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Percutaneous penetration characteristics of amethocaine through porcine and human skin

A.D. Woolfson, D.F. McCafferty and Karen E. McGowan

School of Pharmacy, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL (U.K.)

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Summary

Percutaneous amethocaine gels produce clinically effective local anaesthesia of intact skin. The present study establishes the percutaneous penetration characteristics of amethocaine from such gels through human stratum corneum, epidermis and whole skin barriers. Results are compared with neonate porcine stratum corneum, porcine whole skin and Silastic® as alternative model barrier membranes. Silastic® was shown to overestimate considerably the flux of amethocaine but neonate porcine skin proved to be a good model for the penetration of the drug through human skin. Differences in barrier membrane resistance to amethocaine penetration, and the effect on drug flux of varying the amethocaine concentration in the gel, were analysed separately by a one-way analysis of variance and the Newman-Keuls multiple range test. The main barrier to percutaneous penetration of amethocaine was the stratum corneum. The efficiency of the gel formulation in promoting the percutaneous penetration of amethocaine was reflected in the relatively high fluxes of the drug through both types of stratum corneum. The percutaneous penetration characteristics of amethocaine observed in this study, together with the pharmacological properties of the drug itself, explain the rapid onset and long duration of anaesthesia obtained clinically with the use of amethocaine percutaneous anaesthetic gel.

Introduction

Local anaesthesia of intact skin achieved by the topical application of a percutaneous anaesthetic preparation is of increasing interest in both paediatric practice, and in the area of minor surgical procedures performed on an out-patient basis (Coley, 1989; Woolfson and McCafferty, 1989). A percutaneous amethocaine anaesthetic gel developed by us for this purpose (Woolfson et al., 1988) has been shown to be safe and effective in both paediatric and adult clinical applications (Small et al., 1988; Woolfson et al., 1990a).

The main clinical characteristics of the amethocaine percutaneous anaesthetic preparation are its relatively rapid onset time (30–45 min) and the prolonged duration of anaesthesia produced following removal of the gel (in excess of 3 h). However, the distribution of the drug within the skin, and its overall percutaneous penetration characteristics, have not been established. In particular, no information on flux values through human skin has been reported. Such

Correspondence: A.D. Woolfson, School of Pharmacy, The Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast BT9 7BL, U.K.

values are of importance in predicting the maximum possible systemic absorption of amethocaine via the percutaneous route.

The aims of this investigation were therefore to establish the skin penetration characteristics of amethocaine with respect to (i) human whole skin, (ii) excised human stratum corneum and (iii) human epidermis. Such data are also compared with those obtained using neonate porcine skin and the model barrier membrane Silastic®. Thus, the degree of relevance of these latter two model membranes to human skin can be established in respect of amethocaine. The use of model barrier membranes rather than human skin is currently a topic of some controversy in percutaneous absorption studies (Barry, 1990).

A volunteer concentration-response study has previously been reported for amethocaine (Woolfson et al., 1988). A further objective of the present investigation was therefore to investigate the effect on amethocaine flux through the various barrier membranes of varying the applied drug concentration, and to relate these observations to the in vivo situation. The relevance of in vitro penetration studies, which are sometimes of limited value, can therefore be viewed in the context of a candidate preparation whose clinical properties are well established.

Materials and Methods

Percutaneous anaesthetic preparations

The candidate preparations were aqueous gels containing, respectively, 2, 4 or 6% m/v amethocaine base U.S.P. (Orgamol, Switzerland), and were prepared as previously described (Woolfson et al., 1988). A control preparation was an amethocaine-free gel base only.

Diffusion cell apparatus

Modified Franz diffusion cells (FDC-400, flat flange, 15 mm orifice diameter) were mounted in triplicate on an FDCD-3 diffusion cell drive console providing synchronous stirring at 600 rpm (Crown Glass Co., Somerville, NJ). Temperature maintenance was via water circulation (37°C) through the diffusion cell water jackets. Skin samples were mounted on stainless-steel filter supports (Millipore Corp., Cambridge, MA).

Determination of amethocaine

Amethocaine was determined by ion-pair reverse-phase high-performance liquid chromatography using the system described previously (Woolfson et al., 1990b). Calibration graphs for amethocaine were linear ($r > 0.998$) between 2.0 and 53.4 μg/ml. Chromatographic peak identification was by comparison of retention data with standards.

Preparation of porcine and human whole skin samples

Skin samples were human skin (leg, surplus from surgery) and neonate porcine skin (Faculty of Agriculture, The Queen's University of Belfast). Subcutaneous fat was carefully removed by dissection and the skin was cut into pieces of a suitable size for mounting in the diffusion cell. Prepared skin samples were stored frozen at −18°C until required.

Preparation of porcine and human stratum corneum

Skin samples were prepared as described and incubated at 37°C in trypsin type II: crude (Sigma Ltd, Poole, Dorset) solution (0.1% m/v in pH 7.2 phosphate-buffered saline, PBS, 20 ml). Stratum corneum sheets were obtained by carefully stretching the whole skin sample, stratum corneum side uppermost, and securing by pins to a cork board. The stratum corneum was carefully peeled off the epidermis, rinsed several times in water (reagent grade 1, Milli-Q system, Millipore Corp., Cambridge, MA) and then cut to the required size of the diffusion cell. Prepared stratum corneum samples were stored frozen at −18°C until required.

Preparation of human epidermis

Skin samples were prepared as described. Subcutaneous fat was carefully removed by dissection. The skin samples were placed in aluminium foil and heated for 2 min at 60 °C. The skin samples were secured, dermis uppermost, to a cork mat by pins. The dermis was then easily

removed by dissection as the dermo-epidermal barrier was visually apparent. The epidermal samples were cut to the required size and stored frozen at −18°C until required.

Silastic®

Silastic® sheets were of medical grade polydimethylsiloxane, thickness 0.0125 cm, and were obtained from Dow Corning, Reading, U.K. Each sheet was washed thoroughly with water and blotted dry with absorbent paper before use.

Preparation and experimental procedure for diffusion cell apparatus

The diffusion apparatus consisted of three water-jacketed diffusion cells maintained at 37°C. The cells, of the modified Franz type, were mounted in series on the drive console. The receiving chamber of each cell was filled with PBS (12 ml, pH 7.2) and allowed to equilibrate for 1 h. The skin component (stratum corneum uppermost) or Silastic® barrier, as appropriate, was placed on a filter support. A thin layer (approx. 1 mm) of the candidate gel was spread evenly, using a casting bar technique, across the barrier membrane. An occlusive covering (Parafilm®) was placed over the membrane-gel sandwich thus formed which was then secured between the flat flange of the receiving chamber and the flat flange of the cell top by a metal clamp. The receiving fluid was continuously and synchronously stirred in all three cells. Each experiment was performed simultaneously in triplicate. Barrier membranes for each triplicated run were cut from the same skin source or Silastic® sheet. Samples (0.3 ml) were withdrawn at intervals, replaced in each case with 0.3 ml PBS, and the amethocaine concentration determined.

Controls

Controls were performed by applying amethocaine-free gels to the various barrier membranes and sampling the receiver fluid over the course of the experiment. No chromatographic peaks were recorded when monitoring the eluent at 310 nm.

Treatment of results

Flux values (expressed throughout as $\mu g\ cm^{-2}\ h^{-1}$) were calculated by linear regression analysis as the slopes of the linear sections of the [concentration per unit membrane area] vs time plots. Concentrations per unit area were corrected for the sampling dilution. Each measurement was made in triplicate. Error bars shown are standard deviations. Where no error bar is shown on a plot the standard deviation was too small to be visually represented.

Results

The penetration characteristics of amethocaine through the various barrier membranes studied are shown in Figs 1–3, for 2, 4 and 6% m/v amethocaine percutaneous gels, respectively.

The corresponding flux values of amethocaine through the various barrier membranes are listed in Table 1 for 2, 4 and 6% m/v amethocaine gels, respectively. In general, as the amethocaine concentration increased, a corresponding increase in flux was observed. The greatest fluxes occurred with Silastic® as the barrier membrane, followed by stratum corneum samples. The lowest fluxes were seen with human and porcine whole skin samples.

Application of ANOVA (single factor analysis of variance with repeated measures) to the flux values of amethocaine obtained for each drug concentration through each of the barrier membranes demonstrated that significant differences ($P < 0.05$) existed between (i) concentrations and (ii) barrier performances. Therefore, the Newman-Keuls multiple range test was applied in order to obtain more detailed comparisons between (i) each of the different drug concentrations in the candidate formulations and (ii) each of the barriers. These detailed statistical comparisons are given in Tables 2–5. Amethocaine flux values from a 4% m/v gel, the standard preparation used in clinical studies (Woolfson et al., 1990a), were all significantly different ($P < 0.05$, Table 3), except for those fluxes measured through porcine and human whole skin. The

TABLE 1

Amethocaine fluxes from 2, 4 and 6% m / v gel formulations through various barrier membranes

Barrier membrane	Amethocaine flux ($\mu g\ cm^{-2}\ h^{-1}$)	S.D.
2% m/v amethocaine gel		
Porcine stratum corneum	13.45	1.08
Porcine whole skin	4.19	0.25
Human stratum corneum	39.58	0.22
Human whole skin	8.65	0.42
Silastic	101.60	0.78
4% m/v amethocaine gel		
Porcine stratum corneum	42.38	0.33
Porcine whole skin	13.76	1.11
Human stratum corneum	52.07	1.13
Human whole skin	15.27	0.95
Human epidermis	21.42	0.72
Silastic	107.87	2.31
6% m/v amethocaine gel		
Porcine stratum corneum	32.87	2.09
Porcine whole skin	18.46	0.52
Human stratum corneum	52.00	0.98
Human whole skin	12.14	1.34
Silastic	279.60	5.21

comparisons between amethocaine fluxes through the various barriers for 2 and 6% m/v gels (Tables 2 and 4, respectively) were all significantly different ($P < 0.05$).

The effect of amethocaine concentration is analysed in detail in Table 5. Each of the three formulation concentrations is compared for each barrier membrane in turn. Amethocaine fluxes through human stratum corneum from 4 and 6% m/v gel films were not significantly different

TABLE 2

Comparison of fluxes from 2% m / v amethocaine gel through various barrier membranes (Newman-Keuls multiple range test)

Comparison in terms of membrane barrier	Significance of difference
Silastic vs porcine whole skin	$P < 0.05$
Silastic vs human whole skin	$P < 0.05$
Silastic vs porcine stratum corneum	$P < 0.05$
Silastic vs human stratum corneum	$P < 0.05$
Porcine stratum corneum vs porcine whole skin	$P < 0.05$
Human stratum corneum vs porcine stratum corneum	$P < 0.05$
Human stratum corneum vs porcine whole skin	$P < 0.05$
Human whole skin vs porcine stratum corneum	$P < 0.05$
Human whole skin vs porcine whole skin	$P < 0.05$

TABLE 3

Comparison of fluxes from 4% m / v amethocaine gel through various barrier membranes (Newman-Keuls multiple range test)

Comparison in terms of barrier membrane	Significance of difference
Silastic vs porcine whole skin	$P < 0.05$
Silastic vs human whole skin	$P < 0.05$
Silastic vs porcine stratum corneum	$P < 0.05$
Silastic vs human stratum corneum	$P < 0.05$
Silastic vs human epidermis	$P < 0.05$
Porcine stratum corneum vs porcine whole skin	$P < 0.05$
Human stratum corneum vs porcine stratum corneum	$P < 0.05$
Human stratum corneum vs porcine whole skin	$P < 0.05$
Human stratum corneum vs human whole skin	$P < 0.05$
Human whole skin vs porcine stratum corneum	$P < 0.05$
Human whole skin vs porcine whole skin	$P > 0.05$
Human stratum corneum vs human epidermis	$P < 0.05$
Human whole skin vs human epidermis	$P < 0.05$
Porcine whole skin vs human epidermis	$P < 0.05$
Porcine stratum corneum vs human epidermis	$P < 0.05$

TABLE 4

Comparison of fluxes from 6% m / v amethocaine gel through various barrier membranes (Newman-Keuls multiple range test)

Comparison in terms of membrane barrier	Significance of difference
Silastic vs porcine whole skin	$P < 0.05$
Silastic vs human whole skin	$P < 0.05$
Silastic vs porcine stratum corneum	$P < 0.05$
Silastic vs human stratum corneum	$P < 0.05$
Porcine stratum corneum vs porcine whole skin	$P < 0.05$
Human stratum corneum vs porcine stratum corneum	$P < 0.05$
Human stratum corneum vs porcine whole skin	$P < 0.05$
Human whole skin vs porcine stratum corneum	$P < 0.05$
Human whole skin vs porcine whole skin	$P < 0.05$
Human stratum corneum vs human whole skin	$P < 0.05$

($P > 0.05$). Similarly, amethocaine fluxes through Silastic® from 2 and 4% m/v gels were not significantly different ($P > 0.05$). However, all other comparisons were observed to be significantly different ($P < 0.05$).

TABLE 5

Comparison of fluxes from 2, 4 and 6% m / v amethocaine gels through various barrier membranes (Newman-Keuls multiple range test)

Barrier membrane	Comparison in terms of amethocaine gel concentration (% m/v)	Significance of difference
Porcine stratum corneum	2 vs 4	$P < 0.05$
	2 vs 6	$P < 0.05$
	4 vs 6	$P < 0.05$
Porcine whole skin	2 vs 4	$P < 0.05$
	2 vs 6	$P < 0.05$
	4 vs 6	$P < 0.05$
Human whole skin	2 vs 4	$P < 0.05$
	2 vs 6	$P < 0.05$
	4 vs 6	$P < 0.05$
Human stratum corneum	2 vs 4	$P < 0.05$
	2 vs 6	$P < 0.05$
	4 vs 6	$P > 0.05$
Silastic®	2 vs 4	$P > 0.05$
	2 vs 6	$P < 0.05$
	4 vs 6	$P < 0.05$

Discussion

Silastic® (dimethylpolysiloxane) is an isotropic polymer which has frequently been used as a barrier membrane for in vitro percutaneous penetration studies. Silastic® acts as a simple lipid-like barrier with the diffusion characteristics of suitable penetrants being in accordance with Fick's Laws. The penetration of amethocaine from gel and cream formulations using a flow-through diffusion cell with a large diffusional area has previously been reported (McCafferty et al., 1988).

In this study the fluxes through a thin Silastic® membrane of amethocaine from 2, 4 and 6% m/v amethocaine gels have been determined. Diffusion of amethocaine through the barrier into the receiving fluid was found to follow apparent steady-state kinetics. The highest concentration amethocaine gel gave a flux substantially in excess of that obtained with lower drug loadings, and was significantly different from them. The two lower drug-loaded films had similar fluxes which were not significantly different. This was probably due to inadequate mixing in laboratory manufacture of these two formulations, and was not repeated in later studies using skin or skin components as the barrier. Reproducibility within each set of triplicate experimental runs was satisfactory with standard deviations in the region of 1–1.5%. Flux values, however, were large when compared to those obtained when skin or skin components constituted the barrier (Table 1). Typically, Silastic® yielded amethocaine fluxes about 10–20-times in excess of those for skin. This indicates that, while Silastic® may be suitable for comparative studies during formulation development, the absolute fluxes obtained with this model membrane have no significance for the in vivo situation.

Amethocaine, due primarily to its lipophilicity, penetrates Silastic® with ease. Skin, of course, is a more complex structure. The stratum corneum, for instance, consists of keratinised cellular layers held together by multilaminar lipid sheets. These

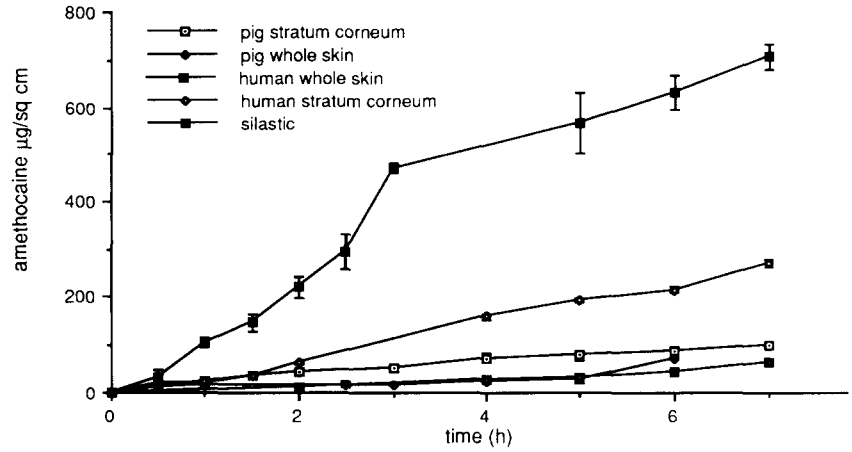

Fig. 1. Comparison of amethocaine penetration through various barrier membranes (2% m/v amethocaine percutaneous anaesthetic gel).

lipid sheets have both hydrophobic and hydrophilic regions with the complete structure forming an effective barrier to the passage of both water and exogenous chemicals. It is therefore not surprising that the absolute flux values through a synthetic membrane bear little relation to those through skin, although the relative values may still be of some interest.

For studies using skin or skin component layers as the barrier membrane, only tissue samples which were large enough to provide three membranes for an individual study in triplicate were used. Again, three amethocaine gel formulations containing 2, 4 and 6% m/v of anaesthetic were used. Generally, as the concentration of amethocaine increased, the flux through each barrier membrane also increased. Given the nature of the formulation, this is unlikely to be due to the conventional increase in concentration gradient resulting in an increased thermodynamic driving force for diffusion. Since these formulations are not suspensions but, in effect, viscous aqueous suspensions of an oily drug, the higher drug concentration results in a greater surface area of

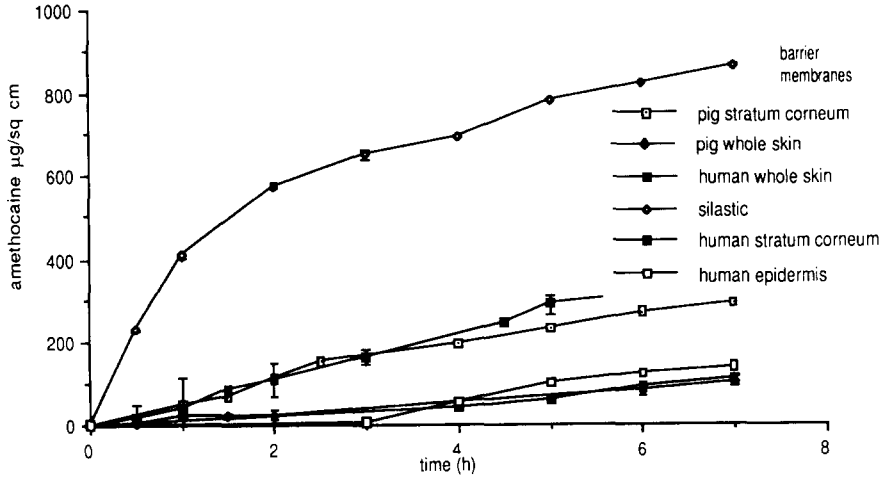

Fig. 2. Comparison of amethocaine penetration through various barrier membranes (4% m/v amethocaine percutaneous anaesthetic gel).

drug being in contact with the barrier, and hence an increased flux. Thus, increasing amethocaine concentration from 2 to 4% m/v in the gel produced a corresponding increase in flux through human stratum corneum from 39.58 to 52.07 μg cm^{-2} h^{-1} (Table 1). However, a further increase to 6% m/v amethocaine in the gel did not produce such a large increase in flux, suggesting that surface area packing of the drug is nearing a maximum and that the penetration process is, in effect, saturable. It should also be borne in mind that tissue variation will affect flux values inter-experiment, although intra-experiment values were obtained using single-source membranes. This is inevitable given the size of skin samples obtainable, particularly of human skin, and the amount required for an extensive study.

Since porcine skin is more readily obtainable than human skin, it was of interest to repeat measurements obtained using human whole skin and stratum corneum. Porcine skin samples used in this study were from neonatal pigs which were either stillborn or died shortly after birth, in the latter case as a result of accidental crushing by the mother. Both human and porcine samples were treated identically after harvesting. Amethocaine fluxes from the various formulations through both porcine whole skin and stratum corneum were much closer to the values obtained with human skin than was observed in the case of Silastic®. Although the differences in fluxes between equivalent human and porcine skin barriers were significant ($P < 0.05$) as determined by a multiple range test (Tables 2–4), the orders of magnitude were similar in each case. Comparison of the effect of drug concentration on flux followed essentially the same pattern between human and porcine barriers (Table 5). Thus, amethocaine fluxes from a standard 4% m/v amethocaine gel were 13.76 μg cm^{-2} h^{-1} (porcine whole skin) and 15.27 μg cm^{-2} h^{-1} (human whole skin). For stratum corneum the flux values were 42.38 μg cm^{-2} h^{-1} (porcine) and 52.07 μg cm^{-2} h^{-1} (human). These results suggest that studies using porcine skin are a good model with respect to the percutaneous penetration of amethocaine.

A further barrier membrane investigated was human epidermis, i.e. the outermost non-vascular region of the skin which includes the stratum corneum as the external layer. The epidermis is approx. 200 μm in thickness. Beneath the epidermis, the dermis is some 10–20-times thicker and possesses a rich blood supply. The hair follicles and various glands such as the apocrine glands all originate in the dermis. Epidermal samples were prepared by heating the whole skin at 60°C for 2

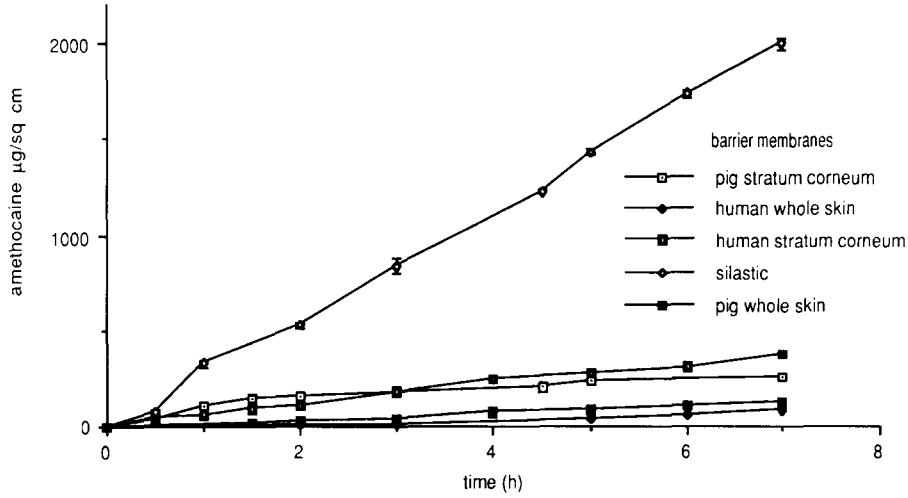

Fig. 3. Comparison of amethocaine penetration through various barrier membranes (6% m/v amethocaine percutaneous anaesthetic gel).

min, followed by careful dissection to remove the dermis. For this study only the standard 4% m/v amethocaine gel was used due to difficulty in obtaining further skin samples. The epidermal flux value for amethocaine through human epidermis (21.42 μg cm^{-2} h^{-1}) was found to be less than for human stratum corneum and greater than that for human whole skin, as might be expected. Comparing the fluxes through the various skin layers, it is clear that, although allowing the largest flux of amethocaine, the stratum corneum provides the main barrier to penetration of exogenous chemicals, given that it is only some 15 μm thick. The extended passage through the much thicker epidermis and dermis affords substantial opportunity for loss of drug due to biotransformation and/or protein binding (Woolfson et al., 1990b). Hence, fluxes through the epidermis and dermis are lower but this reduction in amethocaine flux does not reflect the comparative thicknesses of these layers compared to stratum corneum, indicating that the epidermis (less stratum corneum) and dermis do not constitute a substantial barrier to amethocaine penetration. Since the target nociceptors for the drug lie at the dermo-epidermal junction (Iggo, 1982), relatively close to the skin surface, the penetration characteristics of the drug established in this study correlate well with the clinical efficacy of an amethocaine percutaneous anaesthetic gel, and with the previously established in vivo concentration-response profile (Woolfson et al., 1988). In particular, the use of a 4% m/v gel for all clinical applications, a practice based on volunteer trials and clinical experience, is confirmed by this study.

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