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Spectroscopic Determination of Skin Viability. A Predictor of Postmortem Interval

REFERENCES: Doukas AG, Bamberg M, Gillies R, Evans R, Kollias N. Spectroscopic determination of skin viability. A predictor of postmortem interval. J Forensic Sci 2000;45(1):36–41.

ABSTRACT: We have demonstrated that skin viability decreases at a measurable rate following death in an animal model. The decreased skin viability was measured by fluorescein diacetate and ethidium bromide using fluorescence emission spectroscopy. There is significant decrease of the fluorescence intensity of the fluorescein diacetate assay between the 1–4 h, the 6–24 h, and the >40 h time points postmortem. For times between 6–24 h and >40 h postmortem the ethidium bromide assay showed consistent and significant increases in signal. The fluorescence measurements in this study showed that under the experimental conditions the time of death could be determined for <4, 6–24, and >40 h potmotrem. The application of these assays in the field will require further study of the environmental factors.

KEYWORDS: forensic science, postmortem interval, time of death, skin viability, fluorescence spectroscopy, fluorescein diacetate, ethidium bromide

The determination of time of death (postmortem interval) has been the subject of many investigations in forensic sciences. A number of different methods, biochemical (1–4), physical (5–8), and histological (9), have been applied to the estimation of time of death with mixed results. To this date, however, there is no satisfactory and generally acceptable method for determining the time of death.

The use of spectroscopy, such as fluorescence, Raman, infrared, or diffused reflectance for the diagnosis of the diseased tissue (10–13), has advanced dramatically in the last few years. This array of methods for characterizing and measuring the physiological state of tissue could be applied to the problem of postmortem interval. For example, there are a number of assays based on spectroscopy that can be used to determine the viability of tissue (14,15). Studies of human cadaver skin, ex vivo, have shown that keratinocytes remain viable for many hours after death (16). Furthermore, skin viability decreases dramatically between 18 and 48 h postmortem. The determination of skin viability over time provides a possible method for the estimation of the postmortem interval.

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In this report, we present a methodology based on the viability of skin of sacrificed pigs to determine the postmortem interval. The decreased skin viability was measured by the fluorescein diacetate and the ethidium bromide assays as well as the rate of conversion of δ-aminolevulinic acid into protoporphyrin IX using fluorescence emission spectroscopy. The sensitivity and non-invasive nature of optical technologies make them ideal for use in the determination of postmortem interval. Furthermore, the compactness of the equipment make them easily transportable to the crime scene.

Materials and Methods

Viability Assays

Fluorescein diacetate (FDA) diffuses into cells where the ester bond is cleaved by esterase enzymes. The conversion of FDA to fluorescein requires cells to be viable. Ethidium bromide (EB), on the other hand, assays the integrity of the cell plasma membrane. EB normally cannot cross the viable plasma membrane. However, when cells die the integrity of the plasma membrane is compromised. EB can enter the cell and intercalate with the DNA in the nucleus. The fluorescence quantum yield of free EB in solution is low while the quantum yield of the intercalated EB increases by as much as three orders of magnitude. Both fluorescein and EB have unique spectroscopic signatures which can be measured by fluorescence emission spectroscopy. The combination of FDA and EB assays provides a quantitative way to determine the ratio of viable and dead cells and is used routinely as a viability assay in vitro.

The conversion of δ-aminolevulinic acid (ALA) into protoporphyrin IX (PpIX) by keratinocytes requires the cells to be viable. ALA is a precursor in the biosynthesis of Heme and the cytochromes. ALA is converted into PpIX in a multi-step biochemical process whose intermediate products include the formation of porphobilinogen, uroporphyrinogen, and coproporphyrinogen (17). The synthesis of ALA is the rate limiting step in the synthesis of Heme. When exogenous ALA is present, however, excess of endogenous PpIX is produced because the rate of ALA conversion to PpIX is faster than the conversion of PpIX to Heme. The formation of PpIX has been shown to correlate in vitro with the incorporation of tritiated thymidine (unpublished data), an established assay of cell proliferation. ALA can be introduced into the epidermis by injection, iontophoresis (18), or occlusion. PpIX as well as many of the other intermediates (e.g., uroporphyrinogen, coproporphyrinogen) have unique fluorescence signatures which can identify and quantify their presence in tissue.

Animal Model and Protocol

The measurements were performed on castrated male York swine 100–150 lb from Tufts School of Veterinary Medicine immediately after sacrifice with 100 mg/kg sodium pentobarbital. A total of five animals were measured. This animal model was chosen because of the similarities of the swine skin with the human skin (19,20). The measurements were performed either on the abdomen or the sides of the animal with the hair gently clipped from skin. Teflon rings with a 1.5 cm internal diameter were used to hold a solution of FDA or EB in DMSO. Two Teflon rings were attached on the skin with vacuum grease (Dow Corning, Midland, MI) on adjacent sites. The rings were filled with 1 mL of solution of FDA (1 mg/mL) or EB (0.26 mg/mL). Both solutions were applied for 3 min, then the excess solution was removed and the sites were blotted for 10 s. The carcass was kept in the dark for 2 h to allow the FDA and EB to diffuse into the tissue and the fluorescence intensity was measured. The application of FDA and EB was repeated every 3 h. Two to three different sites on the surface of the skin were measured for each time point and each experiment.

Experiments were also performed ex vivo, on skin removed from the back of the animals immediately after sacrifice (eight animals). Full thickness skin pieces measuring approximately 10 × 10 cm were stored at room temperature until ready to use. ALA 5% w/v in PBS (phosphate buffer saline) was either injected 0.2 mL intradermally or alternatively introduced by iontophoresis (18) at 0.1 mA for 5 min. Subsequently, the skin was placed in a humidified incubator at 37°C. The skin was removed from the incubator temporarily every 3 h to record the fluorescence emission spectrum of PpIX. Skin that was kept at room temperature instead of 37°C produced low PpIX fluorescence signal. Intradermal delivery of ALA produced more consistent results than iontophoretic delivery, possibly because of changes in the conductivity of the skin over time. The results presented here were obtained with intradermal delivery of ALA.

The fluorescence emission spectrum was measured using a fiber based fluoremeter, FluoroMax, (ISA, Edison, NJ). The excitation wavelength was 468 nm for both FDA and EB, and the emission spectra were recorded from 480 to 720 nm. The peak fluorescence maxima of converted fluorescein and EB are at 518 and 618 nm, respectively. Both bands are easily detected over the background fluorescence emission of the skin. The excitation wavelength for PpIX was 405 nm while the emission spectrum was recorded from 500 to 750 nm with the peak fluorescence maximum at 634 nm. The fluorescence emission spectra of control sites treated with vacuum grease or DMSO solvent were measured under otherwise identical conditions. The fluorescence spectra were indistinguishable from the baseline spectrum of the skin (data not shown).

Results

The emission spectrum of fluorescein at 4 and 24 h postmortem is shown in Fig. 1. The background fluorescence before the application of the FDA solution is also known for comparison. Figure 2 shows the emission spectrum of EB at 18 and 24 h postmortem. The background fluorescence before the application of the EB solution is also shown for comparison. Both FDA and EB emission

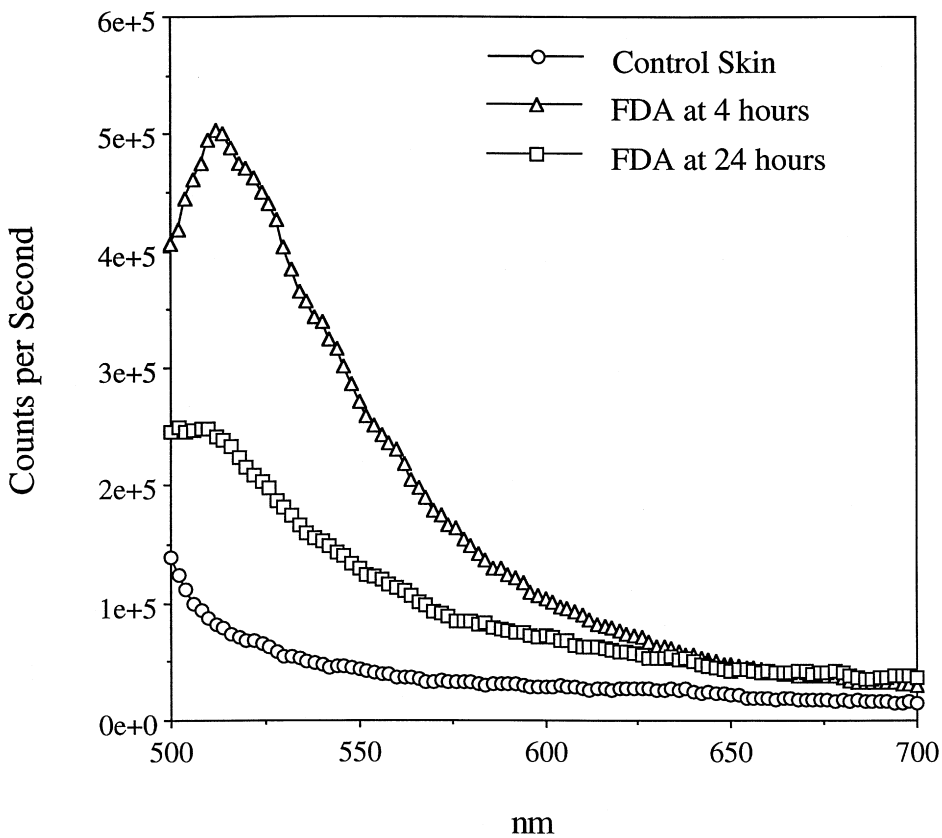

FIG. 1—*Fluorescein emission spectra at 4 and 24 h postmortem. The background fluorescence before the application of the FDA solution is also shown for comparison. The excitation wavelength was 468 nm and the emission spectra were recorded from 480 to 720 nm. The peak fluorescence maximum of converted fluorescein is at 518 nm. The emission spectra were recorded 2 h after the application of the DMSO solution on the surface of the skin.*

spectra were recorded 2 h after the application of the DMSO solution on the surface of the skin. The fluorescence emission spectrum of PpIX at 7 and 13 h postmortem are shown in Fig. 3. The spectrum was taken 3 h after intradermal injection of ALA in the excised full thickness skin.

Figure 4 shows a photograph of the sites where FDA and EB in DMSO were applied. The fluorescence of fluorescein and EB are clearly shown. Fluorescein appears green while EB appears red. The summary of the fluorescence measurements from the five animals is shown in Fig. 5. All data have been placed into three groups, namely 1–4 h, 6–24 h, and >40 h, respectively. Error bars represent standard deviations.

Discussion

In all cases, the fluorescence signature of fluorescein, EB, and PpIX could be easily distinguished from the endogenous fluorescence of the skin, Figs. 1–3. The fluorescence intensity changed significantly over time following the physiological state of the skin. The fluorescence intensity of FDA decreased rapidly during the first 6 h postmortem. On the other hand, the fluorescence intensity of EB remained almost constant during the first 24 h and then increased slowly over time. Thus, both the short and long term changes of the physiological conditions of the skin could be probed. The three assays, FDA, ALA, and EB, measure different cellular functions. While FDA probes cell viability, the conversion of ALA to PpIX correlates with cell proliferation. Therefore, the fluorescence intensity of fluorescein and PpIX is maximum when the keratinocytes are viable. This happens at early times following death. On the other hand, the EB stains cells that are dead. Therefore, the fluorescence intensity reaches maximum at a later time when a large number of cells in the skin are dead.

The change of the fluorescence intensity of fluorescein over time is summarized in Fig. 5. We can identify three time periods, 1–4 h, 6–24 h, and >40 h postmortem. There was dramatic decrease in the fluorescence intensity of fluorescein after a period of 4 h. The fluorescence of the EB, on the other hand, remained stable during the first 24 h and then increased slowly over time. The student's t test (one sided) applied to the FDA assay for the time periods <4 and 6–24 h postmortem, showed that the measured differences were statistically significant ($p < 0.01$). Note that one sided was chosen because we expected the fluorescence intensity to decrease over time. The FDA assay could determine, under the laboratory conditions, whether the time of death had occurred within 4 h or 6 h after the assay was applied.

There was considerable scatter of the data points from different sites and animals. The variability of the measurements was probably due to the variability among the animals as well as the difference of the skin thickness of different sites (21). Furthermore, the environmental conditions can greatly affect the permeability of the stratum corneum. Although we tried to keep the environmental conditions under control, it is quite possible that there were variations both in temperature and humidity over the time (several months) it took to complete the measurements. The ex vivo viability measurements presented additional problems. The skin shrunk and dehydrated during the measurements which resulted in changes of the optical properties of the skin. It should be empha-

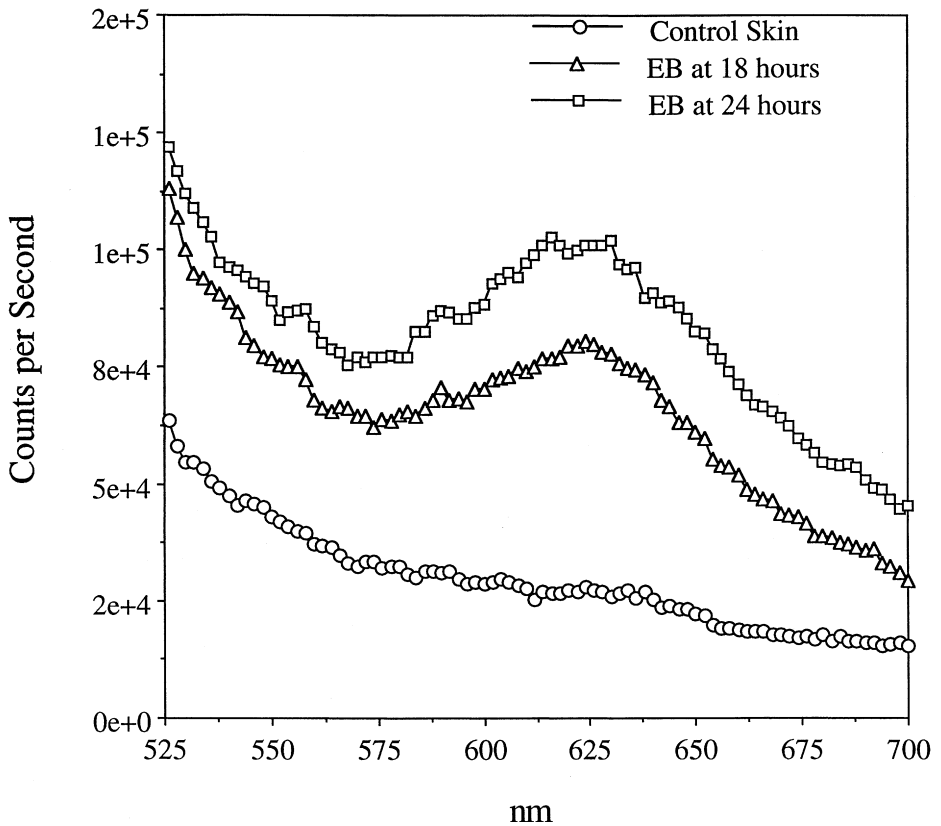

FIG. 2—*Ethidium bromide emission spectra at 18 and 24 h postmotrem. The background fluorescence before the application of the EB solution is also shown for comparison. The excitation wavelength was 468 nm and the emission spectra were recorded from 480 to 720 nm. The peak fluorescence maximum of EB is at 618 nm. The emission spectra were recorded 2 h after the application of the DMSO solution on the surface of the skin.*

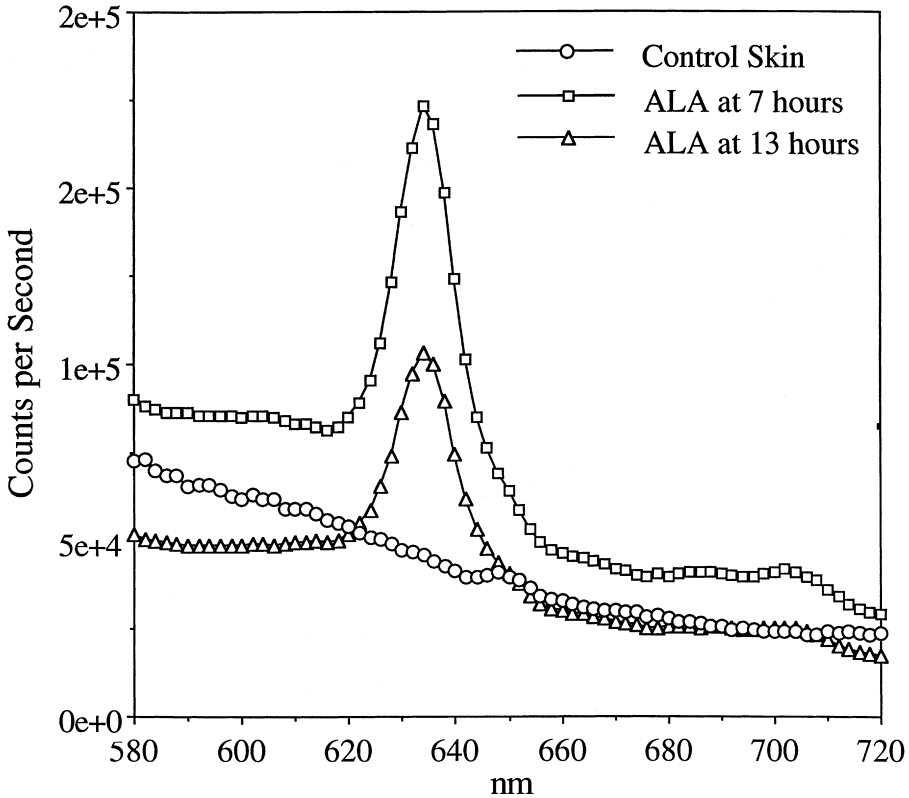

FIG. 3—*Protoporhyrin IX emission spectra at 7 and 13 h postmortem. The background fluorescence before the application of the ALA solution is also shown for comparison. The excitation wavelength was 405 nm and the emission spectra were recorded from 500 to 750 nm. The peak fluorescence maximum of PpIX is at 634 nm. The emission spectra were recorded 3 h after the application of the ALA intradermally.*

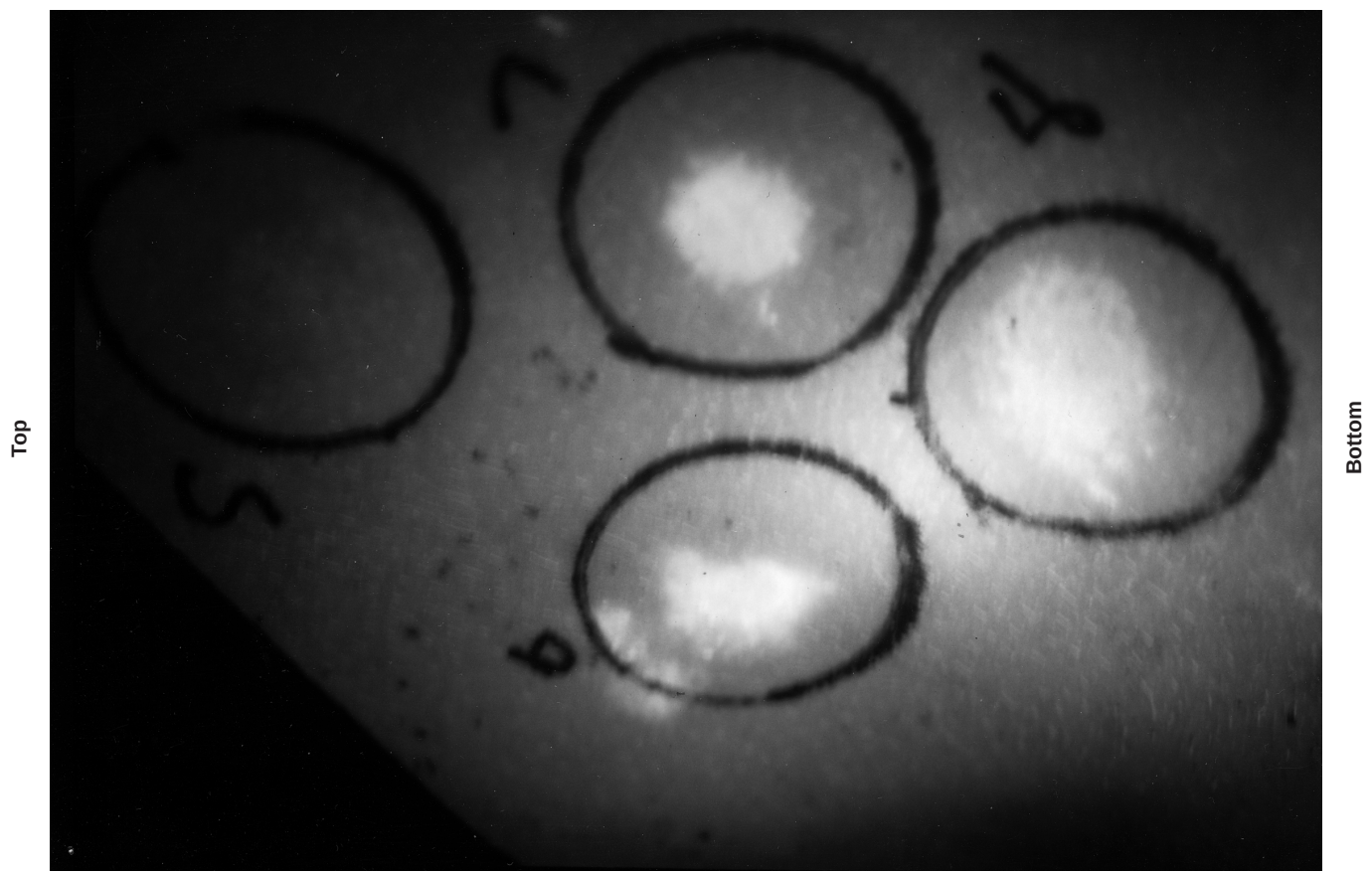

FIG. 4—*Photograph of the back of a swine, 3 h after applications of FDA and EB. The red fluorescence (site# 5&8) corresponds to EB while the blue fluorescence (sites# 6&7) corresponds to FDA. The intense light appearing in the space between the three circles is the reflection of the flash.*

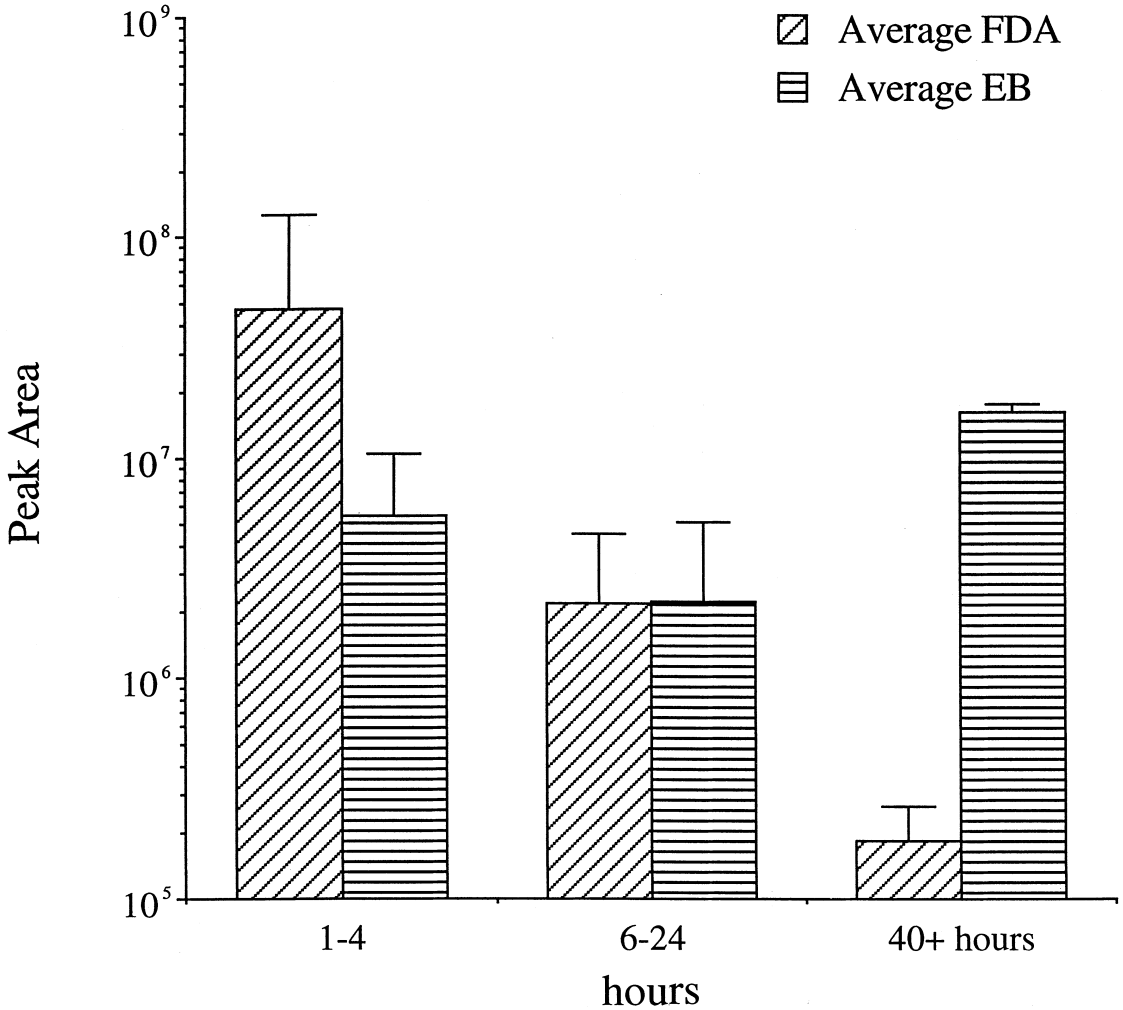

FIG. 5—*Peak intensity of fluorescein diacetate and ethidium bromide. All data points have been placed into three groups 1–4, 6–24, and >40 h postmortem. Notice that the vertical axis is in a logarithmic scale. Error bars represent standard deviations.*

sized that changes in the scattering coefficient of the skin will impact on the transmitted light for the excitation as well as the measured fluorescence intensity.

Although the previous discussion was focused primarily on the FDA and EB assays, the conversion of ALA to PpIX has also shown promise as a viability assay. However, the ALA assay required the tissue to be kept at 37°C which is not always practical with the animal carcasses. This is because the conversion of ALA to PpIX is a multistep process (eight steps) catalyzed by specific enzymes. This process is sensitive to temperature. At room temperature, the enzymatic reactions slow down which results in reduced PpIX formation. On the other hand, the conversion of fluorescein diacetate to fluorescein and the intercalation of ethidium bromide to DNA are single step reactions and are not as sensitive to temperature. The ALA assay, however, may still have a role in forensic sciences for the characterization of mechanically induced trauma in humans. In fact, we have shown that pressure waves cause measurable changes in the viability of keratinocytes, as measured by the conversion of ALA into PpIX, in vivo (unpublished data).

In conclusion, we have shown that under laboratory conditions, the skin viability decreases at a measurable rate following death in an animal model. The decreased skin viability was measured with spectroscopic techniques using a number of different assays. There was a substantial difference in the fluorescence intensity of the FDA assay between 0–4 h postmortem and 6–24 h which could be further elucidated to obtain higher time resolution. These results, however, are affected by environmental and genetic conditions which should be investigated. In addition, the present experiments should be extended to human cadavers in order to assess the full potential of skin viability and spectroscopic methods as a method for determining postmortem interval.

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