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Research Paper

Autoradiographic and Scintillation Analysis of 5-Aminolevulinic Acid Permeation Through Epithelialised Tissue: Implications for Topical Photodynamic Therapy of Superficial Gynaecological Neoplasias

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Purpose. Aminolevulinic acid (5-ALA) diffusion through both keratinised and non-keratinised tissue, used as a model tissue substrates, was evaluated, together with the depth of permeation and the concentration achieved following delivery from bioadhesive patch and proprietary cream formulations.
Materials and Methods. Moisture-activated, bioadhesive patches loaded with 5-ALA at concentrations of 19.0, 38.0 and 50.0 mg cm^{-2} and an o/w cream (20% w/w 5-ALA) were radiolabelled with C14 5-ALA and applied to excised human vaginal tissue and porcine skin. After 1, 2 and 4 h, tissue was sectioned in two orientations and the 5-ALA concentration at specific depths determined using autoradiography and liquid scintillation counting (LSC).
Results. The *stratum corneum* was a significant barrier to 5-ALA permeation, with concentrations in tissue dependent on application time and drug loading. 5-ALA was detected at 6 mm using autoradiography after 2 h, with LSC showing phototoxic concentrations at 2.375 mm after 4 h of application. Inclusion of oleic acid and dimethyl sulphoxide in bioadhesive patches increased 5-ALA significantly in neonate porcine tissue, but only for patches cast from blends containing 5% w/w oleic acid.
Conclusions. The bioadhesive patch described delivered 5-ALA to depths of at least 2.5 mm in tissue types indicative of vulval skin, suggesting that photodynamic therapy of deep vulval intraepithelial neoplasia is feasible using this means of bioadhesive 5-ALA delivery.

KEY WORDS: aminolevulinic acid; bioadhesion; patch; photodynamic therapy; topical.

INTRODUCTION

Aminolevulinic acid HCl (5-ALA, 5-amino-4-oxopentanoic acid hydrochloride) is a commonly used photosensitiser precursor in photodynamic therapy (PDT) and has been used successfully in the topical treatment of various neoplastic lesions, including basal cell carcinoma (1), actinic keratosis (2), Bowen's disease (3), vulval intraepithelial neoplasia (4,5), vulval Paget's disease (6) and vulval squamous cell carcinoma (7). High intracellular concentrations of 5-ALA lead to the biosynthesis of the potent endogenous photosensitiser protoporphyrin IX (PpIX), an effect driven by enzymatic irregularities and seen preferentially in neoplastic cells (8). Subsequent irradiation with photons of visible light leads to singlet oxygen production and destruction of cells rich in mitochondrial PpIX. The clinical manifestation is one of specific and benevolent eradication of superficial lesions without significant scarring or the need for surgical intervention.

The requirement for both visible light delivery and 5-ALA administration has compelled most 5-ALA-based PDT towards the treatment of lesions that are readily accessible, normally those found on skin and mucous membranes. The *stratum corneum (SC)*, the outermost layer of the epidermis, represents the most important barrier to skin penetration of topically applied drugs and its role in PDT is of notable consequence (9). Skin penetration of most small molecular substances can be considered to be a process of passive diffusion. Access via alternative routes, such as down hair follicles or sweat ducts, is believed to be of little importance as they occupy only approximately 0.1% of the total human skin surface (10). Therefore, diffusion for the most part follows a tortuous intercellular pathway through neutral lipids, with lesser contributions from transcellular movements across corneocytes (11,12). Unsurprisingly, lipophilicity and molecular weight are primary determinants for effective transdermal delivery. The hydrochloride salt of 5-ALA itself is a small molecule of low molecular weight (167.6 Da) and whilst this is unlikely to impinge on its ability to penetrate skin, its zwitterionic nature will impair penetration, since it is ionised at both high and low pH. Its octanol:water partition coefficient is 0.03 (13). As a result, topically applied 5-ALA penetrates intact *SC* poorly (9,14). Although disordered epithelial barriers presented by many skin lesions allow enhanced 5-ALA penetration, its low lipophilicity (15) prevents it from penetrating significantly into hyperkeratotic

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or deep nodular lesions (1,16,17). The potential implications are one where insufficient concentrations of 5-ALA are delivered to specific depths beneath the surface of a lesion, followed by poor induction of phototoxic concentrations of PpIX, leading to treatment failure and clinical reoccurrence at some time in the future.

The importance of achieving therapeutic concentrations of 5-ALA across the full lesion thickness is underlined in the photodynamic therapy of vulval intraepithelial neoplasia (VIN). No available treatments, such as those based on laser or surgical interventions, guarantee cure, but several recently published studies have shown encouraging responses to PDT. The superiority of PDT treatment lies in avoiding mutilating surgery and gives the option of repeated treatments, as necessary, without any cumulative effects. The design of a drug delivery system for drug administration to this area is especially problematic, but recent work has described the development of a novel bioadhesive patch as a means to deliver 5-ALA to this region (18). It has been shown that the maximum histologically discernible depth of the great majority of VIN lesions is around 2.5 mm (19). This immediately sets an important objective for successful PDT in that merely achieving subtherapeutic concentrations at this depth from the surface is unlikely to result in sufficient PpIX production at deep sites, *ergo*, a sequel of almost certain reoccurrence from underlying neoplastic cells that have evaded any phototoxic effect. Therefore, one aim of this study was to determine whether 5-ALA, released from a novel bioadhesive patch, was capable of penetrating model tissue to this depth. Importantly, the stratal concentration of 5-ALA at a depth of 2.5 mm in tissue was of interest. It should be remembered that pathological VIN lesions are normally not available for permeation studies and normal vulval tissue is not removed routinely, due to a need to preserve vulval structure and function. Since the outer surfaces of the vulva consist of keratinising epithelial tissue, that is, tissue that is in the process of becoming keratinised (20,21), both keratinised and non-keratinised tissue were used in this study. Non-keratinised vaginal tissue, removed during routine repair operations and possessing no *SC* barrier and keratinised neonate porcine skin were used. It was hoped that this would allow an intermediate approximation of 5-ALA penetration into vulval tissue proper.

Numerous attempts to increase drug permeation across the *SC* are described in the literature, such as the application of Wolf's skin-stripping technique (22) and the use of occlusive formulations (23). Co-formulation with chemical penetration enhancers is a strategy that lends itself to the patch approach in that they can be incorporated with 5-ALA into a bioadhesive matrix. Therefore, a further aim of this work was to investigate factors that would influence 5-ALA permeation and the depth of penetration, such as varying application time, 5-ALA loading in the patch and the incorporation of the penetration enhancers (dimethyl sulphoxide and oleic acid). Autoradiography was chosen as a means to visualise 5-ALA permeation to determine whether 5-ALA simply penetrates through tissue by simple diffusion or whether shunt diffusion occurs through sweat glands or hair follicles, as suggested by Divaris *et al.* (24). Furthermore, this information would provide a static picture of 5-ALA permeation, which would indicate if permeation was homogenous or gives rise to nodes of localised 5-ALA concentrations.

MATERIALS AND METHODS

Materials

Gantrez® AN-139, a copolymer of methy vinyl ether and maleic anhydride (PMVE/MA) was obtained from ISP Co. Ltd. Guildford, UK. Tripropyleneglycol methyl ether (Dowanol®, TPM), oleic acid and dimethyl sulphoxide were obtained from Sigma Aldrich (Dorset, UK) and used without further purification. Plastisol®, a medical grade poly(vinyl chloride) emulsion containing diethylphthalate as plasticiser, was obtained from BASF Coatings Ltd. (Clwyd, UK). Porphin cream® (20% w/w 5-aminolevulinic acid, hydrochloride salt in Unguentum Merck®) and GMP quality 5-ALA, were obtained from Crawford Pharmaceuticals Ltd. (Milton Keynes, UK). Radiolabelled ^{13}C-5-ALA solution, of specific activity 3.7 MBq ml^{-1}, was obtained from PerkinElmer Life Sciences (Beaconsfield, UK). Human tissue samples were collected and used in accordance with ethical approval as authorised by the Queen's University Research Ethics Committee (application no 276/01). Excised vaginal tissue was frozen within 1 h of section by suspension in a liquid nitrogen environment and stored at −70°C until use.

Preparation of 5-ALA-Loaded and Radiolabelled Formulations

Bioadhesive patches were prepared using a casting technique, whereby aqueous blends containing 20% w/w PMVE/MA, 10% w/w TPM and 30% w/w ethanol, all dissolved in distilled water, were poured into bespoke aluminium moulds (10 cm×10 cm×5 mm). Patches of three different 5-ALA loadings, namely, 19.0, 38.0 and 50.0 mg cm^{-2}, were prepared by addition of an appropriate amount of crystalline 5-ALA, dissolved into appropriate amounts of aqueous blend (approximately 2.7 g), before casting. Gels were dried for 4 h under a moving air flow, heated to approximately 40°C and driven by an array of fans. Patches loaded with 38.0 mg cm^{-2} 5-ALA and containing penetration enhancers were prepared by replacing some of the ethanol in the original blend with equal weights of dimethyl sulphoxide (DMSO) or oleic acid (OA). Patches were prepared from blends containing 1, 3, 5 and 10% w/w DMSO and 1, 3 and 5% w/w OA. Finished patches were placed in moisture-impermeable, heat-sealed, poly(ester) foil sachets (Transparent Film Products Ltd., N. Ireland) and stored at −20°C until required.

Patch and cream formulations were spiked with trace radioactivity by addition of radiolabelled 5-ALA solution. Sufficient radioactivity was added to gels, with stirring, to give approximately 5.9×10^5 disintegrations per minute (dpm) in each cm^2 of dried patch. Porphin® cream was used a proprietary control and spiked with radiolabelled 5-ALA so that the amount added was chosen to match the available dpm per cm^{-2} of applied patch formulation. It was assumed that the mass of cream applied to tissue was 2.0 mm thick. Radiolabelled Porphin® cream was stored in plastic syringes at −20°C prior to use.

Evaluation of Static 5-ALA Distribution in Vaginal Tissue

The distribution of drug in vaginal tissue with respect to time was determined using a Franz diffusion cell-based determination (FDC-400 flat flange, 15 mm orifice diameter, mounted in triplicate on an FDCD diffusion drive console providing synchronous stirring at 600 rpm, Crown Glass Co. Inc., USA). Excised tissue was cut cylindrically, approximately 6 mm in diameter and 4 mm deep, using a proprietary dermatological punch (Keyes Biopsy Punch, Stiefel Laboratories Ltd, UK). Tests were performed five times at constant temperature (37°C) and under synchronous stirring (600 rpm).

The tissue was mounted in a purpose-built stainless steel washer, again 4 mm thick, with a circular hole, 5 mm in diameter, machined through its centre. The tissue-washer assembly was supported on top of a stainless steel filter support grid Stainless (Millipore, UK) that was placed across the top of the Franz cell receiver phase. This reservoir was filled with approximately 10 ml of phosphate buffered saline pH 7.4, sufficient to bring the fluid level up to the grid and expel traces of air. At the beginning of the experiment, a disc-shaped sample of patch, 5 mm in diameter and containing both radiolabelled and unlabelled 5-ALA was applied to the uppermost layer of the tissue slab, with the aid of a 5 μl drop of water. A glass cup, of diameter 8 mm, was placed over the assembly and secured with vacuum grease before the upper glass ring of the Franz cell was clamped on, all shown in Fig. 1. The patch and tissue were separated when the penetration time had elapsed, with the latter flash-frozen in a liquid nitrogen atmosphere prior to sectioning.

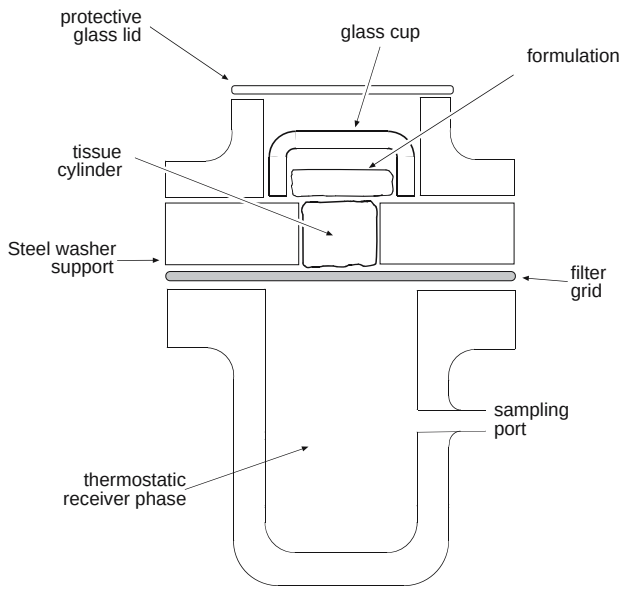

Fig. 1. The cross-sectional arrangement of Franz diffusion cell, showing position of steel washer used to hold tissue samples in position. A glass cup is positioned over the patch or cream and prevents dehydration of the formulation. The filter grid allows the receiver phase to make contact with the lower surface of the tissue, maintaining tissue hydration and temperature. A sampling port is available to allow access to the receiver phase, but was not used in this work

Microtome Procedure and Determination of Radiolabelled 5-ALA

Cylinders of frozen tissue, pre-exposed to 5-ALA-loaded patches, were mounted on the stage of the cryostatic microtome using tissue embedding fluid (Tissue-TEK®, Sakura Finetech, The Netherlands). Frozen tissue cylinders were sectioned progressively, in 50 μm steps, and in a parallel orientation to the tissue surface, such that the cumulative depth of slicing into the tissue was 2.5 mm. Five consecutive slices were taken, dissolved in 1.0 ml tissue solubiliser (NCS®-II Amersham Biosciences, UK) for 24 h at 37°C and added to 10 ml scintillation cocktail (Ultima Gold®, PerkinElmer Life Sciences, Bucks, UK). Vials were stored in darkness for 2 h prior to analysis to reduce chemiluminescence to less than 1% in respect of the total count. Samples were counted (LKB Wallac 1215 Rack Beta II, LKB, Sweden) for 10 min and conversion to dpm achieved against appropriate quench correction. Mean dpm values from three replicate experiments were converted to mean 5-ALA concentrations. These mean concentrations were plotted against mean penetration depths. Mean penetration depths were the average depths for a given set of five consecutive slices, such that if 5×50 μm slices were taken from the top of a tissue sample, then the mean depth plotted against the mean 5-ALA concentration would be 0.125 mm.

Autoradiography Procedures

In contrast to scintillation counting determination, blocks of vaginal tissue were cut into cuboidal sections. These tissue blocks had approximate upper facial dimensions of 20×20 mm and were approximately 6 mm deep. Using 10 μl drops of water, sections of patches, 20×20 mm were secured to the upper surfaces of the tissue blocks, which were mounted in silicone moulds cut to the exact dimensions of the blocks. The moulds were placed on top of the filter support grips on the Franz cell apparatus. The upper glass rings of the Franz cells were clamped on and covered with laboratory film (Parafilm®). Only radiolabelled patches containing 38.0 mg 5-ALA cm^{-2} were used for this study. Application times were 1, 2 and 4 h.

The patches and tissue blocks were separated when the penetration time had elapsed, with the latter flash-frozen in a liquid nitrogen atmosphere, prior to sectioning. Tissue blocks were positioned on the microtome stage so that slices were taken in the plane of drug diffusion, as shown in Fig. 2. Slices were cut at 50 μm thickness and stored in a liquid nitrogen atmosphere until use. Slices were exposed to autoradiography film (Biomax® MR, Eastman Kodak, New York) by allowing frozen sections to momentarily thaw, less than 2 s, onto the film surface. Films were immediately frozen in a liquid nitrogen atmosphere and stored at −20°C for 7 days before development.

Image Development

Biomax® MR films were developed using Kodak GBX® developer and replenisher at 20°C for 2 min under safe-lighting conditions (Kodak GBX-2 filter). The slides were then fixed using Kodak GBX® fixer and replenisher, also for

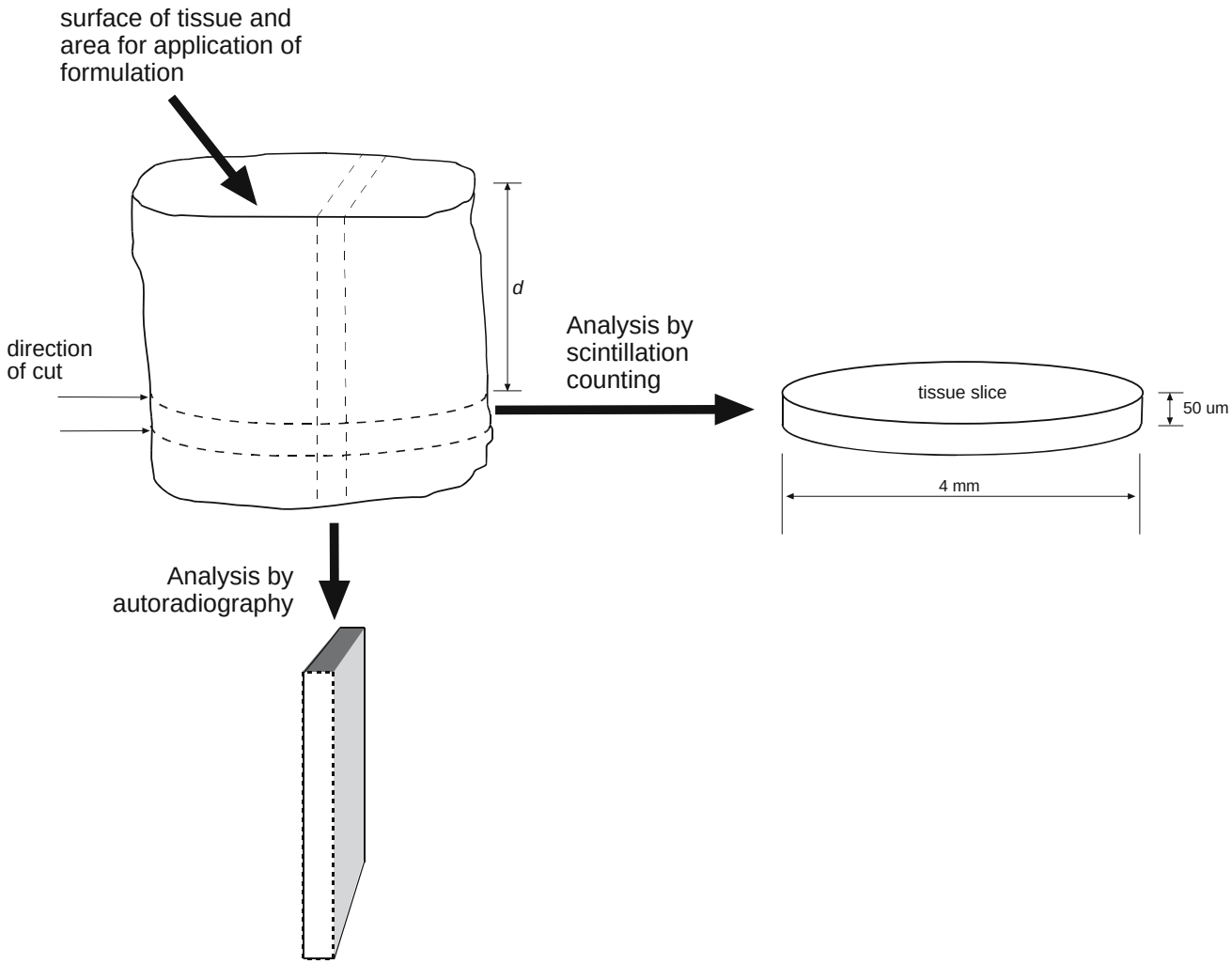

Fig. 2. The direction of cutting used for both scintillation analysis and autoradiographic procedures. The patch is applied to the upper face of the tissue block, which is then cut into slices taken orthogonally to the plane of drug diffusion, which provide samples for scintillation analysis. Blocks of tissue, similarly exposed, are cut normal to the surface and in line with the direction of drug diffusion. These slices provide samples for autoradiography

Table I. Loading Properties of Bioadhesive Patches Used in the Study

[a]Formulation	5-ALA Loading/mg cm^{-2}		Radioactivity/dpm cm^{-2}	
	Theoretical	[c]Determined	Theoretical	Determined
[b]Porphin® cream	38	39.10±3.83	2.9×10^5	2.6×10^5
20/10/30	19	19.13±2.22	5.9×10^5	3.7×10^5
20/10/30	38	38.12±2.59	5.9×10^5	6.5×10^5
20/10/30	50	50.91±0.76	5.9×10^5	5.6×10^5
20/10/29/1 DMSO	38	38.69±0.58	5.9×10^5	6.3×10^5
20/10/27/3 DMSO	38	37.92±1.43	5.9×10^5	5.9×10^5
20/10/25/5 DMSO	38	39.05±1.88	5.9×10^5	6.9×10^5
20/10/20/10 DMSO	38	38.11±0.97	5.9×10^5	5.6×10^5
20/10/29/1 OA	38	37.87±0.89	5.9×10^5	6.1×10^5
20/10/27/3 OA	38	39.99±2.17	5.9×10^5	7.2×10^5
20/10/25/5 OA	38	38.08±0.27	5.9×10^5	7.1×10^5

[a] (% PMVE/MA)/(% TPM)/(% ethanol)/(% DMSO or OA) in aqueous blend
[b] Amount of 5-ALA in 190 mg of cream applied per cm^2 of tissue
[c] Mean ± standard deviation (n=3)

Table II. Penetration Properties of 5-Aminolevulinic Acid Into Vaginal Epithelial Tissue

Formulation	Application Time/h	[a]5-ALA Concentration at 2.375 mm/mg cm^{-3}	[a]Percent of Total 5-ALA Loading Released Into Tissue
Porphin® cream	1	3.18±0.14	8.09±0.74
Porphin® cream	2	5.65±1.52	7.93±0.86
Porphin® cream	4	9.42±4.41	15.28±4.06
19 mg cm^{-2} patch	1	0.36±0.08	0.72±0.08
19 mg cm^{-2} patch	2	2.58±0.96	8.46±3.00
19 mg cm^{-2} patch	4	4.93±1.21	13.46±4.74
38 mg cm^{-2} patch	1	2.31±1.16	8.50±2.80
38 mg cm^{-2} patch	2	6.00±2.56	18.53±3.75
38 mg cm^{-2} patch	4	10.93±0.24	17.65±3.00
50 mg cm^{-2} patch	1	0.70±0.41	0.57±0.07
50 mg cm^{-2} patch	2	8.76±2.14	12.04±0.44
50 mg cm^{-2} patch	4	13.52±1.49	9.13±2.05

[a] Mean ± standard deviation (n=3)

2 min at 20°C, before gently rinsing with water. Developed Biomax® MR films were washed with water to remove the attached tissue. Dried images were then scanned using a scanning densitometer (BIO-RAD GS-800 calibrated densitometer with Quantity One® quantitation software, Bio-Rad Laboratories Inc., California) and raster pictures visualised with three dimensional contouring computer software.

Statistical Analysis

Data concerning the physical properties of bioadhesive films were analysed using a one-way Analysis of Variance (ANOVA). Post-hoc comparisons were made using Fisher's PLSD test. Comparison between cream and patch formulations and between vaginal epithelial tissue and neonate porcine skin, in terms of 5-ALA penetration, were made using the Mann–Whitney U test. The Kruskal–Wallis test was used to analyse the effects of increasing 5-ALA loadings, increasing application times and addition of penetration enhancers on 5-ALA penetration. In all cases, $p<0.05$ denoted significance.

RESULTS

In this study, small sections were taken from a larger formulation and used to deliver 5-ALA to suitably mounted keratinised and non-keratinised tissue. Verification of reproducibility in drug loading across the area of the formulation, both of 5-ALA and radiolabelled 5-ALA, is shown in Table I. Samples extracted from three random locations across the patch formulation confirm that distribution of both payloads, by direct addition and spiking, was reproducible and acceptably close to the nominal loading. Although total RA loading showed greater variance from the theoretical loadings, this was accounted for in calculations between the ratio between 5-ALA and its radiolabelled tracer.

Table II presents the data relating to the penetration of 5-ALA into vaginal epithelial tissue. As expected, increasing the application time increased significantly the 5-ALA concentration at a mean depth of 2.375 mm in tissue, an observation seen for all formulation types. Application of the proprietary Porphin® cream for 4 h led to an 5-ALA concentration at 2.375 mm that was not significantly different (p=0.5127) to that due to the patch containing 38 mg 5-ALA cm^{-2}, which is designed to deliver the same amount of drug per unit area and which was also applied for 4 h. The penetration profiles arising from these two formulations, following for a 4-h application period, are shown in Fig. 3a and illustrates the similar linear nature of both through vaginal tissue. Further consideration of shorter time applications (data not shown),

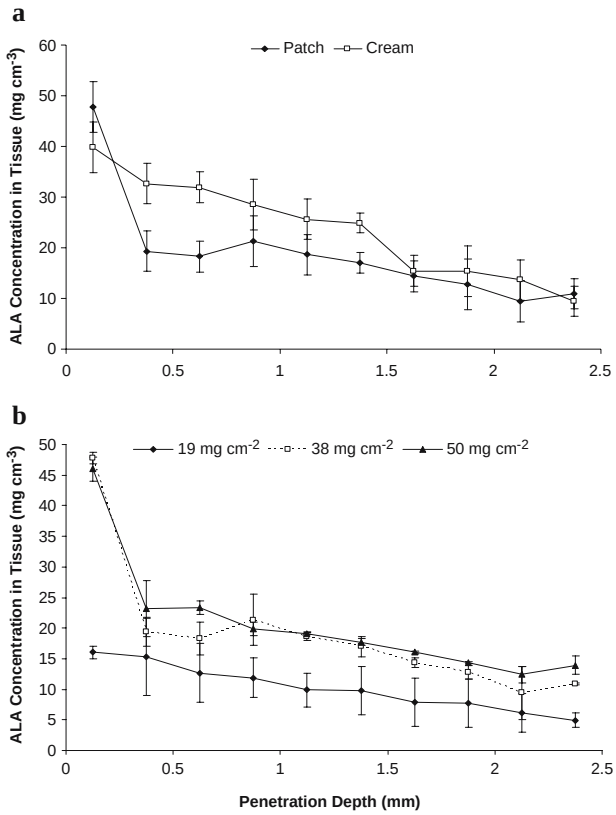

Fig. 3. Influence of **a** formulation type and **b** drug loading on the penetration of 5-ALA into vaginal epithelial tissue. The penetration profiles shown in **a** are those of a patch containing 38 mg 5-ALA cm^{-2} and the proprietary Porphin® cream (20% w/w 5-ALA in Unguentum Merck®) (means±S.D., n=3). Both formulations were applied for 4 h before removal

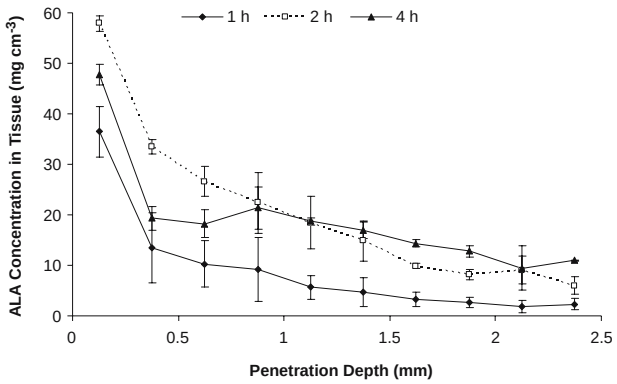

Fig. 4. Influence of application time on penetration of 5-aminolevulinic acid (5-ALA) into vaginal epithelial tissue. The penetration profiles shown are those of the patch containing 38 mg 5-ALA cm^{-2} (means±S.D., n=3)

namely for 1 and 2 h, reveal penetration profiles for the proprietary cream and the patch containing 38 mg 5-ALA cm^{-2} that were essentially similar. The 5-ALA concentrations at depths of 2.375 mm in tissue after 1 (p=0.5127) or 2 (p=0.8273) h of cream application were not significantly different to those after 1 or 2 h of application of the 38 mg cm^{-2} patch.

Increasing the drug loading in the patch caused significant increases in the 5-ALA concentrations at mean depths of 2.375 mm in tissue for application times of 2 (p=0.049) and 4 h, respectively (p=0.0273). No discernable trend was observed with regard to the influence of patch loading on the 5-ALA concentration at a mean depth of 2.375 mm after a 1-h patch application. Fig. 3b shows the 4-h penetration profiles for patches with 5-ALA loadings of 19, 38 and 50 mg cm^{-2}. It is clear that at depths in excess of 2 mm, the two highest patch concentrations result in measurable tissue concentrations of 5-ALA extending beyond this important indicator, which, as mentioned *vide supra*, is the distance from the surface that the deepest VIN are normally found.

As mentioned previously and as can be seen from Table II, increasing the application time, for bioadhesive patches of a given loading, significantly increased the concentration of 5-ALA at a mean depth of 2.375 mm in tissue. For example, as may be seen from Fig. 4, increasing the application time of patches containing 38.0 mg 5-ALA cm^{-2} significantly increased (p=0.0273) the 5-ALA concentration at 2.375 mm. This concentration increase determined at this important distance measure is also observed with patches loaded with 19 and 50 mg 5-ALA cm^{-2} (Table II). As is also apparent from Table II, there are no easily interpreted patterns observed in terms of the percentage of 5-ALA loadings released into vaginal epithelial tissue with respect of formulation, drug loading or application times. It is clear though that without any means of permeation enhancement in the formulation, no more than 20% of the total loading is observed to permeate into the tissue. This important observation shows that topical delivery into non-keratinised vaginal tissue is not particularly efficient.

Table III presents the data relating to the penetration of 5-ALA into two tissue types, namely non-keratinised vaginal epithelial tissue, possessing no *SC* barrier, and keratinised neonate porcine skin. The 5-ALA concentration at a mean depth of 2.375 mm is significantly less for neonate porcine skin than for vaginal epithelial tissue, regardless of the type of formulation used. The relationship between concentration and depth is shown in Fig. 5 and it is clear that the concentration of 5-ALA at 2.375 mm was significantly less in neonate porcine skin after a 4-h application of the proprietary cream (p=0.0495), or of the normal patch containing 38.0 mg cm^{-2} (p=0.0495). Decreases were also observed in the percentages of total 5-ALA loadings released into neonate porcine skin compared to vaginal epithelium for both cream (p=0.1266) and normal patch (p=0.0495). However, the decrease was only significant for the patch.

The effect of adding a chemical penetration enhancer is shown in Table III. It is apparent that the addition of DMSO

Table III. Influence of Formulation Factors and Tissue Type on Penetration Properties of 5-Aminolevulinic Acid

Formulation	Tissue Type	5-ALA Concentration at 2.375 mm (mg cm^{-3}; ±S.D.) n=3	Percent of Total 5-ALA Loading Released Into Tissue (±S.D.) n=3
Porphin® cream	Vaginal epithelium	9.42±4.41	15.28±4.06
Porphin® cream	Neonate porcine skin	0.88±0.002	9.60±2.64
38 mg cm^{-2} patch	Vaginal epithelium	10.93±0.24	17.65±3.00
38 mg cm^{-2} patch	Neonate porcine skin	3.01±1.45	3.37±1.02
1% DMSO	Vaginal epithelium	11.62±2.18	9.93±0.18
1% DMSO	Neonate porcine skin	ND	ND
3% DMSO	Vaginal epithelium	11.02±2.57	13.47±1.51
3% DMSO	Neonate porcine skin	ND	ND
5% DMSO	Vaginal epithelium	9.34±0.55	8.96±0.90
5% DMSO	Neonate porcine skin	4.50±1.17	12.29±1.34
10% DMSO	Vaginal epithelium	8.79±0.68	15.28±1.09
10% DMSO	Neonate porcine skin	4.71±1.06	10.64±1.69
1% OA	Vaginal epithelium	8.67±0.53	13.51±1.02
1% OA	Neonate porcine skin	4.00±0.42	17.53±1.06
3% OA	Vaginal epithelium	10.97±2.85	15.82±3.01
3% OA	Neonate porcine skin	4.55±0.27	7.91±0.98
5% OA	Vaginal epithelium	8.21±2.46	13.13±0.74
5% OA	Neonate porcine skin	8.17±2.35	12.47±1.53

The application time used was 4 h in all cases.
ND Not determined

to the bioadhesive matrix of the patch is an ineffective strategy, with no significant effects on 5-ALA concentrations at mean depths of 2.375 mm in vaginal epithelial tissue (p=0.0537). DMSO had no significant influence on 5-ALA concentration at 2.375 mm in neonate porcine skin (p=0.4298). Films cast from blends containing 10% w/w DMSO could not significantly increase the 5-ALA concentration at 2.375 mm. This pattern is observed also following the addition of OA to the patch, with no significant effect on the concentration of 5-ALA at mean depths of 2.375 mm in vaginal epithelial tissue (p=0.0586). Overall analysis of the effect of OA on 5-ALA concentration at 2.375 mm in neonate porcine skin using the Kruskal–Wallis test showed that there was no significant effect (p=0.0770). However, an individual comparison between the mean 5-ALA concentrations at 2.375 mm achieved using the normal patch and achieved using the patches containing OA (Mann–Whitney U test) did reveal some significance in terms of a difference in 5-ALA permeation (p=0.0495) using the patch cast from a blend containing 5% w/w OA, but this is a marginal effect. Fig. 6 shows the neonate porcine skin penetration profiles for the normal patch containing 38.0 mg 5-ALA cm^{-2} and those of patches containing 38.0 mg 5-ALA cm^{-2} and prepared from aqueous blends containing 10% w/w DMSO and 5% w/w OA. This is the maximum loading of both penetration enhancers and the figure does indicate better permeation of 5-ALA when compared to the normal patch, contrary to the findings of the statistical analysis. As was the case with the results presented in Table II, Table III reveals no discernible patterns observed in terms of the percentages of 5-ALA loadings released into vaginal epithelial tissue and neonate porcine skin regardless of formulation. In general, however, more 5-ALA was released into the vaginal epithelial tissue than into the neonate porcine skin.

Autoradiographs, obtained for tissue blocks exposed to the patch for 1, 2 and 4 h, are shown in Figs. 7a, 9a and 11a. Visualisation was improved by converting images to two dimensional contour plots (Figs. 7b, 9b and 11b) using scanning densitometry and analysing the optical density distribution along an axis of drug flux (Figs. 7c, 9c and 11c) and using a raster scan over the entire plane of the tissue section. The raster scans from the 1-, 2- and 4-h penetration

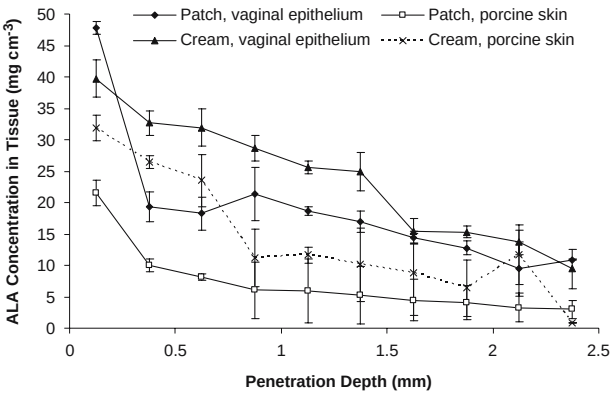

Fig. 5. Influence of formulation factors and tissue type on penetration of 5-aminolevulinic acid (5-ALA). The penetration profiles shown are those of the patch containing 38 mg 5-ALA cm^{-2} and the proprietary Porphin® cream (20% w/w 5-ALA in Unguentum Merck®; means±S.D., n=3). The application time used was 4 h

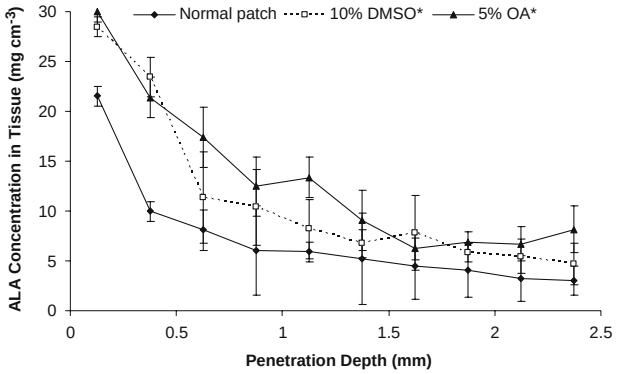

Fig. 6. Influence of penetration enhancers on penetration of 5-aminolevulinic acid (5-ALA) into neonate porcine skin. The penetration profiles shown are those of the normal patch containing 38 mg 5-ALA cm^{-2} and those of patches containing both 38 mg 5-ALA cm^{-2} and the maximum loadings of dimethyl sulphoxide (*DMSO*) and oleic acid (*OA*), respectively (means±S.D., n=3). The application time used was 4 h in each case. *Asterisk* indicates concentrations (% w/w) of DMSO and OA in aqueous blends used to prepare bioadhesive films

autoradiographs were translated into spatial coordinates and visualised with three-dimensional contouring computer software (Delta-Graph®). The contour graphs are shown in (Figs. 8, 10 and 12). Each grey scale value from a raster scan image is translated into a value that forms the z coordinate on the corresponding contour graph. The y coordinate represents the distance along the plane perpendicular to the plane of the patch. The x coordinate is a representation of distance down through the tissue slice, parallel to the plane of the patch and gives an indication of the depth of 5-ALA penetration through the tissue.

The raster scans show that drug penetration moves in a uniform front through tissue and after 1 h, it has reached a depth of approximately 2.0 mm. There is no evidence of 5-ALA at deeper sites. After 2 and 4 h, autoradiography shows that the 5-ALA has permeated through the entire thickness of the tissue section, down to approximately 5 mm. There is no indication of localised hot spots where 5-ALA concentrations are higher than surrounding sites. After 4 h, the graph in Fig. 11c is seen to resemble the corresponding profile seen in Fig. 4, where a steady decline of 5-ALA is seen as distance from the surface is increased.

DISCUSSION

The work described in this paper demonstrated how detailed information regarding the spatial concentration of 5-ALA through epithelial tissue could be assembled using radiographic procedures. This information has an important bearing upon formulation design in that it is an essential requisite that concentrations of 5-ALA delivered to tissue reach a phototoxic threshold before therapeutic outcomes can be expected. These concentrations can be estimated from cell culture experiments, which indicate that concentrations must reach levels between 0.01 and 0.17 mg ml^{-1} before sufficient protoporphyrin IX (PpIX) is produced to cause satisfactory neoplastic cell death upon illumination (25,26). Numerous literature sources report on the penetration of

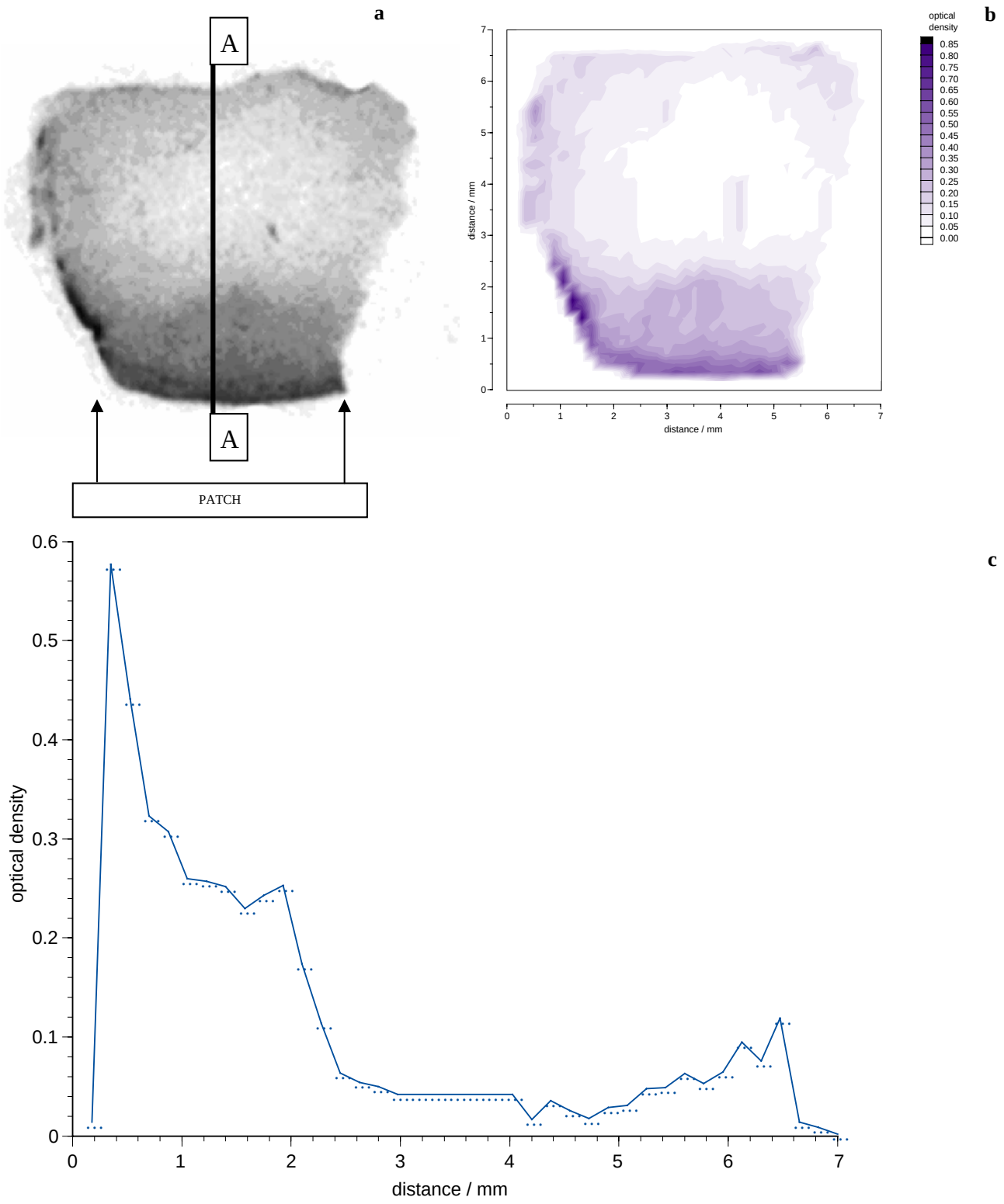

Fig. 7. Autoradiograph **a** of a slice of tissue from a block of vaginal epithelium exposed to a radiolabelled bioadhesive patch for 1 h, **b** the corresponding scanning densitometer trace and **c** variation of optical density taken across the AA axis

topically applied 5-ALA into tissue, both *in vitro* and *in vivo*, but while a wide range of techniques have been used to assess penetration depth, few detail the 5-ALA concentration in tissue at the depths studied. In contrast to the methods used in this work, other work described in the literature has used fluorescence microscopy to investigate the formation of PpIX in tissue, after topical application of 5-ALA. In the majority of cases, an 5-ALA-containing vehicle is applied topically to normal or diseased skin of living subjects, left in place for 4–6 h and then a biopsy is taken at the site of application.

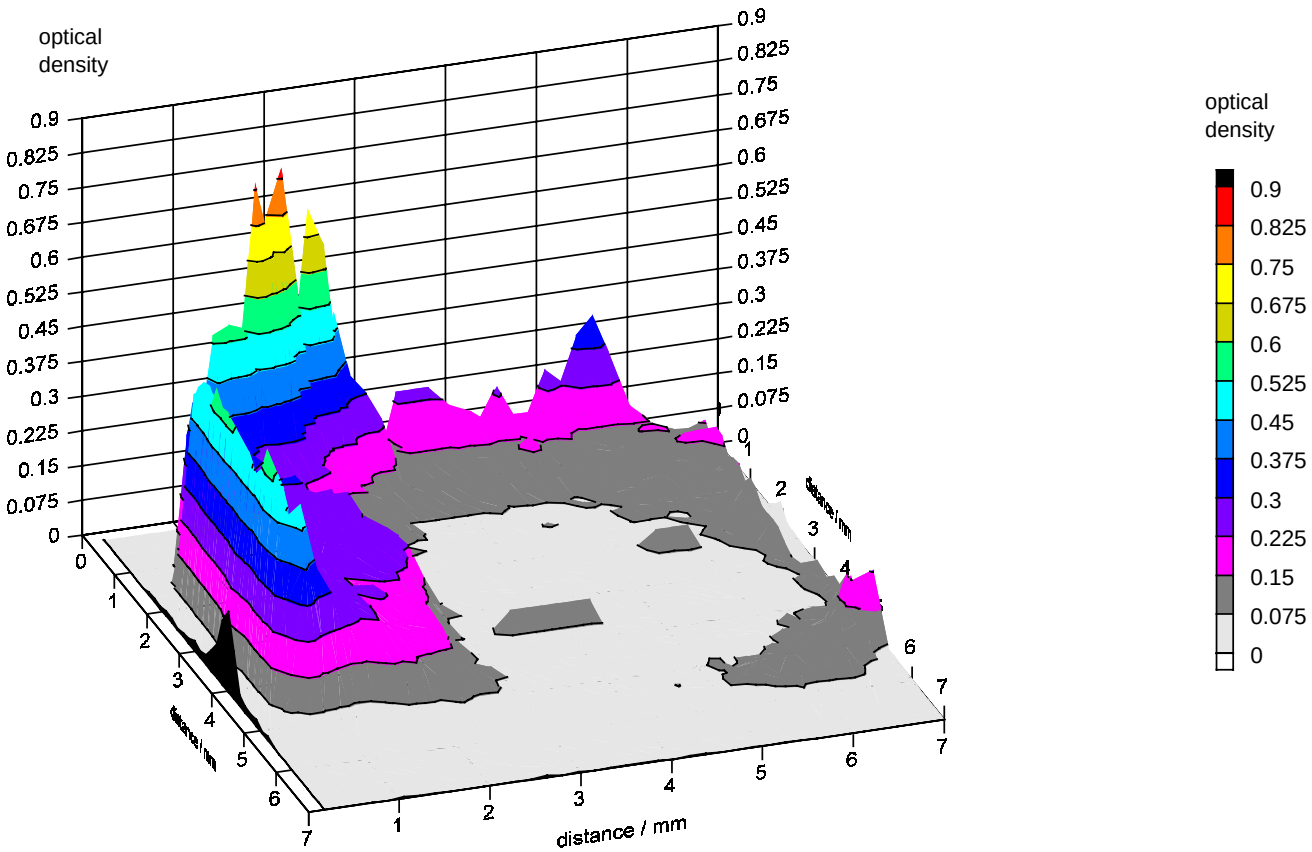

Fig. 8. Raster scan image of an autoradiograph of the slice of tissue (Fig. 7a) taken from a block of vaginal epithelium exposed to a radiolabelled bioadhesive patch for 1 h. The contour plot shows high levels of 5-ALA within the first 2 mm of tissue, but no evidence of 5-ALA beyond this distance

Biopsied samples are frozen and either sectioned using a microtome or dissolved. The fluorescence intensity of PpIX in the sectioned tissue is studied using either fluorescence microscopy (27,28) or determination of PpIX in dissolved tissue samples made using fluorescence spectrophotometry (29–31). These fluorescence-based studies tend to be qualitative, comparing PpIX emission with background auto-fluorescence. They indicate primarily the depth of PpIX formation and, thus by corollary, the 5-ALA penetration in tissue. These types of analysis show that PpIX synthesis is tissue specific and are useful in indicating the maximum depth at which PpIX is observed. Thus, Konig *et al.* (32) reported PpIX fluorescence at a depth of 0.6 mm, 6 h after topical 5-ALA application, in patients with skin tumours. Szeimies *et al.* (33) reported PpIX fluorescence at a depth of 0.3 mm in basal cell carcinomas and Pahernik *et al.* (34) reported PpIX fluorescence at depths as low as 3 mm in hamster skin tumour models.

Methods to investigate 5-ALA levels following topical administration trend to be more complex, especially as some form of tissue extraction is needed. Alternative procedures have been devised to circumvent this problem, such as using microdialysis to quantify 5-ALA in normal skin and basal cell carcinoma after topical application of 5-ALA (20% w/w) in an aqueous gel (35). A microdialysis tube, inserted intracutaneously at a depth of 0.5 mm, showed the 5-ALA to range between 0 and 0.52 mg ml^{-1} under the tumour lesion, but was unable to detect any in normal skin at this depth. The drawback with this approach is that concentrations immediately above and below the 0.5 mm marker cannot be assessed and the total depth is also unknown. The approach used in this work generates a more complete profile of 5-ALA concentrations in a stepwise fashion up to 2.5 mm. As many intraepithelial neoplastic cells can reside at such depths, a study such as the one described in this current work can give a more detailed picture and a better estimation if the formulation approach used is likely to give successful PDT of the target lesion.

Liquid scintillation spectrometry was shown to be an appropriate method for the determination of 5-ALA in tissue samples as sensitivity is excellent and concerns regarding analyte extraction from tissue are minimal. Spiking 5-ALA-loaded aqueous blends and creams with known amounts of radiolabelled 5-ALA before casting produced formulations with uniform activity in unit area. Therefore, the dpm counted during an analysis could be directly related to the 5-ALA recovered from a known volume of tissue, as described for patches spiked with radiolabelled 5-fluorouracil (36). Casas *et al.* (9) using liquid scintillation spectrometry, showed that 5-ALA could penetrate model mouse tumours down to depths of 5 mm, although the majority of drug was found in the upper 2 mm of tissue. Importantly, though, 5-ALA concentrations at the different depths were not reported, in contrast to the present study.

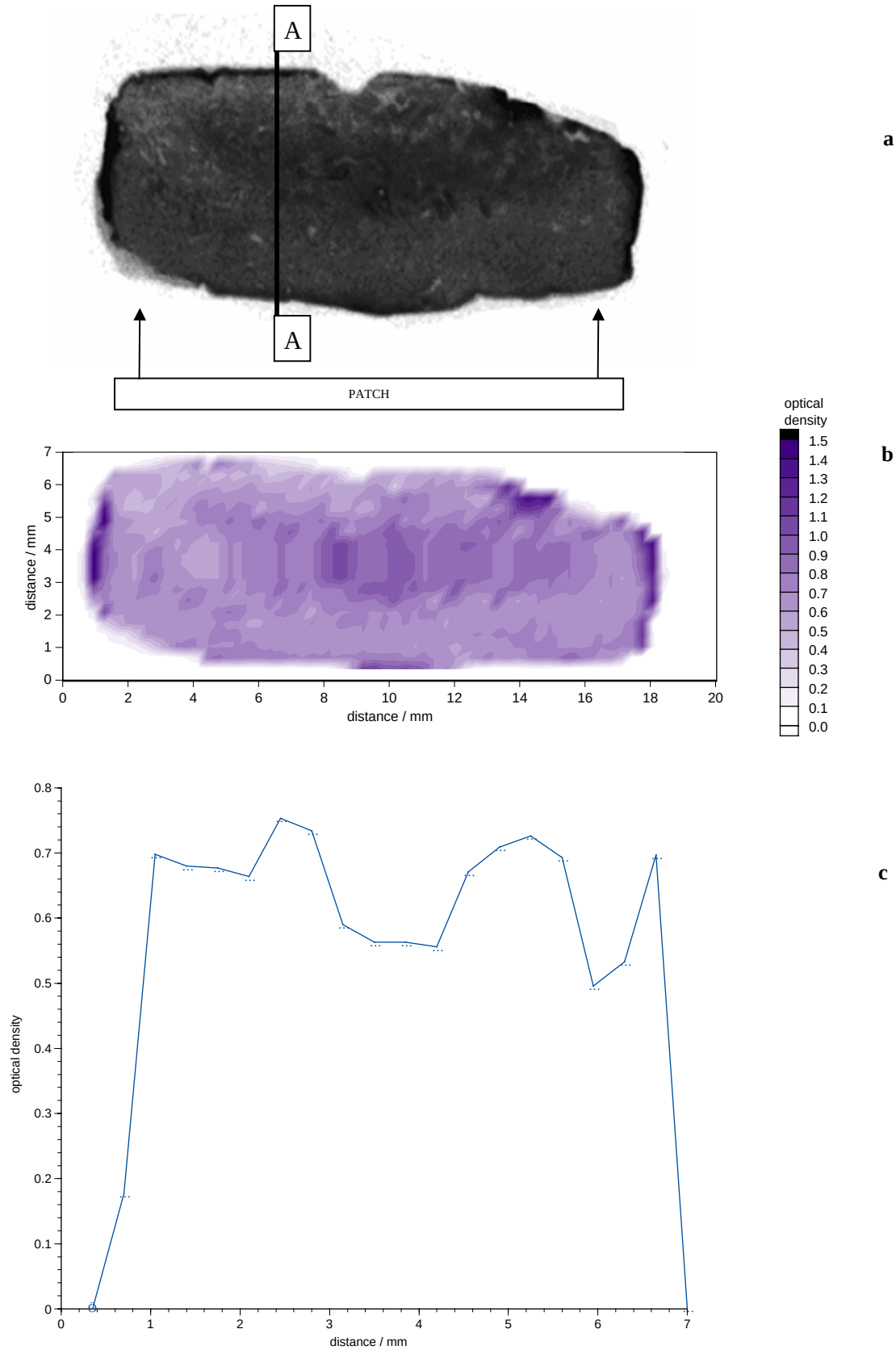

Fig. 9. Autoradiograph of a slice of tissue **a** from a block of vaginal epithelium exposed to a radiolabelled bioadhesive patch for 2 h, with **b** corresponding scanning densitometer trace and **c** variation of optical density taken across the AA axis

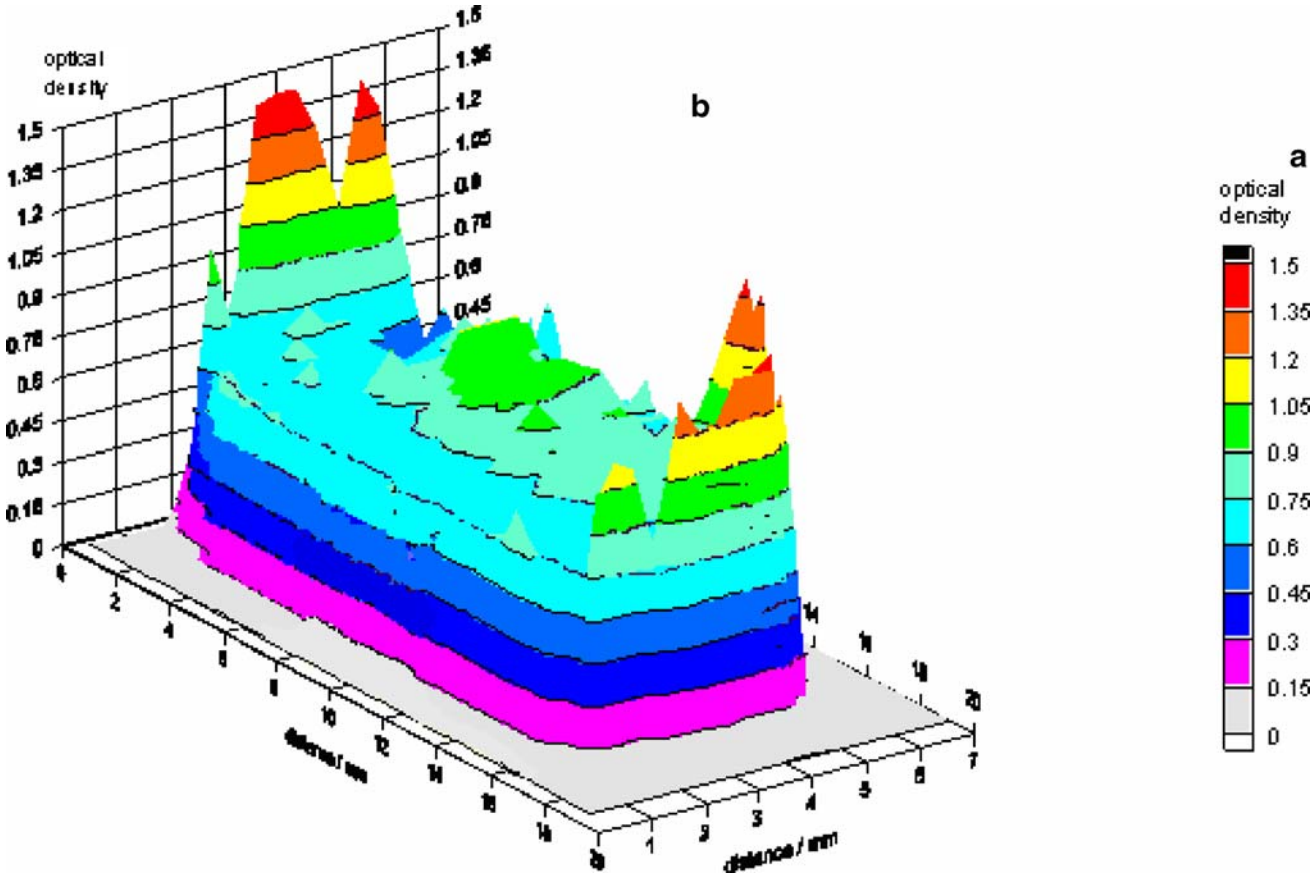

Fig. 10. Raster scan image of an autoradiograph of a slice of tissue from a block of vaginal epithelium exposed to a radiolabelled bioadhesive patch for 2 h (**a**) and the corresponding three-dimensional contour graph (**b**)

Due to the small size of the tissue samples used in this study, 2.5 mm was the maximum thickness of tissue sample that could be microtomed into slices and analysed using scintillation spectrometry. Retrieving the first slice taken at the surface would give an indication of the surface concentration of 5-ALA, but this is not always a pragmatic option as the upper surface is not necessarily flat. If a first slice was taken, it may fail to capture some regions that sit lower in respect to others. Once the second and third slices are taken, this roughness effect is no longer of any consequence. Furthermore, traces of formulation still remaining following the removal of the cream or patch formulation can show as concentration spikes in the first slice. For these reasons, and to reduce the number of vials counted and ensure a significantly high count, five consecutive slices were placed in each scintillation vial. This meant that mean depths and mean 5-ALA concentrations were reported and that the maximum average depth reported was 2.375 mm, even though VIN can occur at depths as low as 2.5 mm. This is unlikely to be a drawback, since the concentrations of 5-ALA observed at a mean depth of 2.375 mm in vaginal epithelial tissue, typically around 5 mg cm^{-3}, were an order of magnitude greater than those required to kill sufficient numbers of neoplastic cells by PDT (25). Hence, it can be expected that 5-ALA concentrations at immediately adjacent depths of 2.5 mm are also high. Indeed, autoradiography showed that 5-ALA can penetrate vaginal epithelial tissue in detectable amounts down to depths of at least 6 mm, even after 2-h application times, as shown in Fig. 9.

From Table II and Fig. 3a, it can be seen that no significant differences were observed between the proprietary Porphin® cream and the patch containing an equivalent amount of 5-ALA, in terms of 5-ALA concentration in vaginal epithelial tissue. From Table III and Figs. 3b and 4 it can seen that drug loading and application time are the most significant factors determining 5-ALA concentration in vaginal epithelial tissue. Vaginal epithelial tissue offers no keratinised *SC* barrier to drug transport, yet the vulva consists of epithelial tissue that is considered to be keratinising. It could be argued that vaginal tissue is not a sufficiently rigorous model for vulval tissue and to address this criticism, neonate porcine skin was selected as a model keratinised tissue type possessing an intact *SC*. It is well known that impairment of the *SC* permeability barrier overlying malignant and premalignant lesions allows increased 5-ALA penetration (37). Indeed, the *SC* has been shown to be the most important barrier to tissue penetration of topically applied 5-ALA (28). It has also been shown, using autoradiography, that the majority of a topically applied 5-ALA dose did not penetrate porcine skin much deeper than the lower reaches of the *SC* (38). The same authors, using scintillation spectrometry, reported that any 5-ALA getting past the *SC* barrier only reached depths of 100–150 μm in tissue. These results are at considerable variance to those in this present work, where in Figs. 5 and 6 there is clear evidence that 5-ALA permeation is able to extend much further. Although the available 5-ALA per cm^2 in the formulations used in the present work have been matched, the patch is thinner and so

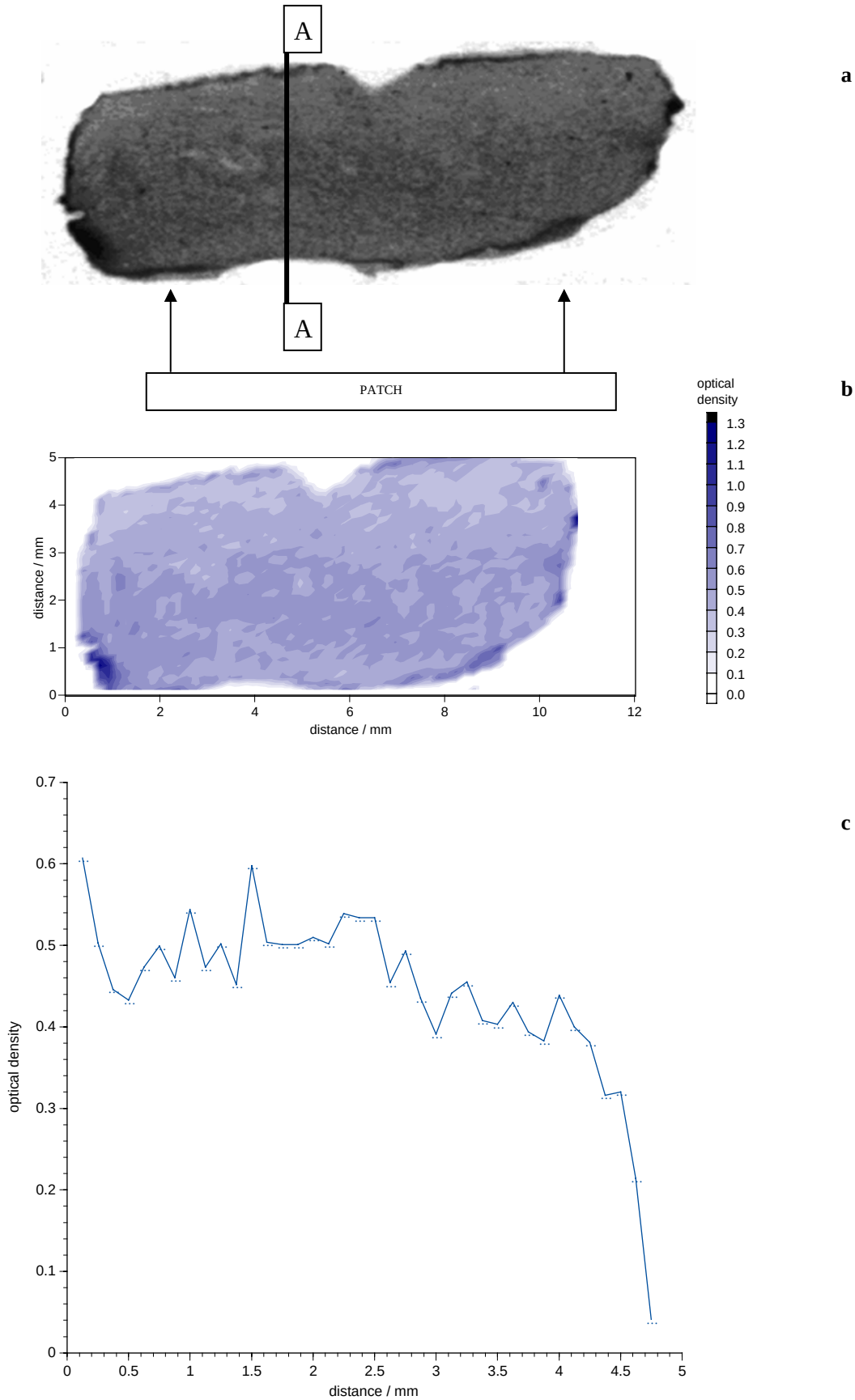

Fig. 11. Autoradiograph of a slice of tissue **a** from a block of vaginal epithelium exposed to a radiolabelled bioadhesive patch for 4 h, with **b** corresponding scanning densitometer trace and **c** variation of optical density taken across the AA axis

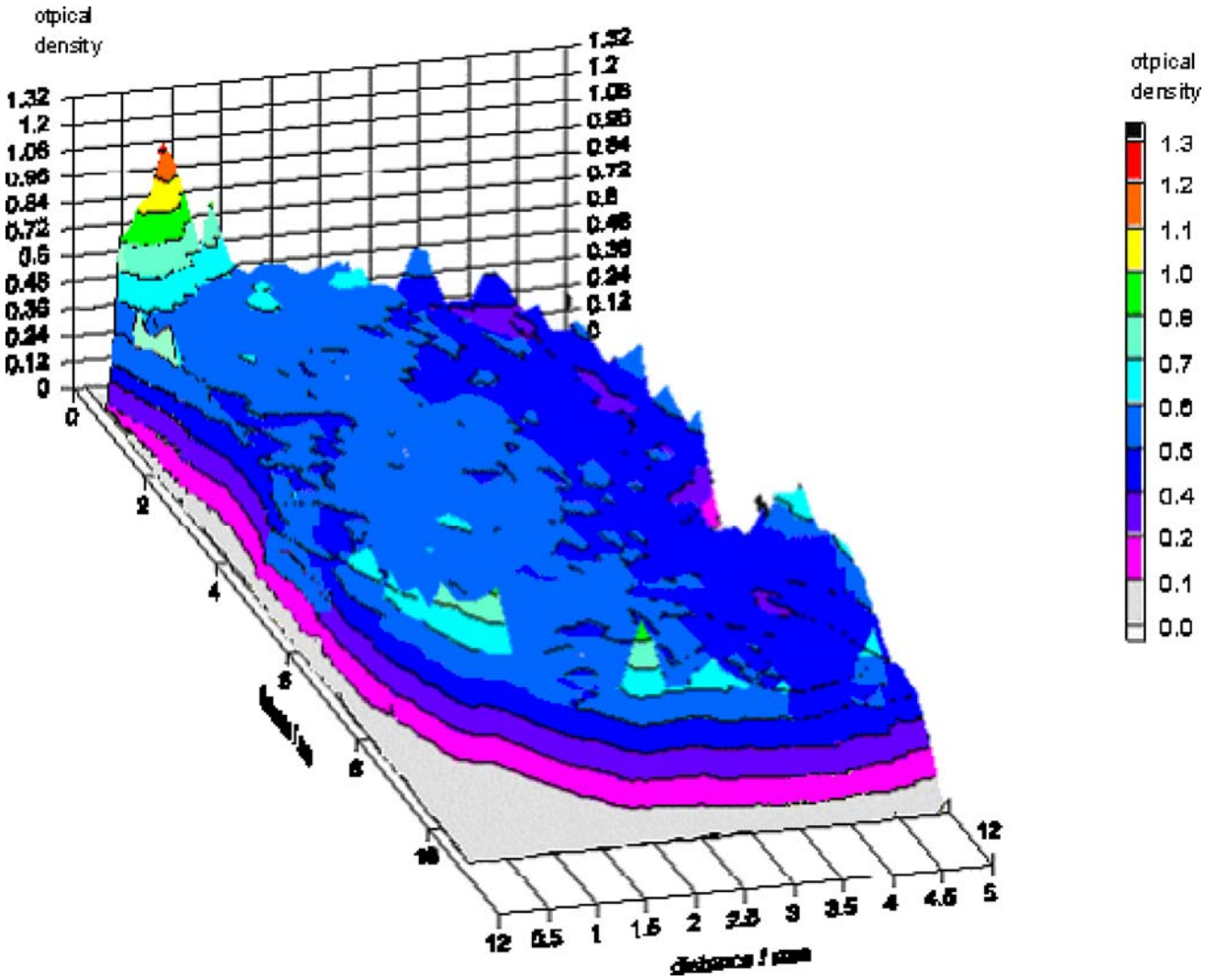

Fig. 12. Raster scan image of an autoradiograph of a slice of tissue from a block of vaginal epithelium exposed to a radiolabelled bioadhesive patch for 4 h

the concentration of 5-ALA will be more per unit volume of formulation matrix. This would also have implications for the length of the diffusional path and the thermodynamic activity of 5-ALA, which may explain, as shown in Table II, how the patch is able to deliver a modest dose increase across the *SC* barrier that that from the cream.

It is not surprising that a number of strategies to enhance 5-ALA penetration through intact *SC* have been devised. One such approach commonplace in clinical practice is occlusion, whereupon an impermeable barrier dressing is applied over the cream formulation once in position over a topical lesion. In this present work, the occlusive approach featured in the experimental design from the outset in that patch formulations possessed an impermeable outer PVC layer. The use of the intimately fitting glass cup, as illustrated in Fig. 1, goes some way to simulate occlusion when cream formulations were evaluated instead. Other more sophisticated strategies include the use of ester derivatives of 5-ALA with enhanced lipophilicity, encapsulation into lipid microparticles or nanoparticles and iontophoretic approaches. Curettage and tape stripping prior to 5-ALA or 5-ALA-ester application have also been used to remove keratinised debris overlying lesions (15). Penetration enhancers, particularly dimethyl sulphoxide (DMSO), have, perhaps, been the most commonly employed tools to enhance tissue penetration of 5-ALA to date (9,37).

In the present study, the adaptability of the patch formulation was demonstrated in that both DMSO and oleic acid could be co-formulated into the bioadhesive matrix in a bid to enhance 5-ALA penetration. It has been proposed that DMSO acts by association with, and solubilisation of, *SC* lipids and proteins, thereby altering the conformation and barrier properties of the skin. Oleic acid (OA) is thought to insert between intercellular lipids, thereby disrupting the ordered bilayer structures. From Table III and Fig. 6, it can be seen clearly that the ability of 5-ALA to penetrate neonate porcine skin is reduced significantly compared to its ability to penetrate vaginal epithelium. This was true for both cream and patch formulations, with the 5-ALA concentration, at a mean depth of 2.375 mm in the porcine tissue, induced by the cream being significantly smaller than for the patch. Inclusion of penetration enhancers in the patch had no significant effect on 5-ALA concentrations in vaginal epithelial tissue. This was to be expected, since both DMSO and

OA have their primary action on the *SC*, a barrier absent from epithelial tissue surfaces. While both DMSO and OA increased 5-ALA concentrations in neonate porcine skin at mean depths of 2.375 mm, this effect was not significant in most cases, regardless of the amount of penetration enhancer present. The one exception was with the patch cast from a blend containing 5% w/w OA, which did cause a significant increase in 5-ALA concentration at 2.375 mm, although as mentioned this effect could be a statistical artefact.

Although findings from this study did not justify the inclusion of DMSO in terms of penetration enhancement alone, it should be remembered that DMSO is known to be a malignant cell differentiator. It has been shown to induce increased activity of 5-ALA synthase, 5-ALA dehydratase and porphobilinogen deaminase in cells in culture (39). In this way, increased PpIX production in treated tissue could be enhanced. Therefore, inclusion of DMSO in patch formulations may be of use in PDT, even if it does not significantly enhance 5-ALA penetration. De Rosa *et al*. (27) showed that only DMSO concentrations of 20% w/w in cream formulations improved 5-ALA penetration and protoporphyrin IX concentrations in deep tissue. In fact, the activity of DMSO is known to be highly concentration dependent and vehicle concentrations of 60% w/w and above are generally required to produce meaningful effects. This has immediate drawbacks in that such high concentrations of DMSO produce erythema, irreversible skin damage, delamination of the *SC* and denaturation of its proteins. Furthermore, such high levels in the patch formulation were not possible due to detrimental effects on the patch properties. Aqueous blends containing 10% w/w DMSO produced patches with reduced tensile strengths and further increases did not allow proper film formation after drying. Films containing excessive OA experienced similar problems, with reduced bioadhesive capabilities and changes in flexibility under ambient storage.

Despite the ineffectiveness of penetration enhancement, 5-ALA concentrations at mean depths of 2.375 mm in neonate porcine skin were an order of magnitude greater than concentrations reported as being required to induce sufficient levels of neoplastic cell death by PDT (25,26). This was true for all formulations investigated. Bearing in mind that this study has shown both non-keratinised and keratinised tissue types accumulated 5-ALA in high concentrations, it is feasible to argue that partially keratinised vulval skin, which harbour VIN, may also accumulate similar effective levels of 5-ALA following topical applications of the vehicles studied. It should also be mentioned that although the tissues used in this study were effectively non-living, it is possible that their living counterparts will show slight differences in diffusivity of 5-ALA. However, given the large excess of 5-ALA found *in vitro*, it is unlikely that diffusivity differences will reduce concentrations to sub-therapeutic amounts during PDT treatment sessions.

The autoradiographs and their three-dimensional rendering resulted in novel representations of static 5-ALA permeation through vaginal epithelial tissue. The plane of the image occupies the x–y coordinate plane and the grey intensity at every point is lifted into the z plane. Figs. 7 and 8, which corresponds to tissue exposed to the radiolabelled patch for 1 h, clearly show the drug gradient acting away from the applied patch. However, beyond 2 mm, a region devoid of 5-ALA is observed. This result shows that 5-ALA permeation through vaginal tissue is rapid, needing only 60 min to reach a reasonable depth. Some leakage is observed around and down the side of the tissue block giving a peripheral artefact. It is interesting to see the contour planes in tissue exposed to radiolabelled patches for 2 and 4 h, as shown in Figs. 10 and 12, respectively. Penetration of 5-ALA down to a depth of at least 6 mm in the tissue is obvious. Additionally, the gradient has become flatter, indicating that the vaginal epithelial tissue, which possesses no *SC* barrier to drug penetration, has effectively been flooded with 5-ALA after such application times. There is no evidence of localised regions of high 5-ALA concentration, bar some peripheral aberrations, suggesting that the bioadhesive patch is an effective delivery vehicle capable of flooding non-keratinised tissue and, most probably, partially keratinised tissue as well.

In summary, it is clear that 5-ALA, released from a bioadhesive patch, penetrates vaginal epithelial tissue to at least 6 mm, as shown using autoradiography. The concentration of 5-ALA in tissue was dependent on application time and drug loading of applied formulations. The presence of a *stratum corneum* barrier significantly reduced the concentration of 5-ALA in tissue after topical application. Inclusion of penetration enhancers, such as oleic acid and dimethyl sulphoxide, in bioadhesive patches increased the concentration of 5-ALA in neonate porcine tissue, but this effect was only significant for patches cast from blends containing 5% w/w oleic acid. Nevertheless, the concentration of 5-ALA in both vaginal epithelial tissue and neonate porcine skin at mean depths of 2.375 mm was still an order of magnitude above concentrations reported as being lethal to sufficient numbers of neoplastic cells during photodynamic therapy (PDT). From this, it can be assumed that the bioadhesive patch described herein could be used to deliver 5-ALA to depths of at least 2.5 mm in vulval skin, at concentrations that would be effective for PDT. This means that this patch should be capable of treating even the deepest lesions of vulval intraepithelial neoplasia successfully. Ideally, lesions of VIN would be included in a similar study and the penetration of 5-ALA into such tissue investigated and compared to drug penetration into healthy vulval skin. However, obtaining such pathological tissue is prohibitively difficult. A final mention should be made of light penetration to depths around 2.5 mm in VIN. Although this study demonstrated that the drug delivery requirement for PDT was able to be met, there is still a need to verify light dosimetry at these specific depths. If the red light used typically in PDT could not penetrate vulval tissue efficiently to 2.5 mm, the 5-ALA present at such a depth and the protoporphyrin IX it generates would be ineffective.

REFERENCES

1. C. A. Morton, R. M. MacKie, C. Whitehurst, J. V. Moore, and J. H. McColl. Photodynamic therapy for basal cell carcinoma: effect of tumour thickness and duration of photosensitizer application on response. *Arch. Dermatol.* **134**:248–249 (1998).
2. E. W. Jeffes, J. L. McCullough, G. P. Weinstein, P. E. Fergin, J. S. Nelson, T. F. Shull, K. R. Simpson, L. M. Bukaty, W. L. Hoffman, and N. L. Fong. Photodynamic therapy of actinic keratosis with

topical 5-aminolevulinic acid: a pilot dose-ranging study. *Arch. Dermatol.* **133**:727–732 (1997).
3. G. I. Stables, M. R. Stringer, D. J. Robinson, and D. V. Ash. Large patches of Bowen's disease treated by topical aminolevulinic acid photodynamic therapy. *Br. J. Dermatol.* **136**:957–960 (1997).
4. P. Hillemanns, M. Untch, C. Dannecker, R. Baumgartner, H. Stepp, J. Diebold, H. Weingandt, F. Prove, and M. Korell. Photodynamic therapy of vulvar intraepithelial neoplasia using 5-aminolevulinic acid. *Int. J. Cancer* **85**:649–653 (2000).
5. P. A. McCarron, R. F. Donnelly, A. D. Woolfson, and A. Zawislak. Photodynamic therapy of vulval intraepithelial neoplasia using bioadhesive patch-based delivery of aminolevulinic acid. *Drug Deliv. Sys. Sci.* **3**:59–64 (2003).
6. A. Zawislak, P. A. McCarron, W. G. McCluggage, J. H. Price, R. F. Donnelly, H. R. McClelland, S. P. Dobbs, and A. D. Woolfson. Successful photodynamic therapy of vulval Paget's disease using a novel patchbased delivery system containing 5-aminolevulinic acid. *Br. J. Obstet. Gynaecol.* **111**:1–3 (2004).
7. P. A. McCarron, R. F. Donnelly, A. D. Woolfson, A. Zawislak, J. H. Price, and P. Maxwell. Photodynamic treatment of lichen sclerosus and squamous hyperplasia using sustained topical delivery of aminolevulinic acid from a novel bioadhesive. *Br. J. Dermatol.* **151**(Suppl. 68):105–106 (2004).
8. S. Collaud, A. Juzeniene, J. Moan, and N. Lange. On the selectivity of 5-aminolevulinic acid-induced protoporphyrin IX formation. *Curr. Med. Chem.-Anti-Cancer Agents* **4**:301–316 (2004).
9. A. Casas, H. Fukuda, G. Di Venosa, and A. M. Del C. Batlle. The influence of the vehicle on the synthesis of porphyrins after topical application of 5-aminolevulinic acid. Implications in cutaneous photodynamic sensitisation. *Br. J. Dermatol.* **143**:564–572 (2000).
10. R. I. Schleuplein. Mechanisms of percutaneous absorption II. Transient diffusion and the relative importance of various routes of skin penetration. *J. Invest. Dermatol.* **48**:79 (1967).
11. P. M. Elias. Structure and function of the *Stratum Corneum* permeability barrier. *Drug Dev. Res.* **13**:97–105 (1988).
12. R. O. Potts. Physical characterisation of the *Stratum Corneum*: the relationship of mechanical and barrier properties to lipid and protein structure. In J. Hadgraft, and R. H. Guy (eds.), *Transdermal Drug Delivery; Developmental Issues and Research Initiatives*, Marcel Dekker, New York, 1989.
13. S. Lieb, R. M. Szeimies, and G. Lee. Self-adhesive thin films for topical delivery of 5-aminolevulinic acid. *Eur. J. Pharm. Biopharm.* **53**:99–106 (2002).
14. Z. Malik, G. Kostenich, L. Roitman, B. Ehrenberg, and A. Orenstein. Topical application of 5-aminolevulinic acid, DMSO and EDTA: protoporphyrin IX accumulation in skin and tumours of mice. *J. Photochem. Photobiol. B Biol.* **28**(3) 213–218 (1995).
15. A. M. Soler, T. Warloe, A. Berner, and K. E. Giercksky. A follow-up study of recurrence and cosmesis in completely responding superficial and nodular basal cell carcinomas treated with methyl 5-aminolevulinate-based photodynamic therapy alone with prior curettage. *Br. J. Dermatol.* **145**:467–471 (2001).
16. A. T. Dijkstra, I. M. L. Majoie, J. W. F. van Dongen, H. van Weelden, and W. A. van Vloten. Photodynamic therapy with violet light and topical 5-aminolevulinic acid in the treatment of actinic keratosis, Bowen's disease and basal cell carcinoma. *J. Eur. Acad. Dermatol. Venereol.* **15**:550–554 (2001).
17. F. Cairnduff, M. R. Stringer, E. J. Hudson, D. V. Ash, and S. B. Brown. Superficial photodynamic therapy with topical 5-aminolevulinic acid for superficial primary and secondary cancer. *Br. J. Cancer* **69**:605–608 (1994).
18. P. A. McCarron, R. F. Donnelly, A. Zawislak, A. D. Woolfson, J. H. Price, and R. McClelland. Evaluation of a water-soluble bioadhesive patch for photodynamic therapy of vulval lesions. *Int. J. Pharm.* **293**:11–23 (2005).
19. P. Shatz, C. Bergeron, E. J. Wilkinson, J. Arseneau, and A. Ferenczy. Vulvar intraepithelial neoplasia and skin appendage involvement. *Obstet. Gynecol.* **74**:769–774 (1989).
20. J. G. Moore, and N. F. Hacker. *Essentials of Obstetrics and Gynecology*, Churchill Livingstone, Philadelphia, 1998, pp. 3–681.
21. L. J. Copeland. *Textbook of Gynecology*, Saunders, Philadelphia, 2000, pp. 1252–1253.
22. J. Wolf. Die Innere Strucktur der Zellen des Stratum Desquamans der Menschlichen Epidermis. *Anat. Forsch.* **46**:170–202 (1939).
23. A. D. Woolfson, and D. F. McCafferty. *Percutaneous Local Anaesthesia*, Ellis Horwood, London, 1993.
24. D. X. G. Divaris, J. C. Kennedy, and R. H. Pottier. Phototoxic damage to sebaceous glands and hair follicles of mice after systemic administration of 5-aminolevulinic acid correlates with localized protoporphyrin IX fluorescence. *Am. J. Pathol* **136**:891–897 (1990).
25. C. Fuchs, R. Riesenberg, J. Siegert, and R. Baumgartner. pH-dependent formation of 5-aminolevulinic acid-induced protoporphyrin IX in fibrosarcoma cells. *J. Photochem. Photobiol. B Biol.* **40**:49–54 (1997).
26. J. Moan, G. Streckyte, S. Bagdonas, O. Bech, and K. Berg. Photobleaching of protoporphyrin IX in cells incubated with 5-aminolevulinic acid. *Int. J. Cancer* **70**:90–97 (1997).
27. F. S. De Rosa, J. M. Marchetti, J. A. Thomazini, A. C. Tedesco, M. V. Lopes, and B. Bentley. A vehicle for photodynamic therapy of skin cancer: influence of dimethylsulphoxide on 5-aminolevulinic acid *in vitro* cutaneous permeation and *in vivo* protoporphyrin IX accumulation determined by confocal microscopy. *J. Control. Release* **65**:359–366 (2000).
28. J. T. H. M. Van den Akker, V. Iani, W. M. Star, H. J. C. M. Sterenborg, and J. Moan. Topical application of 5-aminolevulinic acid hexyl ester and 5-aminolevulinic acid to normal nude mouse skin: differences in protoporphyrin IX fluorescence kinetics and the role of the *Stratum Corneum*. *Photochem. Photobiol.* **72**:681–689 (2000).
29. K. Berg, H. Anholt, O. Bech, and J. Moan. The influence of iron chelators on the accumulation of protoporphyrin IX in 5-aminolevulinic acid-treated cells. *Br. J. Cancer* **74**:688–697 (1996).
30. A. Casas, H. Fukuda, and A. M. Del C. Batlle. Tissue distribution and kinetics of endogenous porphyrins synthesized after topical application of ALA in different vehicles. *Br. J. Cancer* **81**:13–18 (1999).
31. J. C. Tsai, Chen H., W. Wong, and Y. L. Lo. *In vitro*/*in vivo* correlations between transdermal delivery of 5-aminolevulinic acid and cutaneous protoporphyrin IX accumulation and effect of formulation. *Br. J. Dermatol.* **146**:853–862 (2002).
32. K. Konig, A. Kienle, W. H. Boehncke, R. Kaufmann, A. Ruck, T. Meier, and R. Steiner. Photodynamic tumor therapy and online fluorescence spectroscopy after ALA administration using 633 nm light as therapeutic and fluorescence excitation radiation. *Opt. Eng.* **33**:2945–2953 (1994).
33. R. M. Sziemes, T. Sassy, and M. Landthaler. Penetration potency of topical applied 5-aminolevulinic acid for photodynamic therapy of basal cell carcinoma. *Photochem. Photobiol.* **59**:73–76 (1994).
34. S. Pahernik, S. Langer, A. Botzlar, M. Dellian, and A. E. Goetz. Tissue distribution and penetration of 5-ALA induced fluorescence in an amelanotic melanoma after topical application. *Anticancer Res.* **21**:59–64 (2001).
35. A. M. Wennberg, O. Larko, P. Lonnroth, G. Larson, and A. L. Krogstad. 5-aminolevulinic acid in superficial basal cell carcinomas and normal skin—a microdialysis and perfusion study. *Clin. Exp. Dermatol.* **25**:317–322 (2000).
36. A. D. Woolfson, D. F. McCafferty, P. A. McCarron, and J. H. Price. Liquid scintillation spectometry of 5-fluorouracil in cervical tissue following *in vitro* surface application of a bioadhesive cervical patch. *Pharm. Res.* **11**:1315–1319 (1994).
37. Y. Harth, B. Hirshowitz, and B. Kaplan. Modified topical photodynamic therapy of superficial skin tumours, utilizing aminolevulinic acid, penetration enhancers, red light and hyperthermia. *Dermatol. Surg.* **24**:723–726 (1998).
38. P. G. Johnson, S. W. Hui, and A. R. Oseroff. Electrically enhanced percutaneous delivery of 5-aminolevulinic acid using electric pulses and a DC potential. *Photochem. Photobiol.* **75**:534–540 (2002).
39. R. A. Galbraith, S. Sassa, and A. Kappas. Induction of haem synthesis in hep G2 human hepatoma cells by dimethyl sulphoxide. *Biochem. J.* **237**:597–600 (1986).