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The metabolism of amethocaine by porcine and human skin extracts: influence on percutaneous anaesthesia

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Summary

Amethocaine, a local anaesthetic of the ester class, produces effective percutaneous anaesthesia of the skin when applied as a novel preparation. The metabolism of the drug by cutaneous esterases was investigated using enzymatic extracts of porcine and human whole skin, and of human epidermis. Analysis of the incubation mixture indicated the production of *p*-(butyl)aminobenzoic acid, confirming that metabolism of the substrate occurred via cleavage of the ester side chain. In all cases, the rate of metabolic transformation was slow, requiring about 24 h for a 50% reduction in substrate concentration. There was evidence that amethocaine, but not its metabolite, was bound to proteinaceous components of the cutaneous extracts. Given the prolonged in vivo anaesthetic activity of amethocaine percutaneous anaesthetic preparations, the ability of the skin to metabolise the drug is likely to be of significance in determining the duration of anaesthetic activity but is too slow to influence speed of onset of the anaesthetic effect. The lack of systemic toxicity with percutaneous amethocaine may be explained by a combination of slow drug release from the stratum corneum and metabolism by cutaneous esterases.

Introduction

Traditionally, skin has been regarded as a passive, inert structural barrier between the body and its environment. However, it is now clear that the skin also possesses enzyme systems capable of metabolising both endogenous substrates and exogenous chemicals (Pannatier et al., 1978; Mukhtar and Bickers, 1981). Cutaneous enzyme systems include simple, non-specific esterases. The action of skin in hydrolysing short-chain fatty acid esters has been demonstrated by several studies, as reviewed by Findlay (1955). In addition to hydrolysis, skin is capable of other phase 1 functionalisation reactions (oxidation and reduction) and phase 2 conjugation reactions (glucuronide, sulphate and glutathione formation). These reactions have been reviewed by Pannatier et al. (1978). However, quantitative differences can be expected in the metabolic activity of skin from different bodily regions of the same individual, and in the skin of different individuals.

Amethocaine, a local anaesthetic of the ester class, has been shown to produce effective anaesthesia of the skin (McCafferty et al., 1988). Novel amethocaine percutaneous anaesthetic formulations have recently been reported (Woolfson et al., 1988; McCafferty et al., 1989). Findlay (1955) has previously demonstrated the presence

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of non-specific esterases in skin homogenates. Since cutaneous metabolism could have significant effects with regard to both the duration of dermal anaesthesia and potential systemic toxicity, the present study was carried out in order to investigate the susceptibility of amethocaine to cutaneous metabolism by porcine and human skin homogenates.

Materials and Methods

Chemicals

Amethocaine hydrochloride B.P. was obtained from Smith & Nephew Ltd (Romford, U.K.). Pseudocholinesterase (10–20 U/mg protein), PABA (*p*-aminobenzoic acid) and BBA (*p*-(butylamino)benzoic acid) were from Sigma (Poole, U.K.). All other chemicals were of Analar or HPLC quality as appropriate.

Preparation of skin extracts and incubation mixtures

Skin samples were (i) human facial skin, surplus from cosmetic surgery and (ii) neonate porcine abdominal skin (Faculty of Agriculture, Queen's University). Subcutaneous fat was removed from each sample which was then cut into small pieces, crushed under liquid nitrogen, suspended (200 mg 10 ml^{-1}) in PBS (phosphate-buffered saline, pH 7.2), cooled in an ice bath and homogenised (2 min, head no. 18 then 2 min, head no. 10) using an Ultra-Turrax homogeniser (IKA Ltd, F.R.G.). Glass beads (diameter 0.45–0.50 mm, 10 g) were added to the homogenate and the cellular content disrupted (2×5 min, CO_2 as coolant) in a Braun Cell Disintegrator (B. Braun Ltd, F.R.G.). The whole skin extract thus prepared was centrifuged (3000 rpm, 15 min). The supernatant was collected and stored at $-80\,^{\circ}C$ until required.Human epidermis was obtained by carefully stretching the whole skin sample and securing by pins to a cork board, dermis side uppermost. The epidermis-dermis boundary was visually apparent and the dermis was removed by dissection. The remaining epidermal layer was approx 250 μm thick. Homogenates were then prepared as previously described.

In each case, the prepared skin extract (9.0 ml) was incubated with a solution of amethocaine hydrochloride (1.0 ml, 1 mg ml^{-1} in PBS) at $37\,^{\circ}C$. At intervals, samples (0.3 ml) were withdrawn for analysis. Control experiments were performed in all cases, the skin homogenate being replaced by an equivalent volume of PBS.

To determine the effect of a non-specific plasma esterase, amethocaine hydrochloride (2 ml, 200 μg ml^{-1} in PBS) was incubated at $37\,^{\circ}C$ with 50 U pseudocholinesterase. Samples were withdrawn at 15-min intervals and analysed by HPLC.

Determination of amethocaine and its metabolite, p-(butylamino)aminobenzoic acid (BBA)

Amethocaine and its metabolite were determined by reverse-phase ion-pair high-performance liquid chromatography. A Spherisorb 5 μm ODS column (25 cm) fitted with a 5 cm guard column was used in conjunction with a mobile phase of acetonitrile-water 8 : 2 containing 0.005 mol l^{-1} 1-hexanesulphonic acid ion pair reagent (Waters Ltd, Harrow, U.K.).

Instrumentation (Anachem Ltd, Luton, U.K.) comprised a Gilson 302 pump and 802 manometric module fitted with a Rheodyne 7125 injector and 20 μl loop. Detection was at 310 nm using an LKB 2151 variable-wavelength detector (LKB, Bromma, Sweden) operating at 0.04 absorbance units full-scale deflection. Chromatograms were recorded on a Hewlett-Packard 3390A integrator (Hewlett-Packard, Wokingham, U.K.).

Calibration graphs were linear for amethocaine ($r > 0.997$) between 5.14 and 46.30 μg ml^{-1}, and for BBA ($r > 0.998$) between 2.62 and 13.05 μg ml^{-1}. For analysis, samples (0.3 ml) were mixed 1 : 1 with internal standard solution (procaine hydrochloride, 100 μg ml^{-1} in PBS).

Chromatographic peaks were identified by comparing retention times with suitable standards.

Recoveries of amethocaine and BBA from incubation mixtures

Incubation mixtures of skin homogenate and either amethocaine hydrochloride (103.8 μg ml^{-1}) or BBA (2.24 μg ml^{-1}) were prepared as previously described. In each case five replicate samples were taken immediately, the drug concentra-

tion determined by HPLC and percentage recoveries calculated.

Results and Discussion

Novel amethocaine percutaneous anaesthetic preparations have a short contact time (30 min) with skin in the clinical situation (McCafferty et al., 1989). The cutaneous nociceptors lie just below the stratum corneum, about 15 μm below the skin surface (Woolfson and McCafferty, 1989) and therefore substantial penetration of the cutaneous structure is not required for activity. Given these observations, and the fact that the amount of anaesthetic absorbed into the skin structure is low, it was impractical to measure metabolite concentrations in intact skin samples. Therefore, cutaneous enzyme extracts were prepared after the manner of Findlay (1955).

In order to yield an enzymatically active extract a modified method was found to be necessary in which an initial powdering under liquid nitrogen was followed by homogenisation and cell disintegration phases. To prevent uptake of the drug by cellular debris, the latter was removed by centrifugation following the final cell disruption step to yield the enzymatically active extract. Human skin was more difficult to treat than porcine skin. Powdering under liquid nitrogen could be omitted in the latter case. The presence of non-specific esterases in the prepared skin extracts was confirmed by incubation with a standard esterase substrate (*p*-nitrophenyl acetate) and subsequent spectrophotometry.

The pattern of amethocaine skin metabolism was shown to be identical to that experienced when amethocaine was incubated with pseudocholinesterase. In both cases a peak with retention time 2.65 min appeared on the chromatogram, gradually increasing in area with increasing incubation time. Standard BBA and PABA solutions gave retention times of 1.99 min ($s = 0.01$) and 2.66 min ($s = 0.01$). Thus, the metabolism of amethocaine by skin homogenates appears to proceed via simple hydrolysis of the ester side chain, yielding BBA and dimethylaminoethanol (not detected) as the products (Fig. 1). Unlike systemic

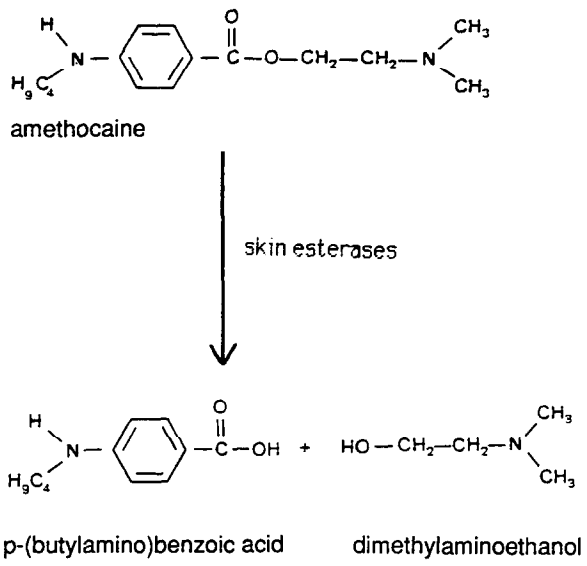

Fig. 1. Reaction scheme for the metabolism of amethocaine by cutaneous esterases.

metabolism, there was no evidence of further transformation to yield PABA as the final product. The chromatographic separation of amethocaine, BBA and procaine (internal standard) is shown in Fig. 2.

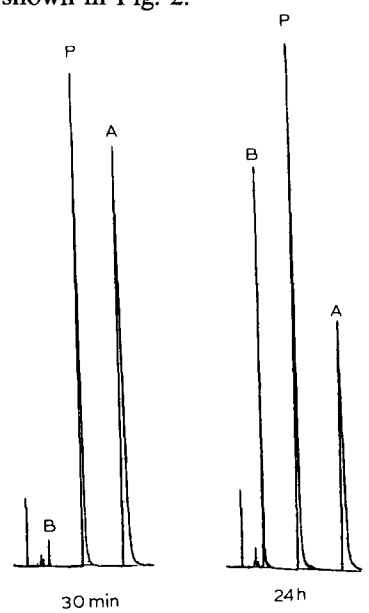

Fig. 2. Chromatograms for the metabolism of amethocaine by human whole skin extracts following 30 min and 24 h incubation at 37 °C. A, amethocaine; P, procaine internal standard; B, *p*-(butylamino)benzoic acid (metabolite).

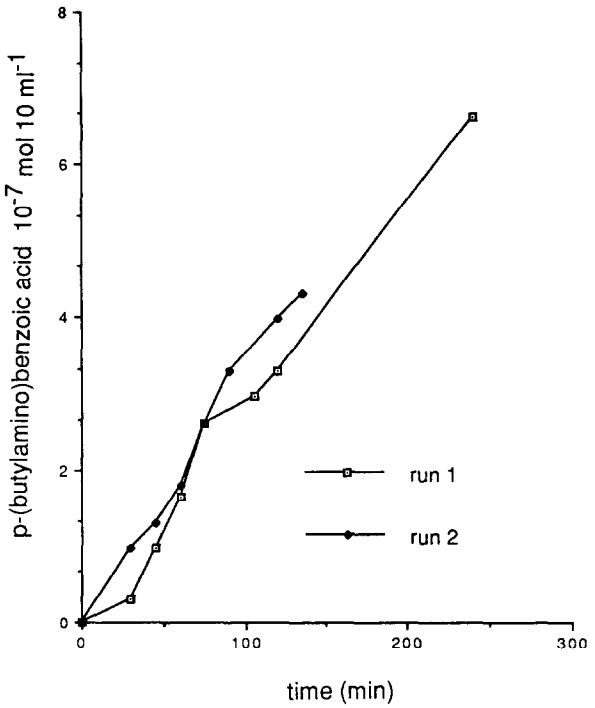

Fig. 3. Increasing concentration of *p*-(butylamino)benzoic acid with time during incubation of amethocaine with porcine whole skin extracts.

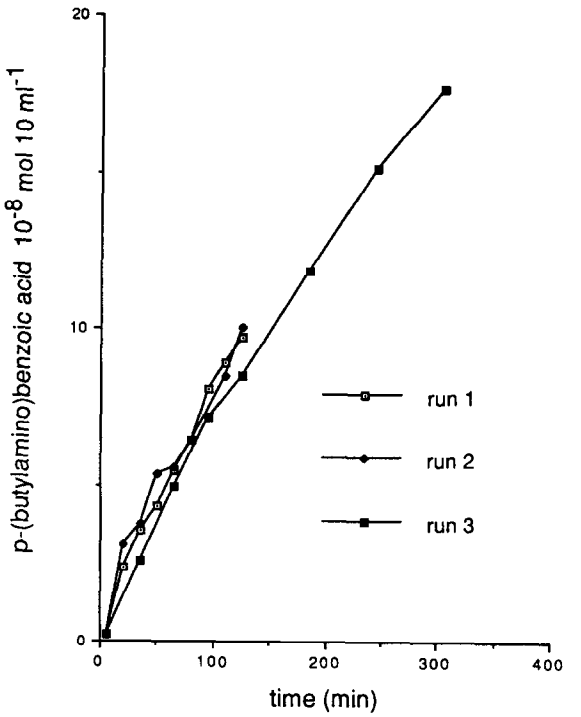

Fig. 4. Increasing concentration of *p*-(butylamino)benzoic acid with time during incubation of amethocaine with human whole skin extracts.

The metabolism of amethocaine by both porcine (Fig. 3) and human (Fig. 4) whole skin extracts was investigated. Control samples in which the skin extracts were replaced by an equivalent volume of PBS showed no significant changes in substrate concentration, nor any metabolite production, during 48 h incubation, confirming that amethocaine was stable under the chosen incubation conditions. For both porcine and human whole skin extracts, the losses of amethocaine over the initial period were within the limits of error of the HPLC assay. In fact, losses of amethocaine could only confidently be observed after 24 h (Fig. 2). Therefore, it was necessary to follow the reaction by monitoring the increasing concentration of the metabolite, *p*-(butylamino)benzoic acid, with time. The data obtained for porcine and human skin (Table 1) showed a good fit to a simple linear model, enabling zero-order rate constants to be calculated from the slopes of the regression lines. Table 2 compares the observed losses of amethocaine following incubation with human whole skin homogenates with that predicted on the basis of the experimental data. Considering the variations in skin enzyme concentrations, and

TABLE 1

Rate constants for the metabolism of amethocaine by human and porcine skin homogenates

Sample no.	Rate constant (mol 10 ml^{-1} min^{-1})	Linearity	Standard error
Human whole skin			
1	7.647×10^{-10}	$r=0.994$	3.152×10^{-11}
2	7.183×10^{-10}	$r=0.979$	6.033×10^{-11}
3	5.729×10^{-10}	$r=0.995$	2.427×10^{-11}
Porcine whole skin			
1	2.854×10^{-9}	$r=0.990$	1.656×10^{-10}
2	3.334×10^{-9}	$r=0.993$	1.606×10^{-10}
Human epidermis			
1	5.900×10^{-10}	$r=0.991$	2.086×10^{-11}
2	4.453×10^{-10}	$r=0.997$	3.331×10^{-11}
3	4.723×10^{-10}	$r=0.946$	2.947×10^{-11}

the difficulties inherent in preparing the extracts, the agreement between the observed and calculated values is reasonable.

There was little difference, either qualitatively or quantitatively, between the results obtained with human and porcine skin extracts. However, human skin was notably more difficult to homogenise than porcine, and this may have reduced enzyme activity somewhat with respect to the human skin samples.

The location of enzyme systems within the skin has been widely studied (Finnen et al., 1985). Skin can be considered to consist of three layers. The outermost layer is the epidermis, the surface of which consists of dead, flat, keratinised cells forming the stratum corneum, the major barrier to drug penetration of the skin. The viable epidermis itself consists of a basal layer of actively dividing cells slowly progressing upwards towards the stratum corneum. The second layer, the dermis, lies below the epidermis and is a connective tissue containing many nerve fibres and blood vessels. The final layer is the hypodermis, an insulating layer of connective tissue containing adipose cells. The viable epidermis has been proposed (Montagna, 1955; Meyer and Neurand, 1976; Bickers et al., 1982) as the major source of cutaneous metabolism. The metabolic activity specifically associated with the epidermal layer of human skin was therefore investigated.

Removal of the dermis from human skin samples was achieved by dissection using a scalpel. The boundary between epidermis and dermis is visually clear. Attempts at cutting the skin with a microtome failed since fixative techniques could not be used due to the risk of inactivating the enzymes present. The epidermal layer left after dissection was approximately 250 μm thick. The pattern of amethocaine metabolism due to human epidermal extracts was both qualitatively (Fig. 5) and quantitatively (Table 1) similar to that obtained with whole skin. Given that the weights of skin and substrate used were the same in both cases, this suggests that the esterase activity in both layers is about equal. However, in practice any drug penetrating into the dermis is rapidly cleared via the dermal capillaries. It is therefore unlikely that penetration will take place beyond the upper 100–200 μm of the dermis. Considering that this layer varies in thickness between 2000 and 5000 μm it is unlikely that metabolism in the dermis is of any practical consequence (Martin et al., 1987).

It was noted that the initial samples taken from the incubation mixture (human whole skin homogenates) yielded a lower analysis for amethocaine than the true concentration. Thus, a recovery experiment was performed, for both amethocaine

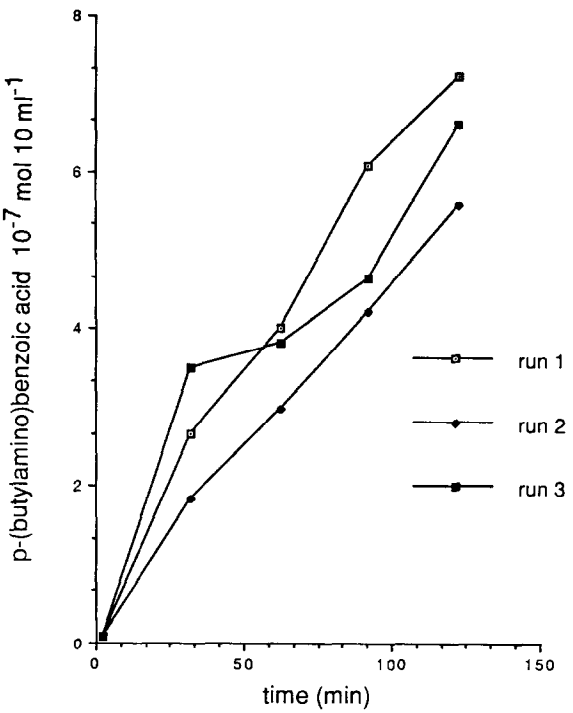

Fig. 5. Increasing concentration of *p*-(butylamino)benzoic acid with time during incubation of amethocaine with human epidermal skin extracts.

TABLE 2

Comparison between predicted and observed losses of amethocaine (AMC) during incubation with human skin homogenates

Sample no.	Time (min)	Observed loss of AMC (μg)	Predicted loss of AMC (μg)
1	2880	546	582
2	1440	309	274
3	1320	253	199

and 4-(butylamino)benzoic acid, the latter by spiking the initial incubation mixture. Results for amethocaine (5 replicate samples) indicated a mean recovery of 60.5% ($s = 4.5$), whereas there was no observable loss in the recovery of the metabolite from spiked samples. The loss of amethocaine is probably due to binding of the drug with proteinaceous material present in the skin extract, including non-esterase enzymes. Amethocaine is known to be strongly protein bound in plasma. This either causes loss of the drug in the guard column or, more likely, prevents detection via masking of the chromophore. However, the validity of measuring loss of drug relative to the initial recovered value is not affected, since all protein binding sites are initially saturated with the drug. Thereafter, loss of amethocaine must be due to metabolic activity.

The observed slow rates of amethocaine metabolism by all skin extracts may be explained by a low affinity of the substrate for the non-specific esterases present. Amethocaine is a comparatively bulky molecule compared to simple model esterase substrates such as *p*-nitrophenyl acetate, which was found to be rapidly hydrolysed by the skin extracts. Percutaneous amethocaine preparations are applied to the skin for just 30 min, and are then removed prior to commencement of the surgical procedure. Profound anaesthesia persists for several hours (Woolfson et al., 1988). Given the slow cutaneous metabolism of the drug, it is unlikely that there will be any demonstrable effect on the onset time for anaesthesia. However, the drug appears to have a comparatively long residence time in the epidermal layers, as demonstrated by the tendency to bind to skin proteins in addition to the persistent anaesthetic effect. It would therefore appear that a significant amount of the drug is eventually lost due to cutaneous metabolism. This may explain the lack of systemic toxicity via the percutaneous route when even large areas of skin have been treated for prolonged periods (Small et al., 1988).

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