

Stability of 5-Aminolevulinic Acid in Novel Non-Aqueous Gel and Patch-Type Systems Intended for Topical Application

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ABSTRACT: Aminolevulinic acid (ALA) stability within topical formulations intended for photodynamic therapy (PDT) is poor due to dimerisation to pyrazine-2,5-dipropionic acid (PY). Most strategies to improve stability use low pH vehicles, which can cause cutaneous irritancy. To overcome this problem, a novel approach is investigated that uses a non-aqueous vehicle to retard proton-induced charge separation across the 4-carbonyl group on ALA and lessen nucleophilic attack that leads to condensation dimerisation. Bioadhesive anhydrous vehicles based on methylvinylether-maleic anhydride copolymer patches and poly(ethyleneglycol) or glycerol thickened poly(acrylic acid) gels were formulated. ALA stability fell below pharmaceutically acceptable levels after 6 months, with bioadhesive patches stored at 5°C demonstrating the best stability by maintaining 86.2% of their original loading. Glycerol-based gels maintained 40.2% in similar conditions. However, ALA loss did not correspond to expected increases in PY, indicating the presence of another degradative process that prevented dimerisation. Nuclear magnetic resonance (NMR) analysis was inconclusive in respect of the mechanism observed in the patch system, but showed clearly that an esterification reaction involving ALA and both glycerol and poly(ethyleneglycol) was occurring. This was especially marked in the glycerol gels, where only 2.21% of the total expected PY was detected after 204 days at 5°C. Non-specific esterase hydrolysis demonstrated that ALA was recoverable from the gel systems, further supporting esterified binding within the gel matrices. It is conceivable that skin esterases could duplicate this finding upon topical application of the gel and convert these derivatives back to ALA *in situ*, provided skin penetration is not affected adversely. © 2005 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 94:1756–1771, 2005

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INTRODUCTION

Photodynamic therapy (PDT) combines the irradiance of visible light with administration of a sensitising drug to bring about selective destruc-

tion of neoplastic cells undergoing rapid metabolism.¹ The action of a light dose of suitable wavelength produces excitation of the photosensitiser within intracellular space.^{2,3} Energy relaxation *via* the transition of molecular oxygen to its singlet state is believed to be the primary cytotoxic species that causes irreversible cellular damage.⁴ The most commonly used agent in PDT is 5-aminolevulinic acid (ALA), which is a naturally occurring precursor in the biosynthetic pathway of haem. Elevated intracellular

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concentrations of haem exert a negative feedback control over its biosynthesis. Administration of excess exogenous ALA avoids this process and, in so doing, induces a back accumulation of protoporphyrin IX (PpIX) due to the limited capacity of ferrochelatase. This enzyme is the final biosynthetic step in the pathway and inserts iron into the PpIX molecule.^{2,5,6} Accumulation is pronounced in rapidly proliferating cells, which not only have reduced ferrochelatase activity, but also enhanced porphobilinogen deaminase activity.^{3,7,8} The end result is photosensitisation resulting from elevated intracellular PpIX concentrations within selected cell populations.

The stability of ALA in non-biological, aqueous systems, such as drug delivery vehicles with a pH of 6 and above, is notoriously poor. It has been shown to be dependent on pH, concentration, temperature and degree of oxygenation of the solution.^{9–12} The reaction, shown in Figure 1a, proposed by Novo Rodriguez et al.,¹³ illustrates the acid-base equilibria leading to formation of 3,6-dihydropyrazine 2,5-dipropionic acid (DHPY). Deprotonation of the amino group allows cyclisation with the ketone group of a neighbouring molecule.¹⁴ Further oxidation leads to pyrazine-2,5-dipropionic acid (PY), considered to be the major degradation product in aerated solutions.⁹

Condensation reactions of NH_2 containing compounds with carbonyl compounds usually require an optimised amount of acid catalyst. The reaction mechanism is shown in Figure 1b

and reveals that nucleophilic attack by the lone pair of electrons is only possible when the nitrogen is unprotonated. Similarly, it is enhanced when the carbonyl is protonated. Clearly, concentrations of these species are affected oppositely by pH and a maximum reaction rate is achieved only when both are present in sufficient numbers to give a reasonable reaction rate. Consequently, a reduction in pH enhances stability of ALA in solution by precluding cyclisation as the amino group is extensively protonated. This happens in spite of protonation on the carbonyl group making it susceptible to attack. Thus, Elfsson et al.¹⁵ found that solutions of ALA, buffered to pH 2.35, were stable over a period of 37 days, even when stored at 50°C. Adjusting the pH to approximately 2.0 is not, however, appropriate to topical dosage form design as cutaneous irritancy is probable. Addition of ethylene diamine tetra acetic acid^{12,15} and antioxidants¹⁶ to ALA solutions has resulted in disappointing improvements in stability. Further, the ever expanding range of potential neoplasias that are being treated with PDT, such as oral cancer¹⁷ and gynaecological neoplasias,¹⁸ has necessitated new approaches for drug delivery to these more challenging sites.

The aim of this study is to evaluate a novel alternative to enhancing the stability of ALA that is more applicable to topical delivery systems. Inspection of the mechanism in Figure 1b suggests that formulation of ALA-containing, non-aqueous systems, where the acid catalyst is not

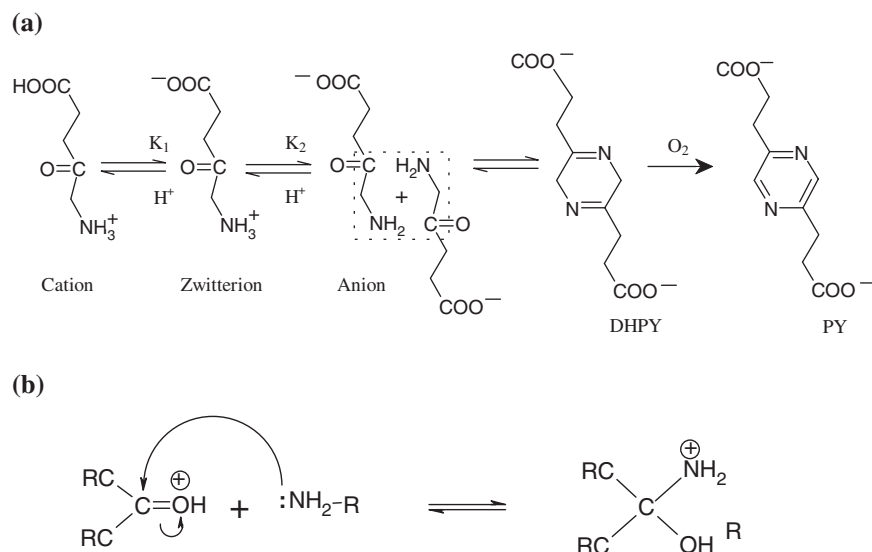


Figure 1. (a) pH-dependent equilibria occurring in aqueous solutions of 5-aminolevulinic acid (ALA). (b) Mechanism of nucleophilic attack by a primary amine on a carbonyl in the presence of an acid catalyst.

present, may enhance stability by suppressing nucleophilic attack at the carbonyl carbon, even though the lone pair of electrons on the nitrogen are available. As such, non-aqueous bioadhesive gels or patches that are known to adhere well to topical sites are investigated in this study. This work evaluates the kinetics of ALA loss, the formation of a potential derivative with a hydroxyl-containing excipient and its intact recovery using enzyme-catalysed hydrolysis. Furthermore, the concentration of the major ALA degradation product, PY, is evaluated in bioadhesive dosage forms that have been formulated to contain no or low levels of water.

MATERIALS AND METHODS

Materials

5-ALA, hydrochloride salt (ALA) was obtained from Crawford Pharmaceuticals, Milton Keynes, UK. Gantrez[®] AN-139, a copolymer of methylvinylether and maleic anhydride (PMVE/MA) was provided by ISP Co. Ltd, Guildford, UK. Tripropyleneglycol methyl ether (Dowanol[™] TPM), glycerol, poly(ethyleneglycol) 400 and butyryl cholinesterase (11.4 U/mg solid) were obtained from Sigma Aldrich, Dorset, UK. Plastisol[®] medical grade poly(vinyl chloride) emulsion, containing diethylphthalate as plasticiser, was provided by BASF Coatings Ltd., Clwyd, UK. Caffeine citrate was obtained from Halewood Chemicals, Middlesex, UK. Carbopol[®] ETD 2050, an "easy-to-disperse" cross-linked poly(acrylic acid) polymer, was provided by Noveon Europe B.V.B.A., Brussels, Belgium. All other chemicals were of analytical reagent grade. Buffer solutions were prepared according to details in the British Pharmacopoeia (BP 2001) and the Pharmacopoeia Helvetica (Ph. Helv.VI).

Preparation of ALA-Containing Formulations

Organogels, consisting of 5% w/w Carbopol[®] ETD 2050 dissolved in anhydrous glycerol or anhydrous poly(ethyleneglycol) 400 (PEG 400), were prepared by addition of the dry polymer to the vigorously stirred solvents. Resulting gels were refrigerated overnight to remove air bubbles and ALA dissolved in with the aid of gentle stirring. The final ALA loading in each of the two gels was adjusted to 20% w/w.

Bioadhesive patches containing ALA were prepared, as described previously¹⁹ from aqueous blends containing 20% w/w poly(methylvinylether/maleic anhydride) (PMVE/MA), 10% tripropylene glycol methyl ether (TPM) and 30% w/w ethanol. Appropriate amounts of ALA were added to the blends, such that the final patch, lined with a medical grade PVC backing layer, contained 38 mg ALA/cm².

Determination of Extraction Efficiencies of ALA

Samples of non-aqueous gels (50 mg) containing ALA were dissolved or dispersed in 12.5 mL of 0.1M borate buffer pH 5 (*Pharmacopoeia Helvetica*). Sections of bioadhesive patches (0.54 cm²) containing ALA were dissolved in 10 mL of buffer and aliquots (1.0 mL) further diluted to 10 mL with buffer. In both cases, the ALA concentration was determined immediately upon dosage form dissolution.

The ALA contents of the solutions produced were determined by HPLC, employing pre-column derivatisation with acetyl acetone and formaldehyde and fluorescence detection.²⁰ ALA (300 µg/mL) in the borate buffer was used as an external standard. Solutions containing ALA-acetyl acetone/formaldehyde reagent derivative, were injected (10 µL) onto a Spherisorb[®] column (Waters Spherisorb[®] HPLC column; 250 mm × 4.6 mm, C₁₈ ODS₂ with 5 µm packing) equipped with a Spherisorb[®] guard column (S5 guard, 10 mm × 4.6 mm, C₁₈ ODS₂ with 5 µm packing). The mobile phase was 49.5% methanol per 49.5% water per 1% glacial acetic acid v/v/v running at 1.5 mL/min. Detection was by fluorescence detection (Shimadzu RF-535 fluorescence detector) with excitation at 370 nm and emission at 460 nm. The chromatograms obtained were analysed using proprietary Shimadzu Class VP[™] software.

Extraction efficiencies of ALA from the various formulations were determined by comparison of experimentally determined ALA loadings to theoretical loadings. Results were expressed as means (±SD) of five determinations.

Determination of Extraction Efficiencies of PY

Samples of non-aqueous gels and bioadhesive patches, containing ALA in the loadings described above, were spiked with appropriate high and low levels of PY, synthesised by the method described by Bunke et al.¹⁰ PY was dissolved or dispersed in the gels using gentle stirring, while it was

incorporated into the aqueous blends used to produce bioadhesive patches prior to patch preparation. Since the amount of PY formed from ALA under the various formulation and storage conditions could not be predicted, both high and low levels of PY were used to assess its extraction efficiency from the dosage forms. The formulations, in the same quantities as described above, were dissolved in the same volumes of buffer as described above for determination of ALA extraction efficiencies.

The PY contents of the solutions produced were determined by HPLC, using UV detection. Solutions containing PY were injected (10 μ L) onto a Nova-Pak[®] column (Waters Nova-Pak[®], 150 mm $\times$ 3.9 mm, C₁₈ ODS₂ with 4 μ m packing), equipped with a Nova-Pak[®] guard column (Waters Nova-Pak[®] guard column; 20 mm $\times$ 3.9 mm, C₁₈ ODS₂ with 4 μ m packing). The mobile phase was 97% acetate buffer pH 2.8 BP/3% acetonitrile v/v running at 0.5 mL/min. Detection was by ultraviolet absorption (Shimadzu SPD-6A UV spectrophotometric detector) at 275 nm. Caffeine citrate (62.5 μ g/mL) was used as internal standard.

The extraction efficiencies of PY from the various formulations were determined by comparison of experimental to theoretical PY loadings. Results were expressed as means ($\pm$ SD) of five determinations.

Stability Study

Formulations containing ALA were stored at 5°C, room temperature, 37°C and 60°C. Patches stored at room temperature were divided into two groups. One group was packaged in heat-sealed, moisture impermeable, poly(ester) foils. The other group was left open to the atmosphere. Patches stored under the other conditions were packaged in foils. Gels were stored in stoppered glass vials. The ALA contents of gels and patches were determined by HPLC following dissolution or dispersion of the same quantities of formulation in the same volumes of buffer as described above. Determinations were performed on the day of preparation and at regular time intervals up to 6 months after preparation. Both ALA and PY contents were reported as means ($\pm$ SD) of five replicates.

The mean loss kinetics of ALA in each of the various formulations were modelled according to the first, second and zero-order reaction rate equations, in order to explicate the mechanism of ALA loss from these formulations.

Nuclear Magnetic Resonance (NMR) Studies

Determination of potential esterification reactions between ALA and the various components of the formulations were investigated using NMR studies. Solid ALA was dispersed in TPM, PEG 400 or glycerol (10% w/w). Similarly, ALA was dissolved in a solution of PMVE/MAC (20% w/w) in D₂O and used to model the possible interaction with the matrix of the patch. In each case, mixtures or solutions were heated to 60°C for 72 h and analysed (General Electric QC500 NMR spectrometer) using a 5 mm probe and operating at 126 MHz for carbon, with D₂O as solvent. NMR spectra were recorded at 25°C.

Esterase Catalysed Hydrolysis

Aliquots of poly(acrylic acid) gel formulation (50 mg), prepared with either glycerol or PEG 400 as the hydrogen bonding cross-linking agent, were removed from formulations, previously stressed at 60°C for 168 h. These were dissolved in PBS (pH 7.4, 12.5 mL) containing 20 mg (228 U) of butyrylcholinesterase and stored in an orbital incubator at 37°C. Likewise, bioadhesive films (0.5 cm²), stressed similarly, were dissolved in 20 mL PBS (pH 7.4) containing 32 mg (364.8 U) of butyrylcholinesterase. Samples (1 mL) were taken at defined time intervals (5, 10, 15, 30, 60, 120, 180, 240, 300 and 360 min) and ALA concentration determined using pre-column derivatisation using acetyl acetone and formaldehyde, as described previously. Analysis was by HPLC with fluorescence detection. Samples were also assayed for PY content, as described previously.

Statistical Analysis

Where appropriate, data were analysed using a one-way analysis of variance (ANOVA). *Post-hoc* comparisons were made using the Tukey/Kramer test. In all cases, $p < 0.05$ denoted significance. Least squares linear regression together with correlation analysis was used to model ALA loss according to first-, second- and zero-order kinetics arising in each formulation under the various storage conditions tested.

RESULTS

Extraction Efficiencies

Determination of extraction efficiencies of ALA and PY from the various formulations investi-

gated in this study were performed immediately after preparation and are shown in Table 1. Extraction efficiencies of ALA and PY from gel formulations were high, with low variability and standard deviations below 5% in all cases. However, greater variability was observed from the bioadhesive films, often approaching 7%.

ALA Stability and Loss Kinetics

Bioadhesive patches stored at 5°C and room temperature showed evidence of ALA crystallisation after approximately 1 month of storage. They remained colourless throughout the duration of the study. Films stored at 37°C and 60°C turned yellow, then orange-brown, finally becoming insoluble in water and swelling to many times their original weights. Similarly, PEG 400 gels stored at 5°C began to turn a pale yellow colour towards the end of the study. Those stored at room temperature and 37°C turned orange over time and those stored at 60°C became dark brown. Increasingly greater cohesion was observed within gels stored at elevated temperatures, making sampling difficult. Although glycerol gels stored at room temperature, 37°C and 60°C showed the characteristic orange palette of colour changes, those stored at 5°C remained clear and colourless throughout the study. As with PEG 400 gels, a temperature and time related increase in cohesion made gels tacky and difficult to manipulate.

Figure 2 shows the influence of time and storage conditions on the ALA loadings in thin bioadhesive

films and the gel formulations. An almost constant and slow rate of loss from packaged patches stored at 5°C was observed. After 196 days, the ALA loading had declined to approximately 86% (33.0 mg/cm²) of the original loading. Films stored at higher temperatures showed accelerated loss, however, so that those at 60°C had decreased to 17% (6.5 mg/cm²) of the original loading after 180 days of storage. The rate of loss of ALA from PEG 400 gels displayed a similar profile (Figure 2b). Drug loss from gels stored at 5°C for 204 days dropped to approximately 44.1% of the original loading, which was significantly higher ($p=0.0001$) from that of gels stored at room temperature (26.8%). Gels stored at higher temperatures showed extensive loss of ALA, where only 5.0% of the original loading was recovered from gels stored at 60°C after 128 days. This temperature related degradative pattern was reflected in the glycerol gels, as shown in Figure 2c, where rate of loss was found to exceed that in the PEG 400 systems. Indeed, only 35.4% of an original ALA loading was recovered from glycerol gels stored at room temperature.

Table 2 details the loss kinetics as fitted to zero-, first- and second-order rate processes. In contrast to patches stored at 5°C, which showed an almost zero-order coefficient, the ALA loss from films stored at higher temperatures approached second-order kinetics. ALA loss from PEG 400 gels stored at 5°C or RTP temperatures fitted the first-order model most closely in both cases. The loss of ALA from gels stored at 37°C was not adequately described by any of the models, while that from gels stored at 60°C fitted the second-order order

Table 1. Extraction Efficiencies of 5-Aminolevulinic Acid (ALA) and Pyrazine 2,5-Dipropionic Acid (PY) from Gel and Patch Formulations (Means $\pm$ SD, $n=5$)

Formulation	Theoretical Loading	Determined Loading	Extraction Efficiency (%)
ALA^a			
Bioadhesive patch	38 mg/cm ²	38.06 $\pm$ 2.98 mg/cm ²	100.16 $\pm$ 7.84
Glycerol gel	20% w/w	18.90 $\pm$ 0.66% w/w	94.48 $\pm$ 3.32
PEG 400 gel	20% w/w	19.70 $\pm$ 0.57% w/w	98.50 $\pm$ 2.83
PY^b			
Bioadhesive patch	0.93 mg/cm ²	0.93 $\pm$ 0.03% w/w	99.69 $\pm$ 3.10
Glycerol gel	5.79% w/w	5.70 $\pm$ 0.20% w/w	98.4 $\pm$ 3.41
PEG 400 gel	6.18% w/w	6.05 $\pm$ 0.19% w/w	97.85 $\pm$ 3.03
Bioadhesive patch	10.24 mg/cm ²	10.73 $\pm$ 0.73% w/w	104.81 $\pm$ 7.15
Glycerol gel	12.63% w/w	12.90 $\pm$ 0.27% w/w	102.15 $\pm$ 2.14
PEG 400 gel	12.46% w/w	12.85 $\pm$ 1.10% w/w	103.13 $\pm$ 3.82

^aFormulations containing ALA had no PY added.

^bFormulations containing PY also contained a defined loading of 5-aminolevulinic acid (ALA).

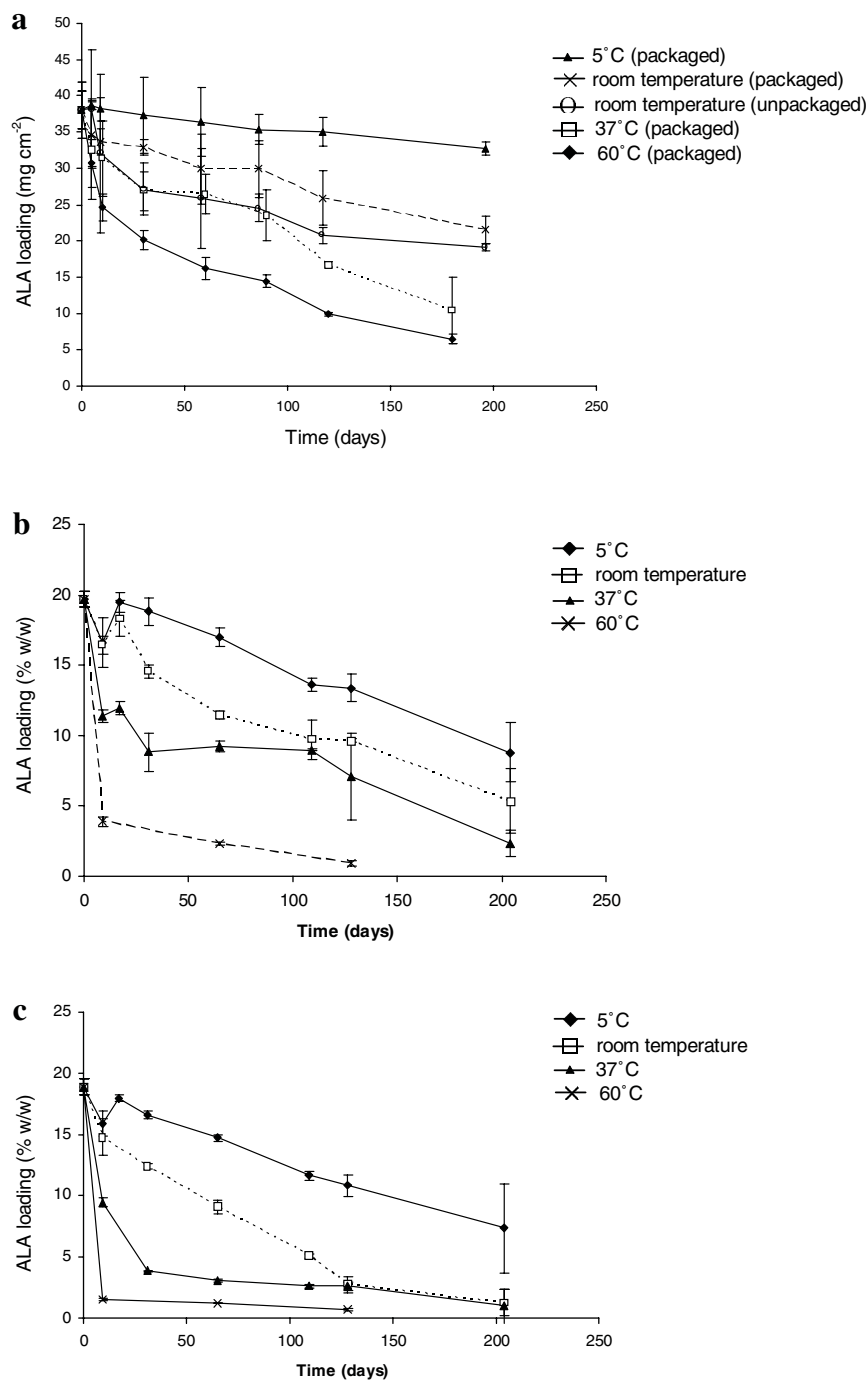


Figure 2. Influence of storage conditions and time on the loadings of 5-ALA in (a) bioadhesive patches, (b) poly(ethyleneglycol) 400 gels and (c) glycerol gels (means $\pm$ SD, $n = 5$).

model most closely. The loss of ALA from glycerol gels stored at 5°C or RTP fitted the first-order model most closely in both cases, whereas that from gels stored at 37°C and 60°C were not adequately described by any model.

PY Formation Studies

Figure 3a and b show the increasing masses of PY formed over time in ALA-loaded bioadhesive patches, stored under various temperatures and

Table 2. Coefficients of Determination (r^2) Values Obtained from Correlation Analysis, Following Linear Regression, of Plots Constructed to Model the Kinetics of ALA Loss from Bioadhesive Patches, PEG 400 and Glycerol Gel Systems According to First, Second and Zero-Order Kinetics

Storage Conditions	Storage Time (days)	Percent of Total ALA Remaining	First-Order Coefficient of Determination	Second-Order Coefficient of Determination	Zero-Order Coefficient of Determination
Bioadhesive patch					
5°C (packaged)	196	86.2	0.9832	0.9864	0.9909
RTP ^a (unpackaged)	196	50.3	0.8483	0.9073	0.7780
RTP ^a (packaged)	196	56.8	0.9596	0.9666	0.9357
37°C (packaged)	180	27.4	0.9316	0.9670	0.9327
60°C (packaged)	180	17.0	0.9427	0.9808	0.7761
PEG 400					
5°C	204	44.7	0.9165	0.8881	0.9148
RTP	204	27.2	0.9683	0.9354	0.9138
37°C	204	11.6	0.8252	0.7322	0.6936
60°C	128	4.6	0.7847	0.9417	0.4609
Glycerol					
5°C	204	40.2	0.9706	0.9459	0.9558
RTP	204	6.9	0.9817	0.8681	0.8971
37°C	204	5.3	0.8255	0.8805	0.519
60°C	128	3.5	0.5545	0.8667	0.3627

^aRoom temperature and pressure.

packaging conditions. No PY was detectable in films prior to storage. Figure 3a demonstrates that packaging status has a significant effect ($p = 0.0093$) on stability as shown by the elevated levels of PY detected in unpackaged films stored at room temperature. PY formation in patches stored at 5°C showed an initial rapid increase, which then proceeded no further. Storage at 37°C and 60°C (Figure 3b), showed almost linear rates of PY formation of an order of magnitude greater than that observed in Figure 3a. Patches stored at 60°C contained significantly ($p < 0.0001$) more PY than those stored at 37°C after 180 days.

Figure 4a shows the time-resolved mass of PY formed in PEG 400 gels containing ALA. Initially, the rate of PY formation was low, but after 109 days, a marked and almost linear increase was observed in all cases. Figure 4b shows a similar pattern of increasing masses of PY formed over time, although a clear pattern arising because of the temperature variations was difficult to discern.

Table 3 compares the actual amounts of PY formed with that expected theoretically as calculated from the bimolecular reaction of ALA to form 1 molecule of PY *via* a series of steps.¹⁶ The amount of PY actually formed in bioadhesive patches is much less than that which would be produced if all of the ALA lost was converted to the degradation

product (PY) alone. This was true in all cases, regardless of storage conditions. It is also clear that this finding is observed for ALA loss in both PEG 400 and glycerol gels. Given the chemical functionality of both ALA and the hydroxyl-containing excipients present in the patch and gel formulations, an esterification reaction is possible that prevents PY formation. NMR analysis of ALA in TPM did not reveal the presence of any such reaction (Figure 5a). However, there is evidence for possible esterification between PEG 400 and ALA (10% w/w) as presented in the ¹³C NMR spectrum shown in Figure 5b. Peaks associated with PEG at 60.72 ppm, 69.99 ppm and 72.13 ppm and also the peaks associated with ALA at 204.34, 175.03, 47.45, 34.63, 30.65 and 27.81 ppm, corresponding to three non-equivalent carbons, are observed. Furthermore, there are additional peaks in the spectrum which support the presence of an ALA-PEG 400 oligomer, formed *via* an esterification reaction. Similarly, the ¹³C spectrum for an ALA-glycerol mixture, after suitable reaction conditions, is shown in Figure 5c. Yet again, there is evidence to suggest that ester formation has occurred with the formation of glyceride derivatives.

Enzyme hydrolysis of gels and patches, previously stressed for 168 s at 60°C, demonstrated a time dependent regeneration of ALA consistent

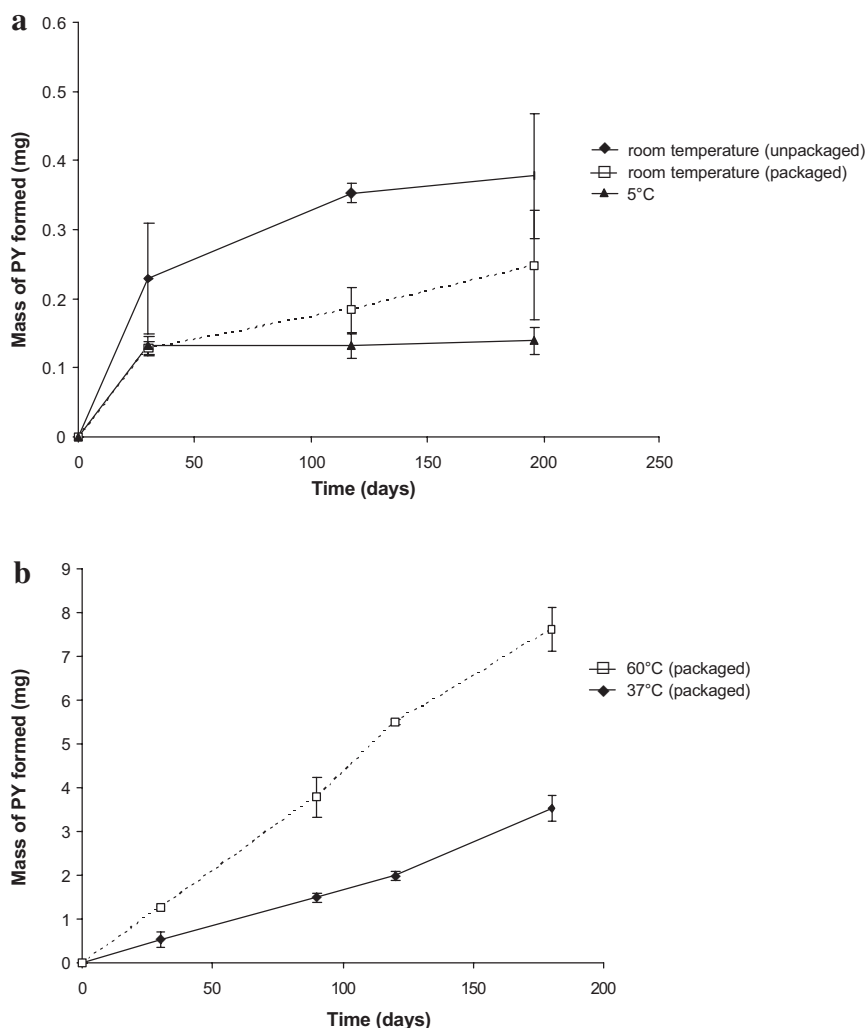


Figure 3. Influence of storage conditions and time on the formation of pyrazine 2,5-dipropionic acid (PY) in bioadhesive patches initially containing approximately 20.4 mg of 5-ALA stored at (a) low and room temperatures and (b) elevated temperatures (means $\pm$ SD, $n = 5$).

with prior esterification to a formulation component. An increase was demonstrated for all formulations, as shown in Figure 6a and b. Control experiments, using no enzyme, showed that ALA remained strongly esterified within the glycerol gel and patch systems, but was sufficiently labile in the PEG-based gel to produce a noticeable rise. Of particular note is the level of recovery achieved in the PEG system, where hydrolysis, be it enzyme-catalysed or not, is able to return the formulation to almost 80% of its theoretical loading. Enzyme treatment had no significant effect on PY content in any of the formulations (data not shown). Additional, unidentified, peaks were present in the chromatograms, eluting later

than PY and possibly relating to presently unidentified cyclic products.

DISCUSSION

The instability of ALA in drug delivery has remained a constant problem as the compound readily dimerises. Not only, are the potentials for both a photodynamic and clinical effect reduced, delivery vehicles buffered above pH 4 takes on an unpleasant yellow-orange appearance. The yellow colouration is attributed to DHPY, based on UV studies of deaerated solutions,⁹ while the red colour is attributed to PY.¹⁶ One strategy adopted

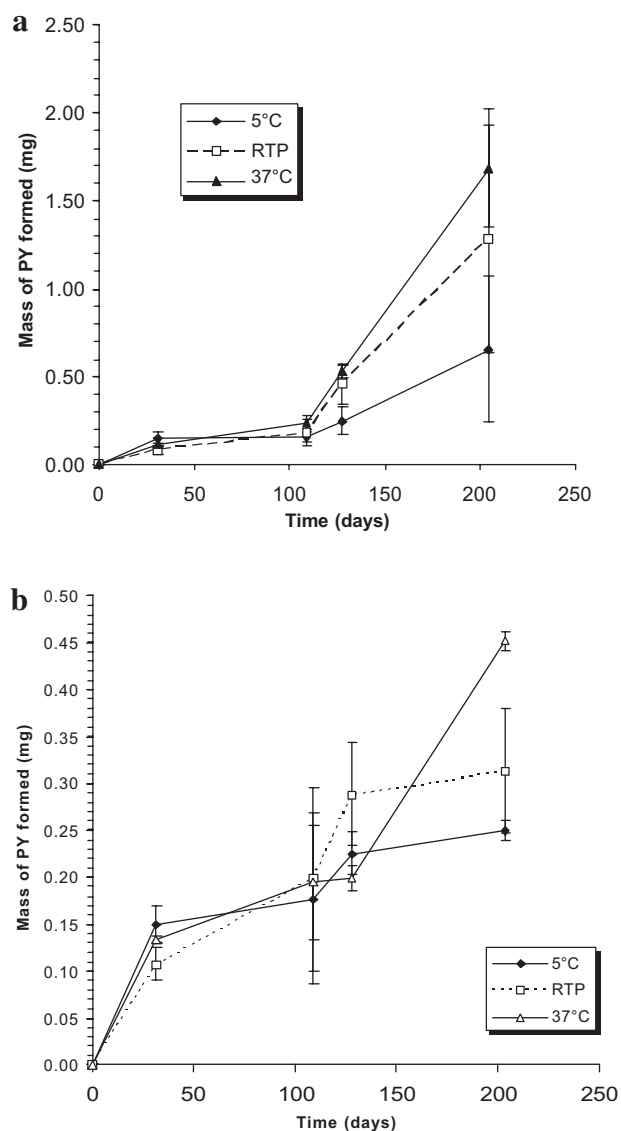


Figure 4. Influence of storage conditions and time on the formation of PY in (a) approximately 50 mg aliquots of poly(ethyleneglycol) 400 gels initially containing approximately 10 mg of 5-ALA and (b) in approximately 50 mg aliquots of glycerol gels initially containing approximately 10 mg of ALA (means $\pm$ SD, $n = 5$).

to overcome this loss is to maintain ALA in the solid state within an anhydrous matrix. An example of this approach is based on direct dispersal of crystalline ALA in a solvent-based pressure sensitive adhesive matrix.²¹ Although ideal for topical application to intact skin, it is doubtful that this system can maintain adhesion in moist environments and would call into question its suitability for mucous areas. Bioadhesive patches and gels, on the other hand, have been shown to adhere well to these types of region, such

as the cervix²² and can overcome specific limitations found with pressure sensitive devices. One such example, namely suitably plasticised poly(methylvinylether maleic anhydride), has been formulated into an anhydrous, yet water-soluble, patch system.¹⁹ Similarly, poly(acrylic acid)-based gel systems have demonstrated bioadhesive potential and can, likewise, be formulated to contain minimal amounts of water.¹⁹ Given their anhydrous nature, these two systems have the potential to enhance ALA stability without the need to reduce pH and yet adhere to mucous membranes and exudative surfaces. For this reason, poly(methylvinylether maleic anhydride)-based patches and non-aqueous hydrogen-bonded poly(acrylic acid)-based gel systems were investigated in this study.

Extraction of ALA and PY from bioadhesive patches was shown to be more variable than those from the gel systems, caused possibly by imperfections on the glass surfaces within the moulds influencing film thickness. Packaged bioadhesive patches, stored at 5°C, achieved ALA contents of 86% of the original loading, almost approaching acceptable levels after 6 months (206 days) storage. In contrast, patches stored under the other conditions lost unacceptable amounts of ALA at this time point and could not be deemed fit for use.^{23,24} To place this ALA loss in context, it must be remembered that the patch concentration is considerably higher than most delivery systems used in PDT, typically about 38% w/w and that the rate of degradation is enhanced by elevated concentration, given the second-order rate kinetics. Considering de Blois et al.¹¹ showed that ALA solutions (pH 5.0), with initial concentrations of 2, 5 and 10% w/w dropped below the 90% limit after 150, 94 and 29 days, respectively, this must suggest that the patch is demonstrating some enhancement of stability at 5°C.

An important finding from this study showed that the quantity of PY formed in bioadhesive films was much less than that predicted based on the amount of ALA lost. Due to the complex nature of the drug-loaded formulation, which contains hydrolysed poly(methylvinylether/maleic anhydride) (PMVE/MAC), tripropyleneglycol methyl ether (TPM) and trace water, it is conceivable that ALA loss is not occurring through conversion to PY, but by a reaction with a film component. Previous work has shown that TPM reacts with PMVE/MAC to produce esters.²⁵ Conceivably, the hydroxyl function on TPM could react with the carboxylic acid group on ALA to yield an ester,

Table 3. Comparison of the Theoretical and Actual Amounts of Pyrazine 2,5-Dipropionic Acid (PY) Formed in Patch and Gel Systems Containing 5-Aminolevulinic Acid (ALA) on Storage (Means $\pm$ SD, $n = 5$)

Storage Conditions	Storage Time (days)	Percent of Total ALA Remaining	Theoretical Amount of PY Formed (mg)	Actual Amount of PY Formed (mg)	Percent of Theoretical
Bioadhesive patch					
5°C (packaged)	196	86.2	1.89 $\pm$ 0.33	0.14 $\pm$ 0.02	7.41
RTP ^a (unpackaged)	196	50.3	6.80 $\pm$ 0.16	0.38 $\pm$ 0.09	5.59
RTP ^a (packaged)	196	56.8	5.91 $\pm$ 0.68	0.25 $\pm$ 0.08	4.23
37°C (packaged)	180	27.4	9.91 $\pm$ 1.64	3.53 $\pm$ 0.31	35.62
60°C (packaged)	180	17.0	11.32 $\pm$ 0.24	7.63 $\pm$ 0.50	67.40
PEG 400					
5°C	204	44.7	2.49 $\pm$ 0.82	0.65 $\pm$ 0.41	26.10
RTP	204	27.2	3.42 $\pm$ 0.61	1.28 $\pm$ 0.64	37.43
37°C	204	11.6	4.14 $\pm$ 1.09	1.69 $\pm$ 0.34	40.82
60°C	128	4.6	2.25 $\pm$ 0.50	1.41 $\pm$ 0.15	62.67
Glycerol					
5°C	204	40.2	3.17 $\pm$ 1.00	0.07 $\pm$ 0.61	2.21
RTP	204	6.9	8.96 $\pm$ 1.44	0.31 $\pm$ 0.07	3.46
37°C	204	5.3	5.32 $\pm$ 1.22	0.45 $\pm$ 0.17	8.46
60°C	128	3.5	3.48 $\pm$ 0.60	0.27 $\pm$ 0.01	7.76

^aRoom temperature and pressure.

hindering its chemical derivatisation with acetyl acetone and formaldehyde on which its quantification depends. NMR analysis did not support this, as shown by the ¹³C spectrum for an ALA/TPM mixture in Figure 5a. The signal from ALA is weak, due mostly to poor solubility in TPM, making detection of ester formation difficult. ALA can also form amides with carboxylic acids under certain conditions.²⁶ However, amide formation between ALA and PMVE/MAC was not observed in NMR analysis (data not shown). Further evidence of a non-dimerisation dependent degradation is supported in the modelling of loss kinetics. It is clear from Table 2 that second-order dependent loss, as would be expected, does occur in patches that show rapid ALA disappearance, whereas that in the 5°C patch appears to be zero-order. Therefore, a degradative pathway that, as yet, remains unresolved is occurring in the patch matrix.

Addition of ALA to glycerol, propylene glycol or dimethyl sulphoxide has been reported to yield yellow solutions, indicative of degradation.¹⁶ This colour change was observed in all gel formulations described in this work, with the exception of glycerol at 5°C. In this case, the colour change was noticeably reduced, almost imperceptible. PEG 400 gels did not possess pharmaceutically acceptable ALA contents after 6 months, regardless of the storage conditions. Yet again, PY contents determined were less than those pre-

dicted on the basis of ALA loss. Ester formation between ALA and poly(ethyleneglycols) has been reported previously²⁶ and may form the basis of a potential explanation for this observation. As PEG 400 is in excess, the acidic functional group on the ALA will react, whereas only one end of the PEG 400 will react. Consequently, there is a high probability of forming only one oligomeric species, as illustrated in Figure 7a. The oligomer formed results in an α -shift effect, as shown, on the terminal methylene group of PEG 400 and a β -shift effect on the second methylene carbon. Consequently, the terminal methylene carbon experiencing an α -shift effect is shifted downfield by approximately +3.75, resulting in a new peak at 64.53 ppm, whereas the second methylene carbon experiencing a β -shift effect, is shifted upfield by approximately -3.32 ppm, generating a new peak at 68.82 ppm.²⁷

Storage at 5°C was unable to prevent the ALA content dropping below pharmaceutically acceptable levels in glycerol gels after 6 months. Storage at elevated temperatures produced similar findings. It is clear from Table 3 that, yet again, large discrepancies exist between the theoretical and actual amounts of PY formed. This could be confirmed visually as glycerol gels appeared to have a slight yellow hue when compared to deeply discoloured PEG 400 gels, indicating that considerably less PY was being formed. Possible esterification with glycerol may explain the ALA

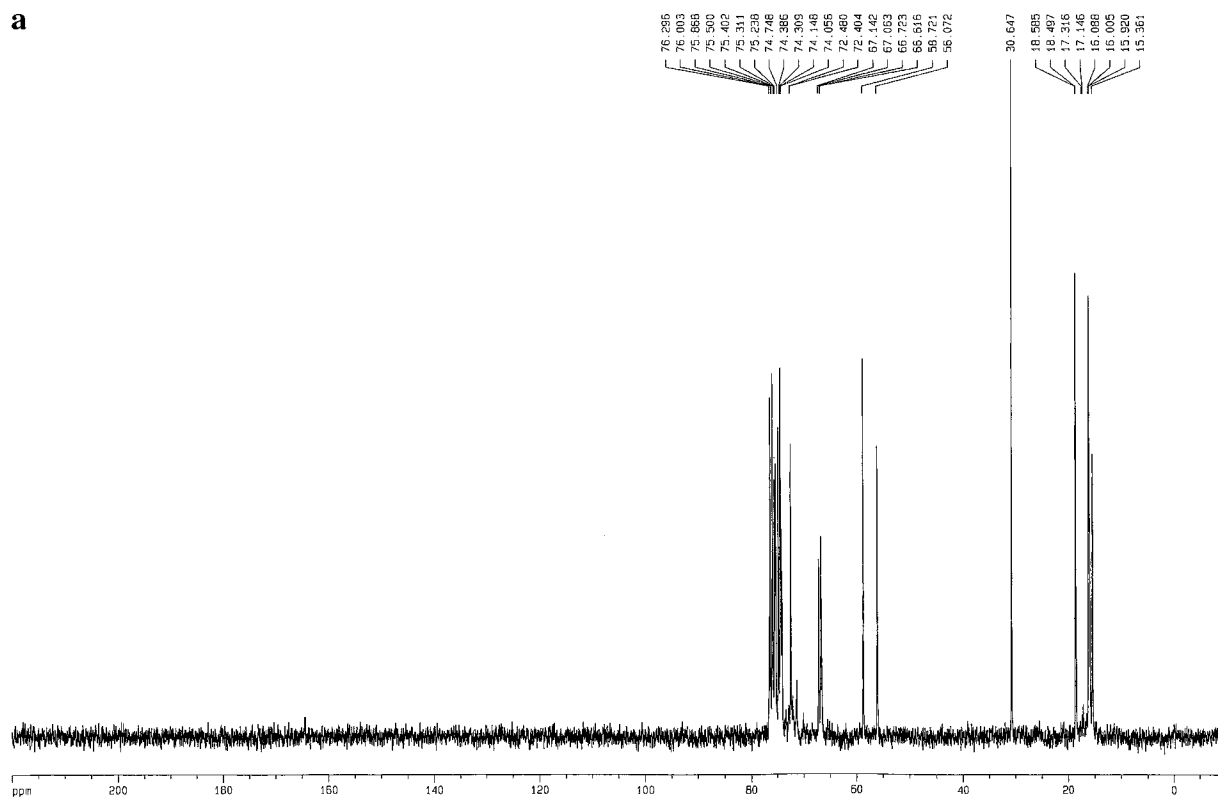
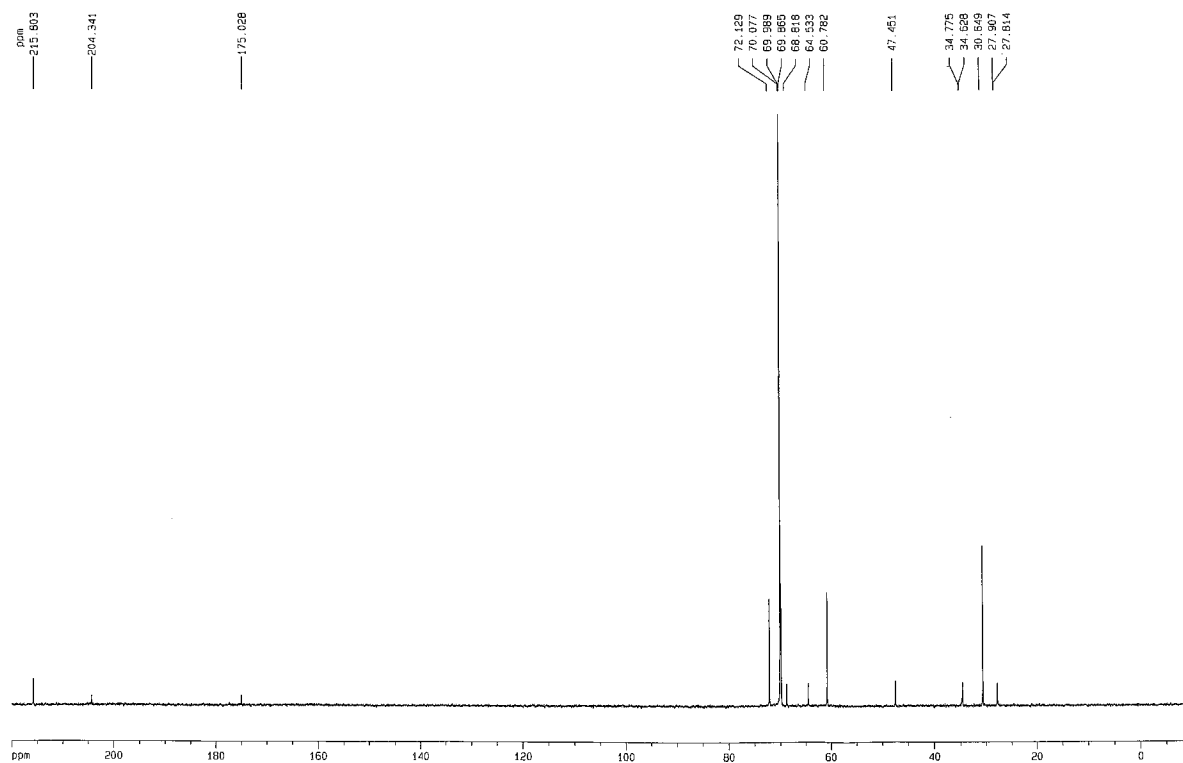
a**b**

Figure 5. ^{13}C NMR spectra for mixtures containing 5-ALA (10% w/w) in (a) tripropyleneglycol methyl ether, (b) poly(ethyleneglycol) 400 and (c) glycerol.

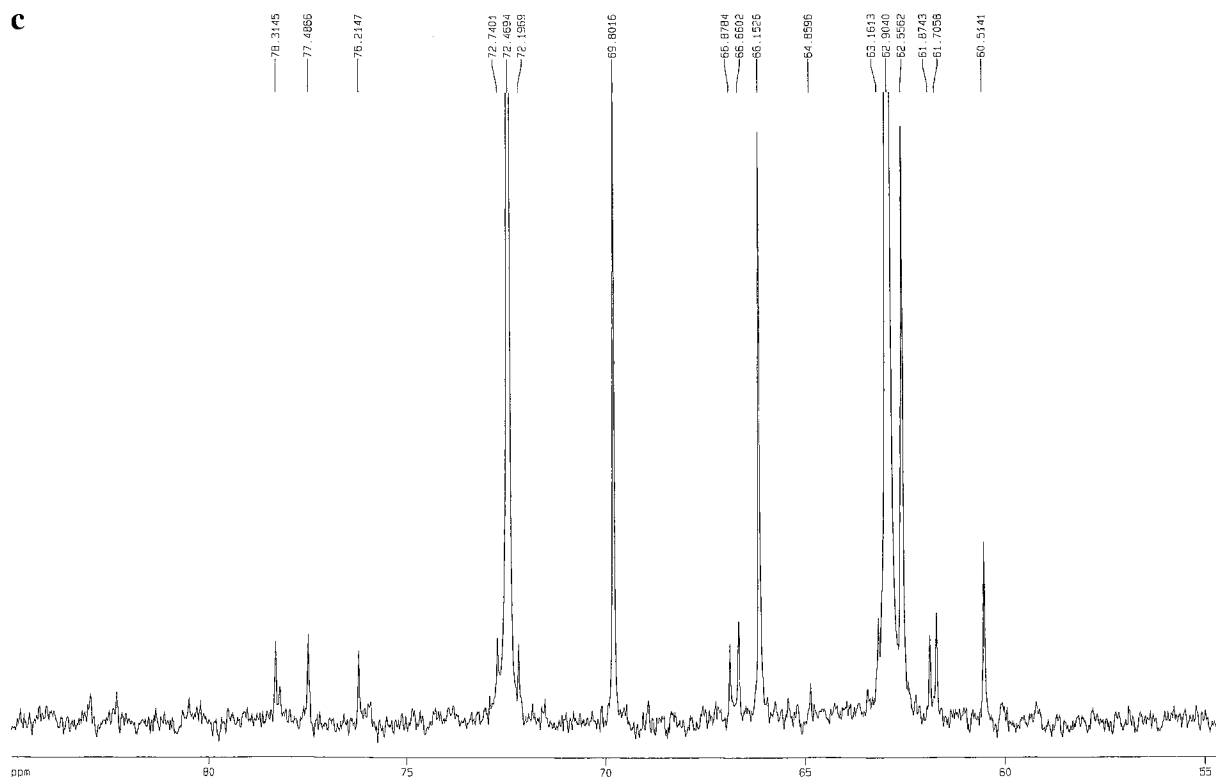


Figure 5. (Continued)

loss^{28,29} with different glyceride residues possible. Given that glycerol is known to be in excess, two different glyceride residues are possible, as shown in Figure 7b. NMR spectroscopy confirmed esterification, as shown in Figure 5c. The reaction of glycerol with ALA at a primary alcohol site generates three non-equivalent carbons on the glycerol residue. One primary alcohol experiences an α -shift effect and the other, at the opposite end of the molecule, experiences a γ -shift effect. Furthermore, the secondary alcohol experiences a β -shift effect. Consequently, the carbon experiencing an α -shift effect is shifted downfield by approximately +3.25 to 66.15 ppm, whereas the other primary carbon at the opposite end of the glycerol molecule experiences a γ -shift effect upfield by approximately -0.34 to 62.56 ppm. The secondary carbon experiencing a β -shift effect is shifted upfield by approximately -2.67 ppm to a position of 69.80 ppm. All of the peaks discussed are apparent on the ^{13}C NMR spectrum of glycerol per ALA. Similarly, reaction at the secondary alcohol functional group generates two peaks from the glycerol molecule, one corresponding to the secondary alcohol experiencing an α -shift effect and the other to the two equivalent primary carbons experiencing β -shift effects. Therefore,

the secondary carbon of glycerol is shifted to a higher frequency carbon upon experiencing a β -shift effect and, thus, is positioned at approximately 76.21 ppm. This represents an increase of approximately 3.74 ppm. The two primary carbons are shifted upfield by approximately -2.39 ppm and are evident at 60.51 ppm. It is interesting to observe that the shift parameters associated with linear and branched substitution differ, in particular an α_2 -shift effect is larger than an α_1 -shift effect, which is complementary to the effects described by Wehrli and Wirthlin.²⁷ The reaction between PY and glycerol could not be confirmed in this study.

ALA ester formation within the three dosage forms is the probable mechanism that explains the discrepancies between the expected PY concentrations and those actually determined. Evidence from NMR studies and enzymatic hydrolysis of the formulations demonstrated that ALA was sequestered into the formulation gel matrix by esterification with either glycerol or PEG. In the glycerol formulations in particular, esterase activity was able to release ALA and return its concentration to approximately 90% of those determined immediately after formulation. Similarly, hydrolysis of extracted patch samples returned the loading per unit area to those typically found immediately

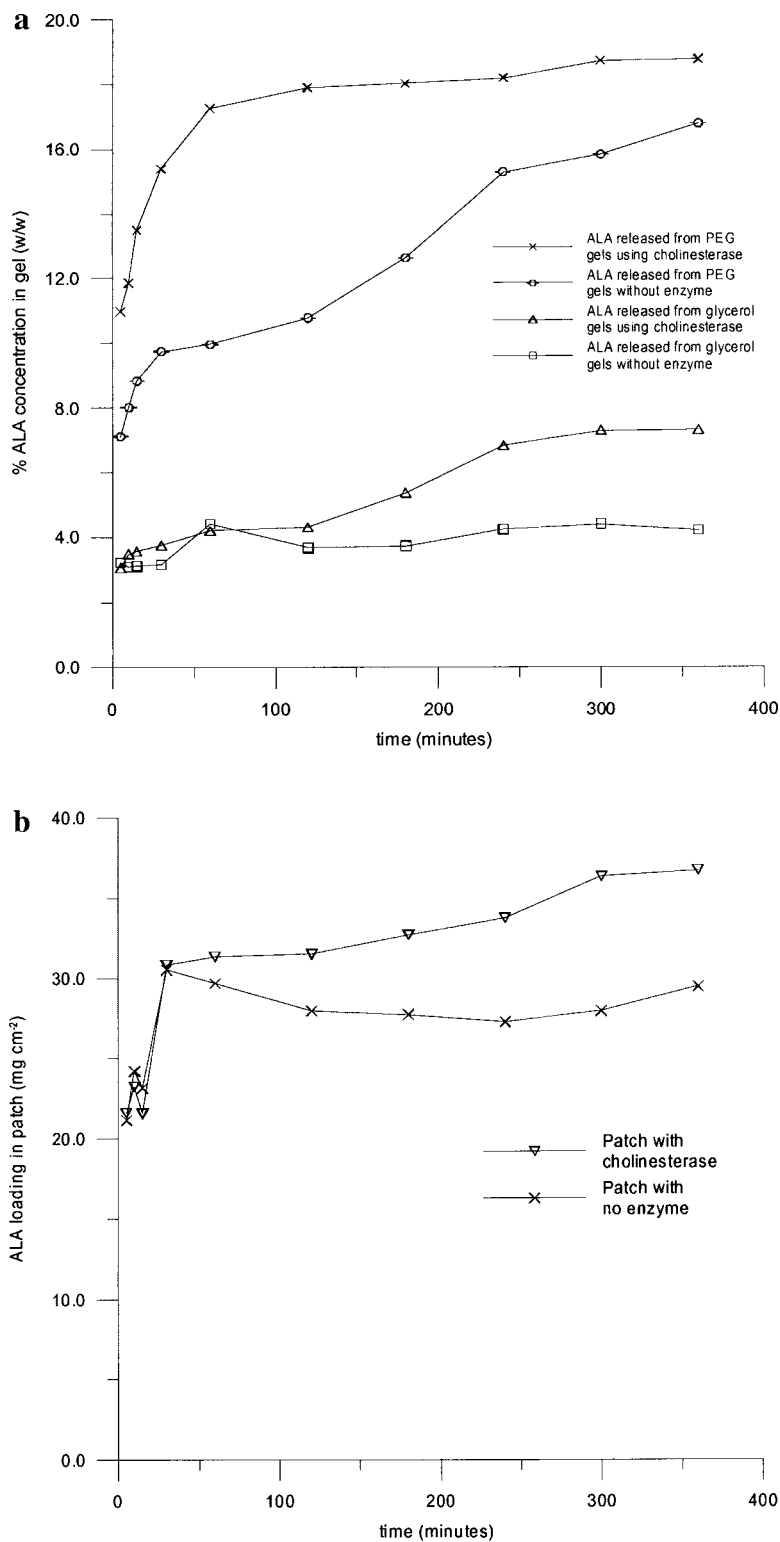


Figure 6. Regeneration of ALA using enzyme-catalysed hydrolysis demonstrated in (a) glycerol and PEG gels and (b) the bioadhesive patch formulation.

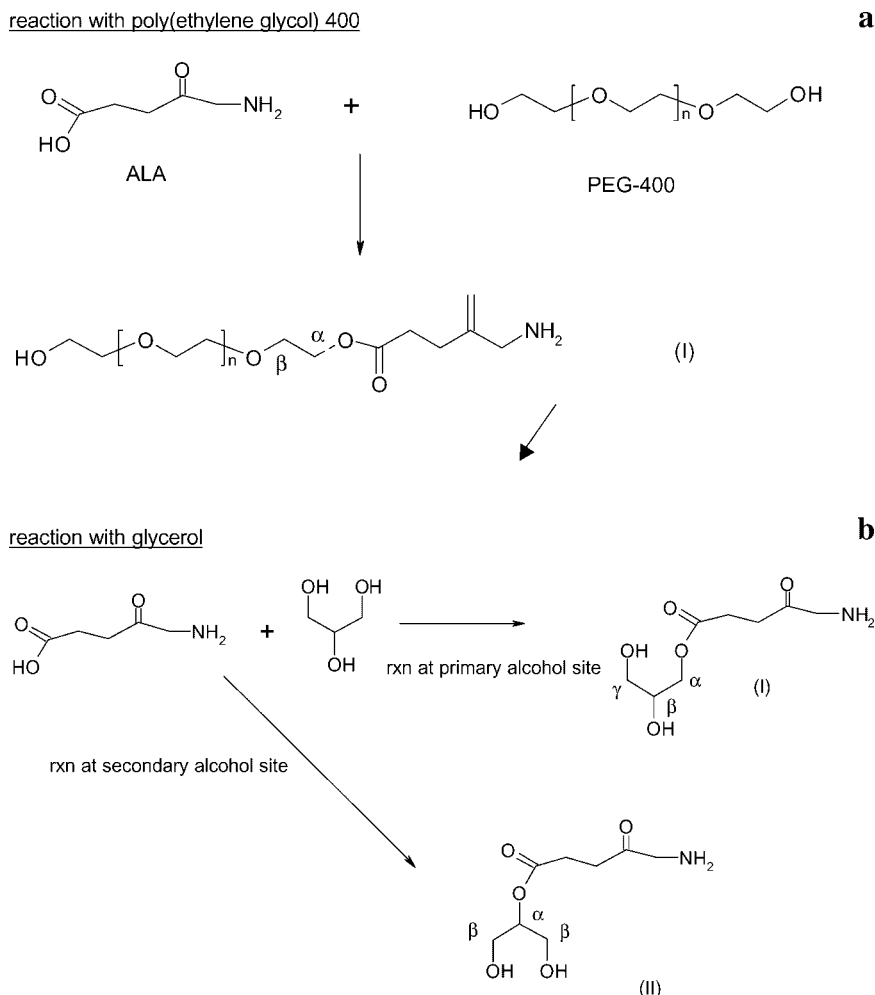


Figure 7. (a) The possible esterification reaction between 5-ALA and poly(ethylene-glycol) 400 (PEG 400) to yield one ester derivative (I). (b) Possible reaction of glycerol and 5-aminolevulinic acid to yield two different glycerides, (I) and (II).

after patch manufacture. The results in Figure 6 also suggest that non-enzyme based hydrolysis is possible, arising from dissolution in the aqueous solvent. This effect was particularly noticeable in the PEG systems, but was not discernable in either glycerol or patch formulations.

In conclusion, ALA contents in all formulations studied had fallen below pharmaceutically acceptable levels after 6 months storage, regardless of storage conditions. Bioadhesive patches stored at 5°C had the highest ALA loadings after 6 months, possessing 86% of their original loading. Therefore, the original hypothesis whereby enhanced stability could be achieved using formulations based on non-aqueous vehicles was not substantiated. More importantly, however, it was demonstrated that ALA loss was not matched by an

expected increase in PY, indicating some other degradative process occurring that prevented dimerisation. This work has shown that ALA esterification to a hydroxyl-containing formulation component, such as glycerol and PEG, prevented condensation dimerisation. The novel feature of such a system is that ALA can be regenerated at a later time point using either enzyme catalysed or aqueous hydrolysis. Interestingly, the non-specific esterase activity present in skin could fulfil this role, giving triggered ALA release upon topical application. This is, of course, dependent upon achieving sufficient cutaneous penetration of the ALA ester. The increased molecular mass may act in conflict to reduced hydrophilicity, thereby making it difficult to predict whether skin penetration will occur in

clinically useful amounts. This novel approach is currently under investigation and the subject of penetration studies using excised skin samples and tissue homogenates.

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REFERENCES

- Gannon MJ, Brown SB. 1999. Photodynamic therapy and its applications in gynaecology. *Br J Obstet Gynaecol* 106:1246–1254.
- Peng Q, Berg K, Moan J, Kongshaug M, Nesland JM. 1997. 5-aminolevulinic acid-based photodynamic therapy: principles and experimental research. *Photochem Photobiol* 65:235–251.
- De Rosa FS, Bentley MVLB. 2000. Photodynamic therapy of skin cancers: sensitizers, clinical studies and future directives. *Pharm Res* 17:1447–1455.
- Fritsch C, Goerz G, Ruzicka T. 1998. Photodynamic therapy in dermatology. *Arch Dermatol* 134:207–214.
- Kennedy JC, Pottier RH, Pross DC. 1990. Photodynamic therapy with endogenous protoporphyrin. 9. Basic principles and present clinical-experience. *J Photochem Photobiol B-Biol* 6:143–148.
- Kennedy JC, Marcus SL, Pottier RH. 1996. Photodynamic therapy (PDT) and photodiagnosis (PD) using endogenous photosensitization induced by 5-aminolevulinic acid (ALA): mechanisms and clinical results. *J Clin Laser Med Surg* 14:289–304.
- Kennedy JC, Pottier RH. 1992. Endogenous protoporphyrin-Ix, a clinically useful photosensitizer for photodynamic therapy. *J Photochem Photobiol B-Biol* 14:275–292.
- Kalka K, Merk H, Mukhtar H. 2000. Photodynamic therapy in dermatology. *J Am Acad Dermatol* 42:389–413.
- Novo M, Huttmann G, Diddens H. 1996. Chemical instability of 5-aminolevulinic acid used in the fluorescence diagnosis of bladder tumours. *J Photochem Photobiol B-Biol* 34:143–148.
- Bunke A, Schmid H, Burmeister G, Merkle HP, Gander B. 2000. Validation of a capillary electrophoresis method for determination of 5-aminolevulinic acid and degradation products. *J Chromatogr A* 883:285–290.
- de Blois AW, Grouls RJE, Ackerman EW, Wijdeven WJA. 2002. Development of a stable solution of 5-aminolevulinic acid for intracutaneous injection in photodynamic therapy. *Lasers Med Sci* 17:208–215.
- Gadmar OB, Moan J, Scheie E, Ma LW, Peng Q. 2002. The stability of 5-aminolevulinic acid in solution. *J Photochem Photobiol B-Biol* 67:187–193.
- Novo Rodriguez M, Huttmann G, Diddens H. 1995. Chemical instability of 5-aminolevulinic acid (ALA) in aqueous solution. *Society of Photo-Optical Instrumentation Engineers* 2371:204–209.
- Butler AR, George S. 1992. The nonenzymatic cyclic dimerization of 5-aminolevulinic acid. *Tetrahedron* 48:7879–7886.
- Elfsson B, Wallin I, Eksborg S, Rudaeus K, Ros AM, Ehrsson H. 1999. Stability of 5-aminolevulinic acid in aqueous solution. *Eur J Pharm Sci* 7:87–91.
- Bunke A, Zerbe O, Schmid H, Burmeister G, Merkle HP, Gander B. 2000. Degradation mechanism and stability of 5-aminolevulinic acid. *J Pharm Sci* 89:1335–1341.
- Hopper C. 1996. The role of photodynamic therapy in the management of oral cancer and precancer. *Oral Oncol-Eur J Cancer Pt B* 32B:71–72.
- Kurwa HA, Barlow RJ, Neill S. 2000. Single-episode photodynamic therapy and vulval intraepithelial neoplasia type III resistant to conventional therapy. *Br J Dermatol* 143:1040–1042.
- McCarron PA, Donnelly RF, Woolfson AD, Zawislak A. 2003. Photodynamic therapy of vulval intraepithelial neoplasia using bioadhesive patch-based delivery of aminolevulinic acid. *Drug Delivery Systems and Sciences* 3:59–64.
- Oishi H, Nomiya H, Nomiya K, Tomokuni K. 1996. Fluorometric HPLC determination of delta-aminolevulinic acid (ALA) in the plasma and urine of lead workers: biological indicators of lead exposure. *J Anal Toxicol* 20:106–110.
- Bretschko E, Szeimies RM, Landthaler M, Lee G. 1996. Topical 5-aminolevulinic acid for photodynamic therapy of basal cell carcinoma. Evaluation of stratum corneum permeability in vitro. *J Control Release* 42:203–208.
- Woolfson AD, Mccafferty DF, Mccarron PA, Price JH. 1995. A bioadhesive patch cervical drug-delivery system for the administration of 5-fluorouracil to cervical tissue. *J Control Release* 35:49–58.
- International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use. 2003. ICH-topic Q6A: test procedures and acceptance criteria for new drug products: chemical substances. Geneva, Switzerland.
- International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human use. 2003. ICH-topic Q6B: test procedures and acceptance criteria for biotechnological/biological products. Geneva, Switzerland.
- McCarron PA, Woolfson AD, Donnelly RF, Andrews GP, Zawislak A, Price JH. 2004. Influence of plasticiser type and storage conditions on the properties of poly(methyl vinyl ether-co-maleic anhydride) bioadhesive films. *J Appl Polym Sci* 91:1576–1589.

26. Berger Y, Greppi A, Siri O, Neier R, Juillerat-Jeanneret L. 2000. Ethylene glycol and amino acid derivatives of 5-aminolevulinic acid as new pPhotosensitising precursors of protoporphyrin IX in cells. *J Med Chem* 43:4738–4746.
27. Wehrli FW, Wirthlin T. 1976. Interpretation of ^{13}C NMR Spectra. Heyden, London.
28. Uraki Y, Hashida K, Watanabe N, Sano Y, Sasaya T, Fujimoto H. 1994. Novel wood processing by maleic acid-glycerol mixture system: improvement of water resistance and mechanical property of cellulose by the processing. *J Wood Chem Technol* 14:429–449.
29. Andrews GP, Fawcett AH, Hania MIM. 2002. Branching polyester oligomers and fractals. In: Cheng HN, English AD, editors. *NMR spectroscopy of polymers in solution and in the solid state*. Washington DC: American Chemical Society.