patches containing 3.5 mg of 5-fluorouracil displayed signs of drug exhaustion after 24 hours. The penetration characteristics of 5-fluorouracil through cervical tissue using the cervical patch delivery system were sufficiently favourable to warrant further clinical investigations.

KEY WORDS: cervical intraepithelial neoplasia; bioadhesive; 5-fluorouracil; liquid scintillation spectrometry.

INTRODUCTION

The human cervix, a cylindrical-shaped organ with a slight taper at its inferior extremity, is composed of dense collogenic tissue, together with approximately 15% of smooth muscle fibre. The cervical canal, approximately 2.5 to 3.0 cm in length, is continuous with the uterine cavity and is lined with columnar epithelium, in contrast to the squamous epithelial tissue covering that portion of the cervix opening onto the vaginal vault. These two distinct forms of epithelial tissue meet at the squamocolumnar junction. Precancerous dysplastic lesions can occur at this junction. Such lesions are described collectively as cervical intraepithelial neoplasia (CIN), a range of epithelial abnormalities ranging from mild (CIN I), through moderate (CIN II) to severe dysplasia (CIN III).

Cervical intraepithelial neoplasia is, initially, a non-invasive condition identified by smear screening and classified by colposcopy (1). The aim of cytological cervical screening is to reduce the morbidity and mortality of invasive cervical carcinoma by detecting and subsequently treating women with CIN (2). Treatment is based on early identification and elimination of non-regressing lesions by ablative or excisional surgical techniques (3). However, these methods involve specialist intervention and the use of sophisticated equipment. Furthermore, they result in some destruction of the cervical architecture. Hence, both clinical and economic advantages would ensue if an effective local drug treatment for the early stages of the condition, CIN I and II, could be developed.

The external surface of the cervix is an accessible site for a suitably designed drug delivery system (4,5). We have, therefore, developed a novel, bioadhesive cervical patch that allows convenient, local drug delivery to the cervix. The use of a bioadhesive allows the delivery system to be non-invasively secured to the cervical surface, the system being conformable, easy to apply and remove and offering a high degree of patient acceptability. The patch incorporates 5-fluorouracil (5-FU) as the cytotoxic agent in a matrix design (6) for continuous drug delivery over a defined period. This is desirable since there is a rapid cell generation time in cervical carcinoma (7) and the drug is active only against cells in the DNA synthesis phase. A drug-impermeable backing layer prevents drug spill onto the surrounding vaginal epithelium.

A possible complication to effective drug therapy for CIN is that dysplastic lesions can occur, not just at the relatively accessible squamocolumnar junction, but can also originate deep within ridges of the cervical canal. 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 (8). Therefore, an *in vitro* model was developed in order to assess the penetration characteristics of 5-fluorouracil through cervical tissue following its release from the bioadhesive drug delivery system.

MATERIALS AND METHODS

Materials

5-Fluorouracil, Hyamine® hydroxide and 5-fluorouracil-6-^{3}H were obtained from Sigma Chemical Co. (Poole, U.K.). OptiPhase HiSafe 3 liquid scintillation cocktail was obtained from LKB Wallac (Milton Keynes, 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 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

Cervical patches were manufactured by casting, with a conventional casting knife technique, a bioadhesive gel onto medical grade PVC film that was prepared by curing PVC

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emulsion on a glass plate at 160°C for 10 minutes. Gels, containing 2% w/w Carbopol 981 and 1% w/w glycerin as plasticiser, together with cold 5-fluorouracil, were spiked with sufficient 5-fluorouracil-6-^{3}H solution to give approximately 3×10^6 dpm in each square centimetre of patch. The total quantity of 5-fluorouracil added was sufficient to give nominal drug loadings of 3.5, 10 and 20 mg, respectively, per patch. Patches were circular with a diameter of 26 mm, the cast gels being air dried from water/ethanol (70/30) for 24 hours with a forced air flow to produce a bioadhesive layer with a final thickness of 0.1 mm.

In Vitro Permeation Studies

Modified Franz diffusion cells, FDC-400, flat flange, 15 mm orifice diameter, were mounted in triplicate on an FDCD-3 diffusion cell drive console providing synchronous stirring at 600 r.p.m. (Crown Glass Co., Somerville, N.J.). Temperature maintenance was via water circulation (37°C) through the diffusion cell water jackets.

The distribution of drug in cervical tissue was determined after application of the bioadhesive cervical patch to the tissue for a fixed time period. Each determination was performed in triplicate using tissue samples from the same source.

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. 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 bioadhesive cervical patch, the bioadhesive matrix spiked with radiolabelled 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. The patch and tissue were separated when the required penetration time had elapsed. The tissue was then flash-frozen prior to sectioning by exposing it to above a liquid nitrogen atmosphere, without immersion, for 3 minutes.

Sectioning Procedure

Slabs of frozen tissue, pre-exposed to the drug-loaded patch, were mounted on the stage of a cryostatic microtome (Frigocut 2800E, Reichert-Jung, Cambridge, Ma.) using OCTC mounting fluid. The microtome environment and stage operated at −25°C. The tissue slab was positioned such that the epithelial surface was parallel to the slicing motion of the blade. Slice thickness was set at 50 μm. Two or three initial cuts were taken and discarded to remove surface undulations. Ten consecutive tissue slices were then taken and grouped in a pre-weighed scintillation vial prior to liquid scintillation spectrometry. This sectioning was then repeated through an increasing depth of tissue.

Liquid Scintillation Spectrometry

Tissue slices containing radiolabelled 5-fluorouracil were first dissolved in Hyamine® hydroxide (1.0 ml). Ultrasonification was used to accelerate tissue dissolution so that all traces of tissue had dissolved after approximately 2 hours. Scintillation cocktail (10.0 ml) was added and mixed and the vial was then stored in darkness for 2 hours before analysis to reduce chemiluminescence to less than 1% in respect of the total count. Samples were usually counted for two minutes or, if longer, until 10^4 counts had been achieved. Conversion to dpm was achieved against quench correction curves.

Quality Control of Cervical Patches

Adhesion of patches to cervical tissue was determined *in vitro* using a bioadhesion tester based on a linear variable displacement transformer, as previously described (6). Total drug content per patch was verified by ultra-violet spectrophotometry and the actual radioactivity, in dpm cm^{-2}, verified by liquid scintillation spectrometry.

High Performance Liquid Chromatography

Samples of receiving fluid were chromatographed directly on a Spherisorb 5μ ODS column (15 cm) using methanol:water (10:90) at a flow rate (Gilson model 302 pump, Anachem Ltd., Luton, UK) of 1 ml min^{-1}. The internal standard was aqueous thymine (40 mg ml^{-1}) mixed with receiving fluid in the ratio 1:5. The injection volume (Rheodyne 7125 injector) was 20 μl and detection (LKB Bromma, Sweden, model 2151) was at 267 nm. Chromatograms were recorded on a model 3390A integrator (Hewlett-Packard, Wokingham, UK).

RESULTS AND DISCUSSION

Determination of 5-Fluorouracil in Cervical Tissue

Liquid scintillation spectrometry was chosen as the most appropriate method for the determination of 5-fluorouracil in cervical tissue samples. Thus, time-consuming extraction and homogenization procedures, and their attendant errors, were avoided. Following sectioning, only dissolution of the tissue and subsequent addition to a scintillation cocktail were required.

Samples used in this study contained up to 70 mg of tissue, thereby introducing sufficient quantities of proteinaceous material that could quench the energy transfer process. Quench correction curves, constructed using the channels ratio method, were therefore performed each week to accommodate decay in the radiolabelled drug, approximately 1% in 6 months.

The radioactive concentrations of standards were checked before use. It is recommended that 10,000 counts is the minimum taken during the counting time of a vial (9). However, during the analysis of tissue, the count rate was so high that this threshold was substantially exceeded. Spiking the 5-fluorouracil-loaded gel with a known amount of radiolabelled 5-fluorouracil before casting produced films with uniform activity in unit area, as shown in Table I. Therefore, the dpm counted during an analysis could be directly related to the drug recovered from a known mass of tissue.

Samples of receiving fluid were analysed at the conclu-

Table I. Cervical Bioadhesive Patch Parameters (Drug Loading And Radioactivity, Theoretical And Determined)

Total 5-fluorouracil loading per patch (mg), as formulated	5-Fluorouracil loading per patch (mg cm^{-2}), as formulated	5-Fluorouracil loading per patch (mg cm^{-2}), as determined by UV spectrophotometry ± s.d., n= 5	Theoretical radioactivity (dpm cm^{-2})	Determined radioactivity (dpm cm^{-2})
3.50	0.66	0.66 ± 0.03	3.22×10^6	3.77×10^6
10.0	1.88	2.06 ± 0.15	3.33×10^6	3.73×10^6
20.0	3.77	3.72 ± 0.12	3.33×10^6	3.69×10^6

sion of each penetration study. There was no evidence of drug degradation, the chromatograms containing only two peaks with retention data corresponding to intact 5-fluorouracil and thymine, the internal standard.

Penetration Characteristics of 5-Fluorouracil Through Cervical Tissue

The portion of the cervix exposed to the vaginal lumen, the *portio vaginalis,* is lined with an epithelial layer of squamous cells. This cellular layer is the location surface for a bioadhesive layer and, hence, represents the initial barrier to the absorption of 5-fluorouracil. For this reason, it was important to identify this layer during dissection and to cut the tissue specimen so that the epithelium was uppermost. The total thickness of the cut tissue slab was approximately 10 mm. Precancerous changes present in the surface epithelium of the cervix have been shown to extend into clefts reaching approximately 4 mm below the surface (8). Therefore, for a topical drug treatment of CIN to be effective, the cytotoxic agent must penetrate to a depth of at least 4 mm below the surface. To allow a sufficient margin of safety, an arbitrary value of 5.5 mm was taken as the maximum depth at which a therapeutic drug level must be present.

To determine the depth of drug penetration into cervical tissue, it was necessary to secure the tissue in an apparatus that would maintain the sample at 37°C and ensure sink conditions. The Franz cell (10), is a well-known and commonly used system for measuring the penetration of drug molecules through barrier membranes, such as excised skin. To study drug diffusion into and through cervical tissue, the cell required minor adaptation, allowing the tissue slab to be supported on a stainless steel grid. Drug that had permeated through the complete thickness of the cervical tissue slab could enter the cell reservoir. This *in vitro* arrangement allowed the determination of drug concentrations in defined tissue layers at increasing depths from the surface after the application of a bioadhesive patch containing 5-fluorouracil. Similar investigations into drug transport are of particular importance in the therapeutic treatment of certain brain disorders (11). For example, cylindrical punches taken over a three-dimensional grid of brain tissue injected with drug at a central apex yielded drug transport and distribution data that were subsequently fitted to a statistical model (12). This model was mathematically complex and mapped the drug distribution into a confidence ellipsoid. The drug distribution in cervical tissue was of a two-dimensional format, with the concentration in each slice being an integral over its entire plane.

To harvest tissue slices at increasing depths below the surface, a cryostatic microtome was used to section the drug-exposed tissue. The advantages of this technique were two-fold. Firstly, it is common to fix tissue, prior to sectioning, in paraffin wax using successively increasing strengths of aqueous ethanol, which would readily wash 5-fluorouracil from the tissue. Secondly, after the penetration experiment, the tissue is immediately frozen and, if it happens to thaw, the drug gradient can dissipate by diffusion. However, cutting tissue frozen at −20°C requires no fixation and overcomes these problems. During the cutting procedure, 10 serial slices, each of 50 μm thickness, were taken, grouped and weighed before dissolution in alkaline detergent and subsequent analysis of 5-fluorouracil by liquid scintillation spectrometry.

The static drug concentrations produced in tissue were studied after exposure to one of three patches containing different concentrations of drug. The patches were prepared to contain, respectively, 3.5, 10.0 and 20.0 mg of 5-fluorouracil in the bioadhesive matrix of a circular patch, diameter 26 mm. This size of patch was considered the most appropriate for clinical use. Security of attachment was confirmed by determining the bioadhesive force of the patch to cervical tissue. The mean force of bioadhesion was 1.39N±0.14 (n=5).

Fig. 1 shows the distribution of 5-fluorouracil through cervical tissue following application of patches with the lowest drug loading, for application periods ranging from 4 to 24 hours. There was evidence of exhaustion of drug from the bioadhesive matrix after a 24 hour continuous application period, as seen by the reduction in drug levels in the surface layers. However, it is important to note that, even after a 4 hour application time, 5-fluorouracil was detectable in cervical tissue layers down to a depth of at least 5.5 mm, the clinically desirable minimum penetration depth. For the intermediate time intervals, the maximum penetration profile was established with little variation between the 8, 12 and 18 hour applications.

Penetration of 5-fluorouracil from both of the higher concentration patches showed a similar pattern, as shown in Figs. 2 and 3. Penetration levels were established at 4 hours and prevailed for the remaining time periods. The distribution was linear, with aberrations shown only in the tissue layers nearest the epithelium. It is most probable that remnants of patch were collected along with the first series of ten

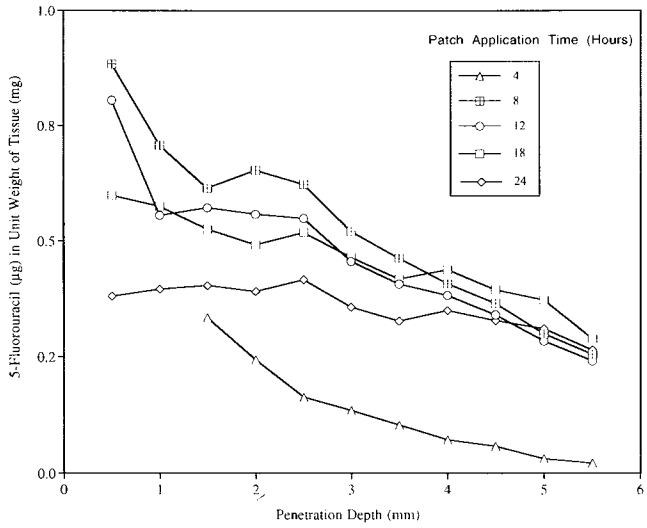

Fig. 1. Penetration characteristics of 5-fluorouracil through cervical tissue following fixed application periods. The drug was delivered from a circular cervical patch, diameter 26 mm, prepared to contain 3.5 mg of 5-fluorouracil in a bioadhesive matrix. Results are the means of triplicate experiments using tissue from a single source in each case.

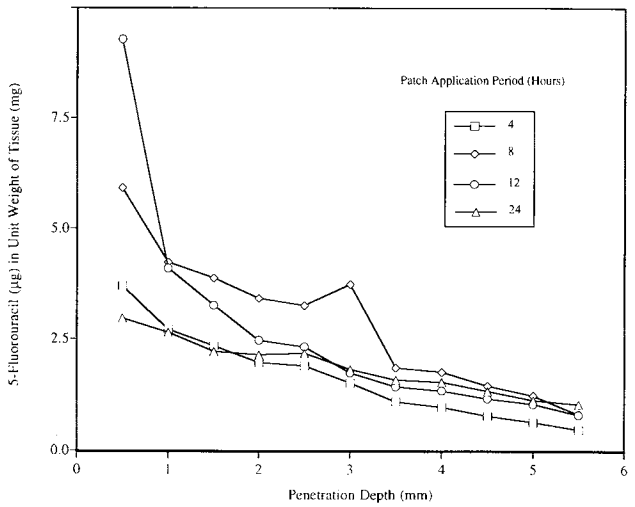

Fig. 3. Penetration characteristics of 5-fluorouracil through cervical tissue following fixed application periods. The drug was delivered from a circular cervical patch, diameter 26 mm, prepared to contain 20 mg of 5-fluorouracil in a bioadhesive matrix. Results are the means of triplicate experiments using tissue from a single source in each case.

slices, thereby giving an erroneously high reading in the uppermost level. The amount of drug in corresponding tissue slices released from a 20 mg patch was approximately twice that found in the 10 mg patch. The results indicated that the penetration of 5-fluorouracil was rapid and extended deep into the tissue. Indeed, 5-fluorouracil was detectable in the reservoirs for all time periods and all patch dosages, indicating that drug penetration had reached to a minimum depth of 10 mm below the surface of the cervical tissue sample. However, at the critical depth of 5.5 mm, 5-fluorouracil concentrations exceeded 0.25 μg per mg of tissue using the lowest patch concentration, which increased to approximately 0.75 μg mg^{-1} for the 10 mg patch and 1.00 μg mg^{-1} for the 20 mg patch. In the latter case, the concentration of 5-fluorouracil was approximately 2000 times in excess of the cytotoxic concentration of the drug determined *in vitro* against HeLa cells (13). The quantitative nature of the data obtained in this study is important since no previous information on 5-fluorouracil penetration through cervical tissue was available. Such information, relating drug concentrations at various tissue depths to changes in both drug loadings and application periods, is essential to the design of a cervical patch for clinical use. Thus, this study has shown that the penetration characteristics of 5-fluorouracil, following its release from a bioadhesive drug-loaded matrix, are such that malignant cells in cervical tissue are likely to be exposed to levels of the drug that will be sufficient to produce a clinical effect. Therefore, further clinical investigation of the cervical patch drug delivery system appears to be justified.

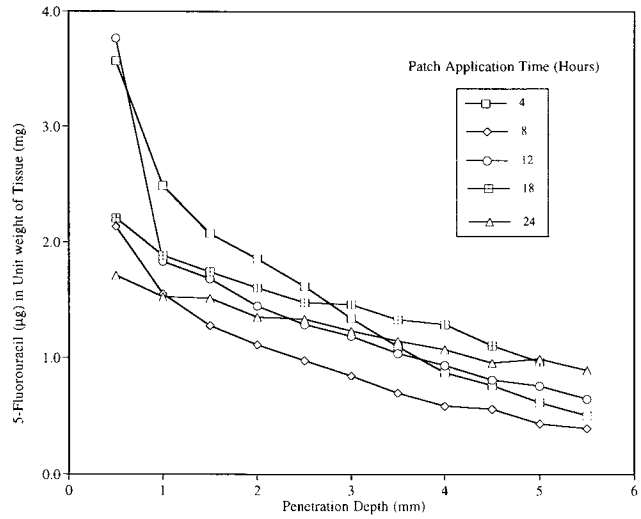

Fig. 2. Penetration characteristics of 5-fluorouracil through cervical tissue following fixed application periods. The drug was delivered from a circular cervical patch, diameter 26 mm, prepared to contain 10 mg of 5-fluorouracil in a bioadhesive matrix. Results are the means of triplicate experiments using tissue from a single source in each case.

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