Toxicology Letters 292 (2018) 63–72

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

A study of inter-individual variability in the Phase II metabolism of xenobiotics in human skin

Sandrine Spriggs[a,*], Richard Cubberley[a], Paul Loadman[b], David Sheffield[a], Antonia Wierzbicki[b]

[a] Unilever, Safety and Environmental Assurance Centre, Colworth Science Park, Sharnbrook, Bedfordshire, MK44 1LQ, United Kingdom
[b] Institute of Cancer Therapeutics, University of Bradford, Bradford, BD7 1DP, United Kingdom

ARTICLE INFO

Keywords:
Phase II metabolism
Ex vivo skin
S9 incubations
Cofactors

ABSTRACT

Understanding skin metabolism is key to improve in vitro to in vivo extrapolations used to inform risk assessments of topically applied products. However, published literature is scarce and usually covers a limited and non-representative number of donors. We developed a protocol to handle and store ex vivo skin samples post-surgery and prepare skin S9 fractions to measure the metabolic activity of Phase II enzymes. Preincubation of an excess of cofactors at 37 °C for fifteen minutes in the S9 before introduction of the testing probe, greatly increased the stability of the enzymes. Using this standardised assay, the rates of sulphation (SULT) and glucuronidation (UGT) of 7-hydroxycoumarin, methylation (COMT) of dopamine and N-acetylation (NAT) of procainamide were measured in the ng/mg protein/h (converted to ng/cm^2/h) range in eighty-seven individuals. Glutathione conjugation (GST) of 1-chloro-2,4-dinitrobenzene was assessed in a smaller pool of fifty donors; the metabolic rate was much faster and measured over six minutes using a different methodology to express rates in µg/mg protein/min (converted to µg/cm^2/min). A comprehensive statistical analysis of these results was carried out, separating donors by age, gender and metabolic rate measured.

1. Introduction

Human skin forms the first protective barrier between our body and the environment (Bouwstra and Ponec, 2006; Madison, 2003). Despite being accepted as a physical barrier, the skin is under constant exposure to xenobiotic chemicals able to diffuse through the skin layers (Berard et al., 2003; Zalko et al., 2011). This could potentially cause toxicity in the skin itself and other parts of the body due to systemic exposure. The second line of defence in the skin's barrier function, often described as a chemical barrier, is its capacity to detoxify and eliminate these compounds via metabolism using phase I and phase II metabolic enzymes (Oesch et al., 2014; Svensson, 2009). Although cutaneous metabolism is actively exploited in the application of dermally applied pro-drugs (Møllgaard et al., 1982), metabolic activation is almost certainly why seemingly unreactive compounds cause skin sensitisation reactions (Schmidt and Khan, 1989). Conversely, it can be argued that the sensitisation risk from a reactive chemical would be reduced if it were metabolised to a non-reactive form in the skin (Manwaring et al., 2015). The redox mechanisms present in the skin might also be involved (Korkina, 2016), though the two phenomena can be difficult to separate in cases such as glutathione metabolism. Thus, skin metabolism needs to be characterised thoroughly to understand its impact on the magnitude of adverse outcomes such as allergic contact dermatitis (ACD).

Published literature regarding cutaneous metabolism is limited and clearance mechanisms within skin are still poorly understood. Studies on the mRNA expression levels in skin have identified the presence of phase I and phase II enzymes, including the phase II enzymes UDP-glucuronyltransferases (UGT), sulphotransferases (SULT), N-acetyltransferases (NAT), catechol-O-methyl transferases (COMT) and glutathione-S-transferases (GST) (Hu et al., 2010; Luu-The et al., 2009; van Eijl et al., 2012). However, mRNA levels often show poor correlation to protein expression levels (Maier et al., 2009) and quantitative measurement of metabolism in skin remains a challenge.

The closest experimental approach under which to study metabolism in skin to compare to in vivo situations, is to use viable ex vivo human skin in culture (Manevski et al., 2015; Zalko et al., 2011). Effectively a living system, the various compartments (both inter and intra cellular) remain relatively intact and tissue viability as well as the levels of some biomarkers are preserved for several days (de Wever et al., 2015; Varani et al., 2007). Several studies have used the incubation of whole skin in media containing concentrations of chemical

Abbreviations: ACD, Allergic contact dermatitis; COMT, catechol-O-methyl transferases; GST, glutathione-S-transferases; HaCaT, Immortalised keratinocytes "Human Adult Low Calcium High Temperature"; NAT, N-acetyltransferases; NHEK, Normal Human Epidermal Keratinocytes; SULT, sulphotransferases; UGT, UDP-glucuronyltransferases

* Corresponding author.
E-mail address: sandrine.spriggs@unilever.com (S. Spriggs).

https://doi.org/10.1016/j.toxlet.2018.04.011
Received 26 October 2017; Received in revised form 2 March 2018; Accepted 12 April 2018
Available online 27 April 2018
0378-4274/

substrate to study activity rates (Eilstein et al., 2014; Manevski et al., 2015), while topical application of the substrate on cultured skin explants or models is favoured in studies covering both skin penetration of the test substrate and identification of its major metabolites (Jacques et al., 2014). Whilst such studies have shown promising results, obtaining fresh ex vivo skin consistently is difficult, and these studies often represent only a small number of donors.

Models commonly used in metabolism studies include primary keratinocytes such as Normal Human Epidermal Keratinocytes (NHEK) or immortalised keratinocytes such as the "Human adult Low Calcium High Temperature" (HaCaT) cell line. The N-acetylated product of 4-amino-2-hydroxytoluene, a common hair dye, has been reported in both ex vivo skin and HaCaT experiments (Goebel et al., 2009). The activity level of NAT1 enzyme in HaCaT cells has been estimated to be 3.4 fold higher than in NHEK (Bonifas et al., 2010) but still comparable to levels expected in the skin. Models more closely related to skin such as reconstructed 3D skin models consisting of keratinocytes-derived layers (e.g. Episkin, EpiDerm, Phenion) and their Full Thickness versions, which include a layer of cultured fibroblasts in collagen under the keratinocytes to mimic the effect of the dermis, have been used, but understanding the differences between all these models and ex vivo skin is still the subject of investigations (Eilstein et al., 2014; Gotz et al., 2012a; Gotz et al., 2012b; Hewitt et al., 2013; Jackh et al., 2011; Wiegand et al., 2014).

When skin culture is not practical, freshly excised skin can be preserved, typically by snap freezing in liquid nitrogen, and stored frozen. However, metabolism studies using frozen skin have yielded mixed results in the past and protocols tend to vary from study to study. We investigated the influence of storage on both fresh skin and S9 fractions and developed approaches to homogenise skin and handle S9 during metabolic assays. We used 7-hydroxycoumarin (7-HC) to study sulphation and glucuronidation (Wang et al., 2005), procainamide for acetylation (Dreyfuss et al., 1972), dopamine for methylation (Mannisto and Kaakkola, 1999) and 1-chloro-2,4-dinitrobenzene (DNCB) for glutathione conjugation (Harris et al., 2002). A standard protocol derived from these experiments was then applied to samples from up to 90 volunteers to generate metabolic rates for each of the five Phase II pathways studied. The metabolic rates obtained for each enzyme were statistically analysed to evaluate similarities within the dataset, mainly by comparing age or gender and creating "high metaboliser" versus" low metaboliser" groups based on one enzyme and comparing with the others. Even without consideration of statistical differences between groups of individuals, the mean metabolic rate for each enzyme can still be a useful piece of information to integrate into in silico models of human skin.

In silico models aiming to extrapolate in vitro data to in vivo exposure scenarios are built using chemicals for which historical data is available such as caffeine (Gajewska et al., 2015) or *para*-phenylenediamine (Manwaring et al., 2015). While some examples have been provided to show that skin metabolism can be a major factor affecting skin bioavailability (Jacques et al., 2010; Jacques et al., 2014), refinements of these in silico models will require a broader use of skin detoxification potential data such as the ones presented here.

2. Materials and methods

2.1. Chemicals and reagents

Dopamine, procainamide, 7-hydroxycoumarin, 1-chloro-2,4-dinitrobenzene, S-adenosyl-methionine (SAM), 3′-Phosphoadenosine-5′-phosphosulphate (PAPS), Acetyl Coenzyme A (AcCoA), Uridine diphosphoglucoronic acid (UDPGA), glutathione (GSH), glutathione disulphide (GSSG), 7-hydroxycoumarin glucuronide (7-HCG), 7-hydroxycoumarin sulphate potassium salt (7-HCS), N-acetylprocainamide hydrochloride (NAPA) were purchased from Sigma Aldrich (Gillingham, UK) and used without further purification. The methylated dopamine standard, i.e. 3-methoxytyramine hydrochloride (3-MT), was purchased from Fisher Scientific Ltd (Loughborough, UK). Deuterated 7-hydroxycoumarin (7HC-d_5) was purchased from BD BioSciences Ltd. All solvents were purchased at the highest purity available (HPLC grade minimum).

A mass labelled internal standard of the glucuronide of 7-HC, i.e. 7HC-d_5-glucuronide (7-HCG-d_5) was created in house by incubating 200 µg/mL 7HC-d_5 in concentrated skin S9 containing 5 mM UDPGA for 4 h at 37 °C. 7-HCG was isolated from a methanol extract by HPLC and stored at −80 °C until use.

Dinitrophenyl-glutathione (DNP-SG), the GSH conjugate formed with DNCB, was created in house by incubating 200 µg/mL DNCB in concentrated skin S9 for 4 h at 37 °C. The mixture was extracted in acetonitrile, the supernatant separated by HPLC, the fraction containing the metabolite DNP-SG obtained frozen at −20 °C until use.

2.2. Human skin samples

All skin samples were sourced from the Bradford Ethical Tissue Bank. The Ethical Tissue Bank was authorized by the Leeds flagged REC to release samples to researchers. The Ethical Tissue Bank also acts on behalf of the Recipient's Institution for the collection, use and storage of Material and associated data. Samples were provided anonymously with only the minimum data set and handled in compliance with the Human Tissue Act. All full thickness skin samples were frozen within 120 min of excision.

2.3. Protocol optimisation for skin S9 preparation

First, freshly excised skin samples were cut to size (triangle of approximately 1 cm side length), weighed and measured (the height and base of each triangle was measured with a ruler and the surface area calculated), homogenised in Phosphate Buffered Saline (PBS) (4 mL/g skin) on ice using an Ultra Turrax blender (IKA) (4 °C, 30 s). The homogenate was then centrifuged at 9000*g* (4 °C, 20 min) and the S9 fraction collected as the supernatant.

As a separate experiment, freshly excised skin triangles were snap frozen in liquid nitrogen and pulverised using a cell crusher (Stratech) using a mallet until the frozen skin had a dust-like powder appearance. PBS was added to the pulverised tissue (4 mL/g skin) and the sample sonicated on ice (4 °C, 6 × 30 s).

The protein content of the resulting S9 was determined using the Bradford Method (Bradford, 1976). We compared the two methods of homogenisation for a single skin sample by submitting fractions of S9 generated by each approach to the same incubations with 7-HC, dopamine and procainamide (with corresponding cofactor).

2.4. Protocol optimisation for preserving enzymatic stability

First, to assess the effect of time on the handling procedure for fresh ex vivo skin (post-surgical procedure), whole skin (one triangle placed in PBS, volume adjusted to the weight of each triangle so that the ratio was 4 mL/g tissue) and freshly prepared S9 fraction (skin homogenised in PBS at a concentration of 4 mL PBS per g of tissue) from the same single donor were incubated at 37 °C or 25 °C for 0, 3, 6 and 24 h, after which the incubated skin was homogenised to prepare S9 for analysis (as above) and the incubated S9 was used as collected. Enzymatic activity was assessed at each time point for all enzymes except GST. The rates were measured in ng metabolite/mg protein/h, the initial rate fixed at 100% activity and all subsequent activities expressed as a percentage depletion of the initial rate.

GST activity is thought to be mostly dependant on the availability of its cofactor, GSH, which is prone to oxidation. To determine the effect of freeze-thawing on GSH levels, whole skin samples from five donors were aliquoted. Half the samples were analysed immediately for GSH and GSSG content (qualitative measurement expressed in peak area),