

Matthew J Traynor¹

Simon C Wilkinson²

Faith M Williams²

Metabolism of Butoxyethanol in excised human skin *in vitro*.

¹ School of Pharmacy, University of Hertfordshire, Hatfield, Hertfordshire, AL10 9AB.

²Medical Toxicology Research Centre and Toxicology Unit, Institute for Research on Environment and Sustainability, Newcastle University, Newcastle Upon Tyne, NE1 7RU, UK.

Email: m.j.traynor@herts.ac.uk

Address for correspondence

Matt Traynor

School of Pharmacy

University of Hertfordshire

College Lane

Hatfield

Hertfordshire

AL10 9AB

Abstract

Glycol ethers are widely used in industrial and household applications because their chemical and physical properties make them versatile solvents, miscible with both water and organic media. Due to the ease with which the glycol ethers are absorbed through the skin and the potential for development of adverse health effects it is important to understand the extent to which local metabolism can contribute to local and systemic toxicity.

Sections of previously frozen, full thickness excised human skin samples were placed on transwell supports and placed with the underside of the skin in contact with receptor fluid. The skin surface was dosed with 115.2 mg of neat butoxyethanol and the absorption and metabolism of butoxyethanol to butoxyacetic acid monitored over time.

In total 64.94 ± 0.04 mg of butoxyethanol or its metabolites were removed from the surface of the skin at 24 hours, representing the equivalent of 56% of the applied dose, the equivalent of 17.5% of the applied dose was recovered from the receiver fluid, 3% from within the skin and the remaining 23.5% of the dose was lost to the atmosphere through evaporation. After 24 hours a total of 31.5 μ g of butoxyacetic acid had been produced representing approximately 0.03% of the applied dose. Therefore approximately 0.16% (31.5 μ g as a percentage of the total amount of butoxyethanol reaching the receiver fluid (20.17 mg) of the absorbed butoxyethanol was metabolised to butoxyacetic acid during its passage through the skin. This suggested that, although enzyme activities capable of converting butoxyethanol to butoxyacetic acid are present in skin, metabolic conversion during percutaneous absorption was small and systemic exposure to the parent compound rather than the metabolite would occur following dermal exposure to butoxyethanol.

This experiment demonstrates that it is possible to maintain metabolic activity in skin samples in an *in vitro* set up for short, but experimentally useful, periods.

Key words: Butoxyethanol, metabolism, *in vitro*, skin absorption, human skin.

Introduction

Glycol ethers are widely used in industrial and household applications as solvents because their chemical and physical properties make them miscible with both water and organic media. It is widely accepted that glycol ethers are easily absorbed through the skin (Johanson *et al*, 1988a, 1988b; Kezic *et al* 1997; Filon *et al*, 1999; Wilkinson and Williams, 2002; Traynor *et al*, 2007). Following a one hour exposure of the human forearm to a 50% butoxyethanol 50% water mixture body levels exceeded the 8 hour threshold limit value for respiratory exposure to butoxyethanol (Jakasa *et al*, 2004). Butoxyethanol has been shown to cause erythrocyte haemolysis (Dartsch *et al*, 1999) following metabolism to butoxyacetaldehyde and butoxyacetic acid by alcohol and aldehyde dehydrogenases (Bartnik *et al*, 1987; Ghanayem *et al* 1987, 1989). While it is known that hepatic metabolism plays a major role in the fate of topically applied compounds (Aasmoe *et al*, 1998), skin has been shown to contain alcohol and aldehyde dehydrogenases (Kao and Carver 1990; Hewitt *et al*, 2000; Lockley *et al* 2004). As glycol ethers can penetrate the skin and have the potential for development of adverse health effects it is important to understand the extent to which local metabolism can contribute to systemic toxicity.

Lockley *et al* (2002; 2004) showed that skin homogenates could metabolise butoxyethanol but did not detect the local metabolism of glycol ethers during dermal penetration *in vitro* and *in vivo* in rat. Lockley *et al*. (2004) measured the absorption and metabolism of 2-butoxyethanol through both human breast skin and rat dorsal skin in a flow through diffusion cell. ¹⁴C butoxyethanol diluted in methanol, equivalent to 15µl/cm² was applied to the skin and rapid absorption of butoxyethanol following a 1 hour lag phase but butoxyacetic acid was not detected by HPLC with radiochemical detection. Lockley *et al*. (2004) suggested that the rapid passage of the solvents through the skin limited access of the parent compound to the enzymes thus preventing metabolism from occurring.

However in a recent study Korinth *et al* (2007) demonstrated dermal metabolism of butoxyethanol using an *in vivo* microdialysis technique in human volunteers. Korinth *et al*. applied an infinite aqueous dose (50% and 90% [v/v] butoxyethanol in water) to the left forearm of four male Caucasian volunteers aged 27-37. Two microdialysis

capillaries were intradermal inserted beneath each donor chamber and perfused with saline as a receptor fluid at a flow rate of 8µl/min. Receptor fluid samples were collected every 30 minutes for 4 hours. Butoxyacetic acid was detected at 4 and 4.5 hours using the method described by Kezic *et al.* (2004).

It is therefore apparent that butoxyethanol is metabolised to butoxyacetic acid during dermal absorption in vivo in man. Therefore the aim of this study was to investigate butoxyethanol during its passage through the skin using human skin in vitro in short term culture and the analytical method of Jakasa *et al.* (2004), which had greater sensitivity for measurement of butoxyacetic acid formation than the method previously reported by Lockley *et al.* (2004).

Materials and methods

¹⁴C 2-butoxyethanol, specific activity 1998 MBq/mmol was obtained from Amersham. Minimum essential medium (Eagle), Retinol and gentamycin were obtained from Sigma; Hi-safe 3 scintillation fluid was obtained from Fisher, and 2-butoxyethanol (99.9% purity) was obtained from Fluka. The water used was sterile water for irrigation. The polydimethylsiloxane membrane (thickness $400 \pm 13 \mu\text{m}$, obtained from SAMCO Silicone Products) was kindly donated by Dr R Chilcott.

Skin Preparation

Human breast skin was obtained after cosmetic surgery from a local hospital, the subcutaneous fat was removed and the skin stored at -70°C until required. Skin was obtained with the patients' consent and the study had approval from the University Hospital of South Durham Ethics Committee.

Skin samples were removed from the -70°C freezer and defrosted for 60 minutes prior to use. Metabolism of butoxyethanol during percutaneous penetration was investigated by a modification of the method used by Cnubben *et al.*, (2002). Full thickness skin was cut to size using a scalpel and a glass ring of internal surface area 0.64 cm^2 was glued to the skin using cyanoacrylate glue. The skin sections were then placed on Costar Netwell supports (pore size $3.0 \mu\text{m}$) in a 12 well plate and placed in to a cell culture plate containing 1.2 ml receptor fluid. (Minimum Essential Medium Eagle with Gentamycin solution ($50 \mu\text{g/ml}$)). The skin was pre-incubated at 37°C for 30 minutes before application of the dose to the skin within the glass ring. The cell culture plate was placed on a rocker within an incubator at 37°C . Samples ($50 \mu\text{l}$) of the receptor fluid were collected from beneath the skin at 0, 4, 8, 12 and 24 hours. The $50 \mu\text{l}$ of receptor fluid was immediately replaced with fresh receptor fluid.

Skin from 5 donors was exposed to a dose of butoxyethanol (115.2 mg) or to ¹⁴C butoxyethanol (115.2mg, equivalent to 56 KBq/cell) which was a sufficient dose to maintain the concentration gradient such that dose depletion does not affect the rate of flux. At the end of the experiment the skin surface was swabbed with tissue and the skin solubilised as previously described (Traynor *et al.* 2007). For radioactivity

measurements the receptor fluid aliquots were analysed by scintillation counting (DPM). As a control skin was treated with 128µl water. Butoxyethanol (128µl) was also applied to polydimethylsiloxane membrane (as a negative control) mounted on a Costar support. Skin from two of the donors was exposed to retinol a competitive inhibitor of ADH (Duester et al. 1991; Molotkov et al. 2002a) (250 or 500 µM in acetone added to the receptor fluid) for 30 minutes prior to application of the butoxyethanol. The inhibitor was left in the receptor fluid for the duration of the experiment. Receptor fluid samples were analysed for butoxyacetic acid.

Derivatisation of butoxyacetic acid

The following method was based on that previously described by Jakasa *et al.*, (2004). The receiver fluid samples obtained at each time point, plus 10µl of ethoxyacetic acid as an internal standard was added to 4-ml open topped glass vials. The vials were placed in a vacuum rotor evaporator at 80°C and evaporated to dryness (approx 15 minutes). The vials were then cooled on ice and 500µl of 1% (v/v) pentafluorobenzylbromide in methanol added. The vials were tightly capped and placed in a heating block at 80°C for 90 minutes. The vials were cooled on ice and the contents transferred to an eppendorf containing 500µl water. The eppendorf was capped and vortex mixed for 10 seconds. Hexane (500µl) was added to the eppendorf which was recapped and vortex mixed for 30 seconds. The hexane (upper phase containing any extracted butoxyacetic acid) was transferred to a GC vial and capped prior to analysis.

Gas chromatography.

GC was carried out with a Hewlett Packard 5890 Series II chromatograph, equipped with an Ultra I column (50m; 0.32mm i.d.; 0.52µm film thickness). A split ratio of 10:1 was employed. The injector temperature was 200°C. Detection was by electron capture detection using hydrogen (130 KPa) and air (270 KPa) with nitrogen (150 KPa) as auxiliary gas. Helium was used as a carrier gas (column head pressure 75 KPa). The detector temperature was 250°C. An injection volume of 1 µl was used. Peak area measurement was by Hewlett Packard HP3396A integrator.

Standard preparation

A range of butoxyacetic acid standards (0-50 $\mu\text{g/ml}$) were prepared for use in quantifying the amount of butoxyacetic acid produced. A stock solution of 1000 $\mu\text{g/ml}$ butoxyacetic acid was prepared in phosphate buffer (pH 7.25). This was then diluted using blank receptor fluid to give standards in the required range (figure 6.4). Aliquots (50 μl) of the standards, and the samples were extracted and derivatised using the method previously described in this paper and elsewhere (Jakasa *et al.*, 2004).

Statistical analysis

All results are presented as mean values \pm SEM with the corresponding n number and total number of skin donors used reported in all cases. Results were statistically analysed using SPSS 14.0 for windows. One-way analysis of variance was performed between each of the groups.

Results

Figure 1 shows the absorption profile (mean \pm SEM, n=10 from a total of 5 donors) of ^{14}C butoxyethanol through full thickness (previously frozen) human skin following application of an infinite dose ($200 \mu\text{l}/\text{cm}^2$) of butoxyethanol to the skin in the cell culture plate. There was linear absorption with time and no measurable lag time. By 24 hours 20.17 ± 0.03 mg of butoxyethanol or its metabolites (mean \pm SEM) had been absorbed to the receptor fluid, representing the equivalent of 17.5% of the applied dose, whilst 64.94 ± 0.04 mg butoxyethanol or its metabolites were removed from the surface of the skin representing the equivalent of 56% of the applied dose. And the equivalent of 3% (3.46 ± 0.01 mg) of the dose was recovered from the skin. It is likely that the remaining 23% of the dose has evaporated during the course of the experiment (Table 1).

Figure 2 shows the amount of butoxyacetic acid recovered in the receptor fluid for breast skin from five human donors (n = 10 samples in total) exposed to a dose equivalent to 115.2 mg of butoxyethanol. There was formation of butoxyacetic acid to a maximum of approximately 8 hours when the rate of production decreased despite there still being unabsorbed butoxyethanol present on the skin surface. The mean production rate of butoxyacetic acid (0 to 8 hours) was $3.07 \pm 0.41 \mu\text{g}/\text{h}$ (mean \pm SEM, n= 10) (Figure 3). It is unclear whether the variability in the amount of butoxyacetic acid produced between the individual donors is as a result of inter-individual variations in the rate of absorption or in the rate of metabolism, indeed it may be a combination of both factors. After 24 hours $31.5 \pm 0.3 \mu\text{g}$ of butoxyacetic acid had been produced representing approximately 0.03% of the applied dose (115.2 mg). Therefore approximately 0.16% ($31.5 \mu\text{g}$ as a percentage of the total amount of butoxyethanol reaching the receiver fluid (20.17 mg) of the absorbed butoxyethanol in the receptor fluid was metabolised during its passage through the skin in to butoxyacetic acid.

Figure 4 shows a comparison of butoxyacetic acid in the receptor fluid following application of an infinite dose of butoxyethanol compared to the same dose in the presence of retinol. Retinol at $250 \mu\text{M}$ caused the maximum rate of production to fall to $1.97 \pm 0.32 \mu\text{g}/\text{h}$ compared to $3.07 \pm 0.41 \mu\text{g}/\text{h}$ for uninhibited ($P < 0.05$) and in the

presence of 500 μM retinol the rate of production fell to $1.39 \pm 0.27 \mu\text{g/h}$ ($P < 0.005$) (Table 2).

When the skin was dosed with water instead of butoxyethanol a small peak was detected on the gas chromatography trace with a similar retention to the butoxyacetic acid derivative in the receptor fluid samples. This was equivalent to a background level of butoxyacetic acid of ($0.39 \mu\text{g/h}$). This figure was deducted from all samples (uninhibited or inhibited) as a background reading. As expected when butoxyethanol was applied to polydimethylsiloxane membrane (negative control), butoxyacetic acid was not detected in the receptor fluid. (table 2, figure 4).

Discussion

In this study using full thickness previously frozen human skin in short term culture metabolism of butoxyethanol to butoxyacetic acid during dermal absorption of butoxyethanol was detected. Previous studies have shown that alcohol and aldehyde dehydrogenase were stable in skin stored at -70C, whilst skin mounted in a static cell has been shown to lose viability (Mac Pherson *et al.*, 1991; Jewell *et al.*, 2000). In this study skin was mounted over tissue culture medium and maintained in a humidified atmosphere to improve viability but the decrease in the rate of formation of butoxyacetic acid by 8 hours is suggestive of loss of viability. Viability of skin in the flow through cell has also shown to be difficult to maintain after 8 hours (Boehnlein *et al.* 1994; Clark *et al.* 1991; Moss *et al.* 2000). This experiment showed that it is possible to detect the products of ADH/ALDH metabolism in skin samples in *in vitro* studies for up to 8 h. Decreased availability of co-factors may have contributed to the decrease in enzyme activity after 8 h.

Following application of an infinite dose of (neat) butoxyethanol 0.03% of the applied dose was converted to butoxyacetic acid during a 24 hour period. This represented 0.16% of the total amount of butoxyethanol absorbed to the receptor fluid. This figure was lower than the 1.9% metabolism reported *in vivo* by Korinth *et al.* (2007), following the dermal absorption of 2-butoxyethanol from a 50% aqueous solution. However it is widely accepted that the rate of absorption of 2-butoxyethanol from a 50% solution is up to 5 times greater than from neat 2-butoxyethanol (Traynor *et al.*, 2007; Kezic *et al.*, 2004).

Retinol is oxidised to retinal by alcohol dehydrogenase and then oxidised to retinoic acid by aldehyde dehydrogenase. Therefore retinol is known to be a competitive inhibitor of both alcohol and aldehyde dehydrogenase (Duester and Duester 1991; Duester *et al.* 1991; Leo *et al.* 1999; Molotkov *et al.* 2002b; Molotkov *et al.* 2002a). The data reported here shows that in the presence of retinol, an inhibitor of ADH4 which is expressed in the skin, there was a dose dependent decrease ($P < 0.05$) in the levels of butoxyacetic acid formed indicating the involvement of dehydrogenases in butoxyacetic acid formation.

Lockley *et al.*, (2004) did not detect butoxyacetic acid formation during dermal absorption of butoxyethanol in skin in a flow through diffusion system. Lockley *et al.*, attempted to quantify the presence of the metabolites in the receptor fluid using a HPLC method with a Ramona LS radiodetector fitted with a calcium fluoride solid scintillation cell. They suggested that the passage of butoxyethanol through the skin was too rapid to allow metabolism to occur. However Korinth *et al.* (2007) detected a low level of butoxyacetic acid in microdialysis fluid following application of butoxyethanol to a volunteer. In light of findings here it is likely that butoxyacetic acid was formed in the Lockley study but was not detected. This is possibly due to the large dilution effect in receptor fluid flowing at 1.5ml/h which with the low levels of metabolism reported both here and by Korinth *et al.* (2007), would have resulted in levels of metabolite below the limit of detection reported by Lockley *et al.* (2004). Similarly metabolism of ethoxyethanol (Lockley *et al.* 2002) and phenoxyethanol (Roper *et al.* 1997) by dehydrogenases was not detected probably due to limitations in the analytical methods utilised. In the study presented here the use of a more sensitive analytical technique in combination with the low volume of receiver fluid used has allowed detection of these metabolites *in vitro* for the first time.

The low level of conversion to butoxyacetic acid during dermal absorption reported both here and by Korinth *et al.* (2007), when the skin contains the oxidative enzymes may be due to rapid diffusion of butoxyethanol through the skin (as suggested by Lockley *et al.* (2004), thus decreasing availability of the substrate to metabolic enzymes in the keratinocytes of the epidermis. The low level of metabolism may also be as a result of the low level of expression of ADH in the skin. In a comparison study (data not shown) the authors of this paper detected the production of $29.56 \pm 2.04 \mu\text{g}$ of butoxyacetic acid per mg of homogenated liver cytosolic protein compared to $3.69 \pm 0.37 \mu\text{g}$ per mg of homogenated skin cytosolic protein.

Metabolism of butoxyethanol in the skin following topical application to man *in vivo* would be small compared to metabolism in the liver and would not contribute to systemic toxicity. However formation of the intermediate toxic metabolite, the aldehyde could exert local toxic effects in the skin by locally binding to protein before further oxidation to the acid. Free aldehydes are rarely measured in skin absorption

experiments because rapid further metabolism, conjugation or protein adduction would likely occur (Smith *et al.*, 2000). The level of metabolism is similar to that reported *in vivo* by Korinth *et al.* (2007), and as suggested by those authors is likely to be related to the low capacity of the enzymes in the skin rather than the absolute amounts of 2-butoxyethanol permeated or the rate of permeation. For butoxyethanol it is unlikely that the small amount of dermal metabolism would result in local toxicity, contribute significantly to the overall systemic burden of toxic metabolites or reduce the bioavailability of the parent.

Acknowledgements

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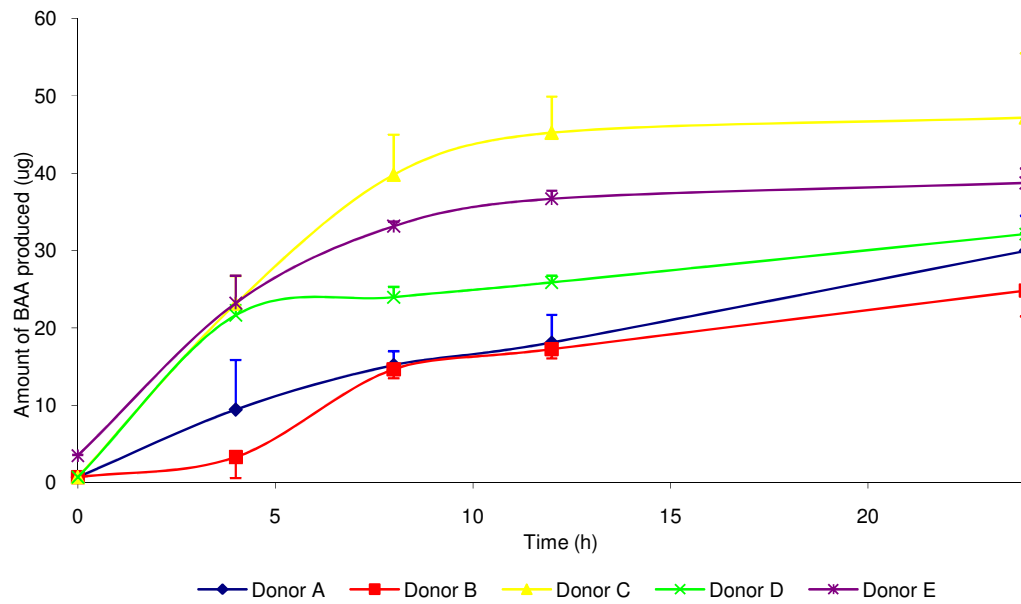
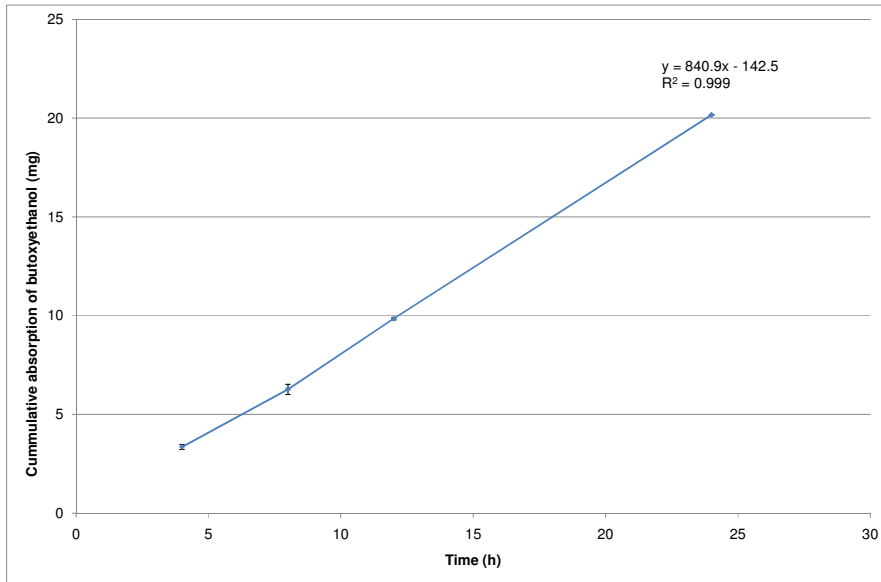
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Receptor Fluid	Removed dose	Within skin	Total recovered
17.5 ± 0.03%	56 ± 0.04%	3 ± 0.01%	76.5 ± 0.03%

Table 1 The distribution profile butoxyethanol (mean ± SEM n = 10 cells, from 5 skin donors) following 24 hour exposure of full thickness human skin to an infinite dose (200µl/cm²) butoxyethanol in short term culture.

Treatment	Number of skin donors (number of samples used)	Maximum rate of production of butoxyacetic acid (µg/h)	Total amount of butoxyacetic acid produced (µg) in 24 h
Uninhibited	5(10)	3.70±0.43	31.5±0.3
Inhibited with 500µM retinol	2(4)	1.97±0.31	23.7±2.7
Inhibited with 250µM retinol	2(4)	1.39±0.26	21.2±3.3
Dosed with water	2(2)	0.00±0.00	0.0±0.0
Skin replaced with PDMS membrane	1(2)	0.00±0.00	0.0±0.0

Table 2 Maximum rate of production (mean ± SEM) of butoxyacetic acid following topical administration of butoxyethanol or water to full thickness human skin or PDMS membrane in cell culture plates.



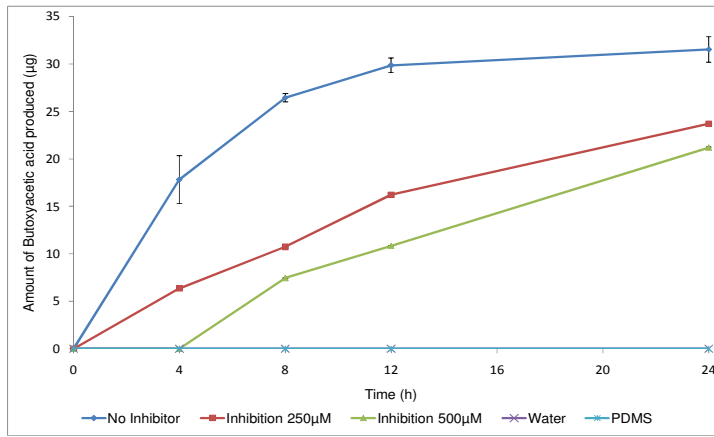
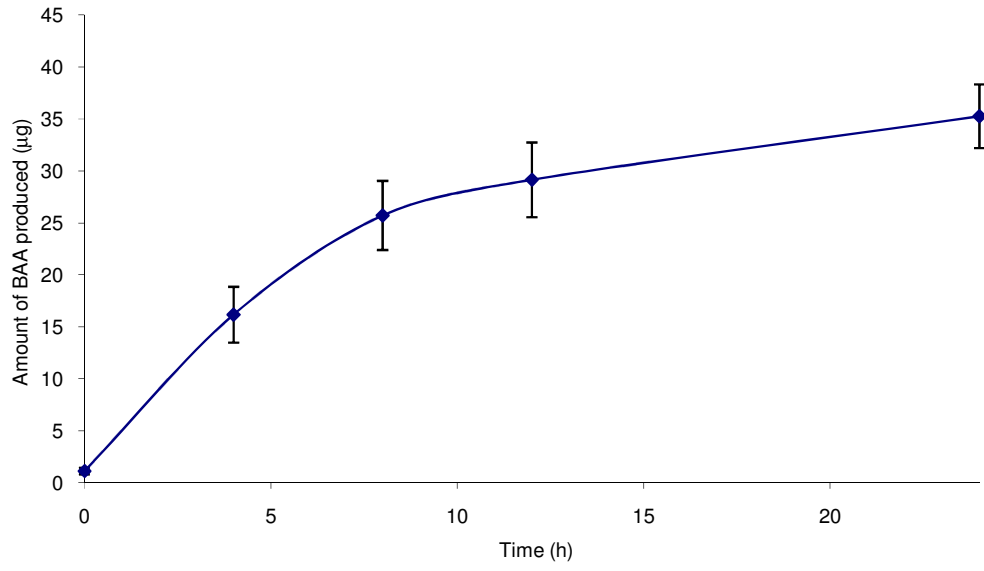


Figure Legends

Figure 1. Mean cumulative absorption (\pm SEM n = 10 from a total of 5 donors) of butoxyethanol through full thickness human skin following topical application of butoxyethanol in a cell culture plate.

Figure 2. Mean cumulative (\pm SEM n = 10 samples from a total of 5 donors) amount of butoxyacetic acid recovered from the receptor fluid following topical administration of butoxyethanol to full thickness human skin sections *in vitro*.

Figure 3. Mean cumulative absorption (\pm SEM n = 10 samples from a total of 5 donors) of butoxyacetic acid in receptor fluid following topical administration of butoxyethanol to full thickness human skin sections *in vitro*.

Figure 4. Cumulative absorption of butoxyacetic acid in receptor fluid following topical administration of butoxyethanol, alone (n = 10 from 5 donors) or in the presence of 250 μ M retinol (n = 4 from 2 donors) or 500 μ M retinol (n = 4 from 2 donors) to full thickness human skin, water to full thickness skin (n = 2 from 2 donors) or butoxyethanol to PDMS membrane (n = 2) in cell culture plates. Figures shown are after deduction of background readings obtained from the water (negative control sample) at each timepoint.