JPP Journal of Pharmacy and Pharmacology

JPP 2009, 61: 159–165

Received August 20, 2008
Accepted October 27, 2008
DOI 10.1211/jpp/61.02.0004
ISSN 0022-3573

Research Paper

# Metabolism of captopril carboxyl ester derivatives for percutaneous absorption

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## Abstract

**Objectives** To determine the metabolism of captopril *n*-carboxyl derivatives and how this may impact on their use as transdermal prodrugs. The pharmacological activity of the ester derivatives was also characterised in order to compare the angiotensin converting enzyme inhibitory potency of the derivatives compared with the parent drug, captopril.

**Methods** The metabolism rates of the ester derivatives were determined *in vitro* (using porcine liver esterase and porcine ear skin) and *in silico* (using molecular modelling to investigate the potential to predict metabolism).

**Key findings** Relatively slow pseudo first-order metabolism of the prodrugs was observed, with the ethyl ester displaying the highest rate of metabolism. A strong relationship was established between in-vitro methods, while in-silico methods support the use of in-vitro methods and highlight the potential of in-silico techniques to predict metabolism. All the prodrugs behaved as angiotensin converting enzyme inhibitors, with the methyl ester displaying optimum inhibition.

**Conclusions** In-vitro porcine liver esterase metabolism rates inform in-vitro skin rates well, and in-silico interaction energies relate well to both. Thus, in-silico methods may be developed that include interaction energies to predict metabolism rates.

**Keywords** AutoDock; captopril; metabolism; percutaneous absorption; transdermal drug delivery

## Introduction

The prodrug strategy has been widely employed to increase and optimise the adsorption, distribution, metabolism and excretion of many drugs, whereby the active compound is released as a metabolite following a biological reaction. This strategy is well established and has been developed to enable targeted drug delivery for oral therapeutics. Recently, this approach has also been applied to the transdermal drug delivery of, for example, ketorolac,[1] temozolomide,[2] 5-fluorouracil,[3] naltrexone[4] and morphine.[5] Captopril is well established as an oral therapeutic that could benefit from transdermal drug delivery via the prodrug strategy. Although it is recognised as the oldest of the angiotensin converting enzyme (ACE) inhibitors and has since been superseded by oral ACE inhibitor prodrugs such as enalapril, there is current clinical interest in transdermal antihypertensive medications not only for patient compliance benefits, but also for situations where oral administration would be unsuitable, particularly during patient intubation, as an adjunctive therapeutic. Currently, the only transdermal antihypertensive is clonidine (Catapres-TTS), which is marketed in the US and has been recognised as a useful therapeutic for long-term management of hypertension.[6] This is not available in the UK and there is no other comparable transdermal antihypertensive currently available on the UK market.

As reported previously, a series of captopril ester derivatives were produced via a quantitative structure–permeability approach, and were shown to have enhanced skin penetration when compared with the parent drug.[7] While it is accepted that a minor side-effect from transdermal preparations of captopril prodrugs could be the characteristic 'captopril cough', such prodrugs would address the present clinical need for a cost-effective transdermal antihypertensive.

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Although no metabolism was observed in this study, skin is known to be a metabolically active organ that contains cytochrome P450 (CYP) and other non-specific esterases,[8,9] and the metabolic reversion of the ester derivatives to captopril facilitated by these enzymes is an essential aspect of the proposed mechanism of drug delivery. Concurrent skin absorption and metabolism of various other ester prodrugs has been reported by other investigators,[10,11] whereby the drug molecules interact with the skin enzymes as permeation occurs, and under conditions of passive diffusion, the timescales of interaction are sufficient for bioconversion to occur. By contrast, Ngawhirunpat *et al*.[12] evaluated the simultaneous permeation and metabolism of methyl nicotinate in human, snake and shed snake skin, the latter from three species. Total flux was only significantly higher for one species of snake skin (*Naja kaouthia*). A significant difference was observed in skin esterase activity across species and the authors concluded that discrepancies observed in transdermal permeation profiles could be attributed to the difference in esterase activity in the skin. While this study may suggest that species variation is significant, Jewell *et al*.[13] investigated the use of skin from the ears and back of minipigs and found similar levels of esterase activity to that found in human breast skin used in in-vitro studies. They suggested that porcine skin is a good model for human skin in investigating the metabolism and pharmacokinetics of ester prodrugs. The findings of this work are supported by other studies such as Abdulmajed *et al*.[14] and Suppasansatorn *et al*.[15] Further, Prusakiewicz *et al*.[16] examined a range of skin from different species. They found that metabolism and kinetics of hydrolysis in the minipig was also comparable with human skin, whereas metabolic activity in the rat was significantly higher by two or three orders of magnitude. Such lack of correlation across species was further re-enforced by Henrikus and Kampffmeyer[17] when investigating hydrolysis of topically applied chemicals in the rabbit. They found little correlation between different types of experiment, including the use of intact skin, skin homogenates and the use of liver esterase. They concluded that the results from isolated rabbit ear perfusion differ quantitatively and qualitatively to those obtained from supernatant of skin homogenate or purified liver esterase.

The aim of this study was to perform a series of experiments using three distinctly different in-vitro techniques in order to gain a better understanding of the metabolism of captopril *n*-carboxyl derivatives, and how this may impact on their use as transdermal prodrugs. In addition, the pharmacological activity of the ester derivatives was characterised in order to compare the ACE inhibitory potency of the derivatives compared with the parent drug, captopril.

## Materials and Methods

### Materials

Captopril was a gift from Bristol-Myers-Squibb, New York, US. Alkyl esters were synthesised as described previously.[7] Characterisation was by gas chromatography–mass spectrometry (GC-MS) liquid chromatography–mass spectrometry (LC-MS), infrared analysis, nuclear magnetic resonance, optical rotation and Raman spectroscopy. Methyl, ethyl, propyl and butyl esters were used for porcine liver esterase (PLE) and in-vitro skin metabolism. PLE and acetylcholinesterase were both acquired from Sigma-Aldrich, St Louis, MO, US. Skin metabolism studies involved using homogenised porcine ear skin as the source of natural non-specific esterases. An Ultra Turrax homogeniser (IKA, Staufen, Germany) was used to macerate and homogenise tissue. Chemical analysis was carried out using LC-MS (Model 1100MSD; Agilent Technologies Ltd, Santa Clara, CA, US). In-silico experiments involved use of a silicon graphics workstation (SGI Indigo 2) with Unix operating system. Insight II (version 4) package (BioSym Technologies, San Diego, CA, US) was used to construct prodrug structures. These included *n*-methyl to *n*-hexyl straight-chain esters. The Research Collaboratory for Structural Bioinformatics Protein Data Bank (http://www.rcsb.org/pdb) was used to download structures of CYP2C9 (code 1og5), RNase (code 2hky), and acetylcholinesterase (code 1b41).

All pharmacological studies were approved by the University of Brighton Ethics Review Committee in accordance with the UK Animals (Scientific Procedures) Act, 1986. Pharmacological studies involved the use of angiotensin I and angiotensin II, both purchased from Tocris Ltd, Bristol, UK. Chemicals required for De Jalon's Ringer solution (sodium chloride, D (+) glucose, potassium chloride, sodium hydrogen carbonate, and calcium chloride) were purchased from Sigma-Aldrich. Contractile tissue (uterus) was dissected from mice maintained under controlled conditions (23 ± 1°C, 12-h light–dark cycle, with food and water provided *ad libitum*) following $CO_2$ asphyxiation.

### Metabolism by porcine liver esterase

Each captopril derivative (10 mg) was dissolved in phosphate buffered saline (PBS, pH 7.4, 10 ml) and equilibrated with stirring in a water bath at 37°C for 45 min. Concentrations of derivatives were well below their saturation point in PBS (Table 1), and were assayed using the British Pharmacopoeal potentiometric titration method.[18]

To initiate the experiment, PLE (50 units, 28 $\mu$l of a suspension in 0.05 M ammonium sulfate) was added, at which point a stop-clock was started. Samples (200 $\mu$l) were taken immediately and then at 20, 40, 60, 80, 100, 120, 180 and 320 min, and also at 16 and 24 h, while the remaining solution was maintained at 37°C in the water bath. Samples were immediately shaken with 300 $\mu$l acetone, to raise the dielectric point and precipitate the enzyme, preventing any further metabolism within the sample. Four replicate samples were prepared for each prodrug, and a blank sample was prepared as above but replacing the PLE with an equal

**Table 1** Aqueous solubility of captopril and ester derivatives

| | Methyl ester | Ethyl ester | Propyl ester | Butyl ester |
|---|---|---|---|---|
| Saturated solubility (mM) | 38.7 | 35.6 | 18.3 | 18.0 |
| Concentration (mM) | 4.32 | 4.07 | 3.86 | 3.66 |

Aqueous solubility at room temperature.

volume of PBS to investigate the spontaneous (non-enzymatic) hydrolysis of the ester prodrugs under the same conditions. The samples were passed through 0.2 $\mu$m Teflon filters to prepare them for analysis by high-performance liquid chromatography (HPLC) using an Agilent 1100MSD system (Agilent Technologies Ltd) with diode array detection set at 220 nm for captopril (as per the British Pharmacopoeal method for HPLC of captopril) and 200 nm for the prodrugs corresponding to previous $\lambda_{max}$ determinations.[7] An ODS (octadecylsilyl, $C_{18}$, Hichrom H5ODS) column was used (125 mm × 4.5 mm) with an ODS guard column. The mobile phase consisted of water : methanol (50 : 50), and the flow rate was set at 0.5 ml/min. A sample size of 50 $\mu$l was used for all assays. External calibration was used to produce standard curves for each prodrug. Each standard curve displayed good linearity ($r^2 > 0.99$) over the concentration range used in the assays. Concentrations of unconverted derivative (ester) were therefore measured over time and plots of log concentration–time were constructed to derive metabolism rates.

## Skin metabolism method

Rates of bioconversion were determined as described previously[19] using skin homogenates as the source of non-specific metabolic enzymes. However, due to the differences in the solubility of the captopril esters compared with those described previously, it was necessary to make slight alterations to experimental conditions. Hence, rather than crushing skin under liquid nitrogen, skin metabolism experiments were performed by taking finely sliced porcine ear skin (3 g, $n = 4$) and homogenising it (three bursts of 12 s) in Dulbecco's phosphate-buffered saline (30 ml) over ice using an Ultra Turrax homogeniser. The homogenised mixture was centrifuged at 906*g* for 20 min, and the supernatant decanted. Metabolism mixtures were then prepared by adding prodrug (600 $\mu$M, 1 ml) to supernatant (5 ml) that had been stirred at 37°C for 30 min. Samples were taken and analysed by HPLC as described above, but using a mobile phase of 50 : 50 ion pair reagent (*tert*-butylammonium chloride, 2 mM) and methanol.

## In-silico methods

### *Preparation of ligand structures*

Methyl to hexyl *n*-esters were built using the fragment database in Insight II version 4.0 (Accelrys Incorporated, San Diego CA, US). Partial charges were assigned using the Gasteiger method.[20] Geometries were optimised using the consistent valence force field function (part of Discover software) within the Insight II version 4.0 program. Ligands were saved as files in the '.mol2' format.

### *Preparation of macro (receptor) molecules*

CYP2C9, RNase and acetylcholinesterase were downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank. Oxygen atoms were discovered within the downloaded molecule as part of residual water molecules remaining from experimental X-ray crystallography studies. These were removed using Insight II version 4.0, and the structure was checked for any missing polar hydrogen atoms, which were subsequently added manually by editing the '.pdb' file. Charges were added using the Gasteiger method and the proteins (macros) were saved as files in the '.mol2' format.

### *Grid creation*

Autogrid, a subprogram of AutoDock, was employed to compute a cube in space within which the protein would fit. Grid spacing and number of grid points were defined for each protein as part of each experiment. Grid parameters used were: 125 × 125 × 125 grid points, with 0.4Å grid spacing and 100 GA (genetic algorithm) runs for CYP2C9; 120 × 120 × 120 grid points, with 0.6Å grid spacing and 100 GA runs for RNase; and 100 × 100 × 100 grid points, with 0.6Å grid spacing and 250 GA runs for acetylcholinesterase.

### *In-silico docking protocol*

The AutoDock (version 3.0.5) program was used to perform automated molecular docking experiments. All investigations were performed with no energy associated with the ligands (i.e. held rigid). Unix commands (given in the AutoDock user manual) were input to: (1) convert the ligand and macro files from '.mol2' to '.pdbq' and '.pdbqs' formats, respectively; (2) to create the grid parameter file containing the appropriate grid size and points spacing; (3) to create the docking parameter file with the appropriate amount of GA runs; (4) to create the grid; and (5) to perform the dock.

### *In-silico analysis protocol*

The 100 lowest docked energy structures (kcal/mol) according to their force field scoring functions were output from the AutoDock program in clusters. Clusters were formed using a 5Å root mean squared cut-off for all non-hydrogen atoms, and were presented in histogram format. Lowest docking energy conformations were investigated by visualisation using Insight II version 4.0. The five lowest energy conformations of captopril and each ester with each protein were interrogated to determine docking sites and the neighbouring residues.

## Pharmacological ACE inhibition activity of derivatives

Freshly excised mouse uterus was dissected and immersed in organ baths (10 ml) that were primed with fresh de Jalon's physiological media solution at 34°C with aeration provided by carbogen (95% carbon dioxide/5% oxygen) and under a passive tension of 0.5 g (recorded by calibrated transducers connected to Chart version 5 software). Contractile responses were recorded from the addition of a series of angiotensin I doses to produce a dose–response curve. From this, the dose at 70% response was determined to be $1 \times 10^{-7}$ M (in-bath) angiotensin I. This dose was used as the standard dose for inhibiting series of captopril and captopril ester ($C_1$ to $C_4$ only) doses, whereby the ACE inhibiting species (either captopril or ester) was added to the organ bath 1 min before addition of angiotensin I standard dose, and contractile responses were recorded, yielding dose–response inhibition curves for each species. Four replicates were carried out for each ACE inhibiting species.

## Statistical analysis

All experiments were conducted four times. For the metabolism by PLE and skin metabolism, plots of log

concentration versus time were constructed to derive metabolism rates. A comparison of the slopes and intercepts of the linear regression lines were performed using the method described by Zar[21] (equivalent to analysis of covariance) using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, US). $P < 0.05$ was considered statistically significant in all comparisons. When the slopes were considered to be statistically significant no further analysis was conducted. The magnitude of the slopes and therefore the metabolism rate are displayed in the appropriate figure along with 95% confidence limits.

## Results

### Porcine liver esterase and skin metabolism results

The results of the PLE and skin metabolism of captopril ester derivatives are shown in Figure 1. In both cases, the slopes of the regression lines and therefore the metabolism rates were shown to be significantly different ($P < 0.05$). The magnitude of the metabolism rate and the 95% confidence limits are also shown in Figure 1. In both cases, the ethyl ester metabolism rate was shown to be significantly higher. The half-lives of the ester derivatives were determined to be between 10 and 12 h.

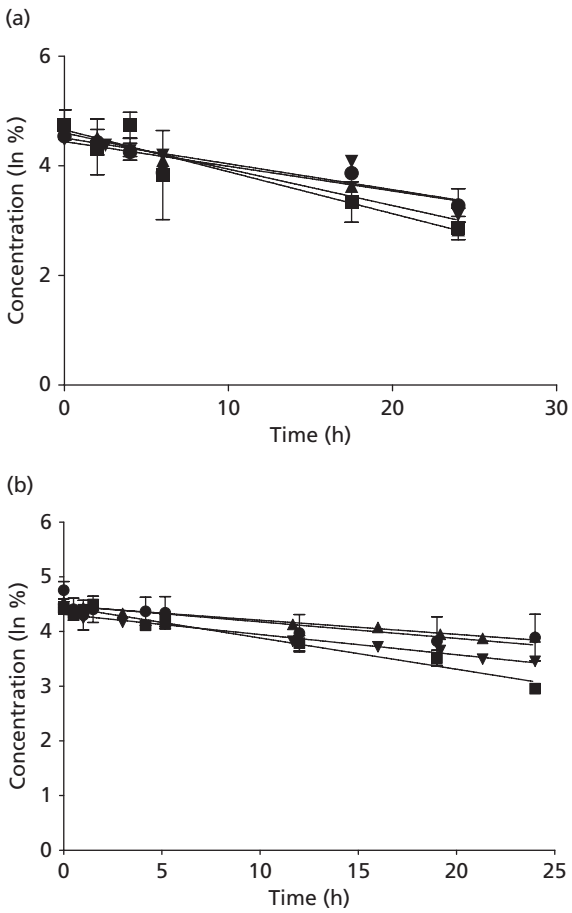



**Figure 1** In-vitro porcine liver esterase and skin metabolism rate results. Concentration of captopril esters decreased over time when incubated with porcine liver esterase (a) or skin (b) at 37°C ($n$ = 4). Where (●) Methyl, (■) Ethyl, (▲) Propyl and (▼) Butyl.

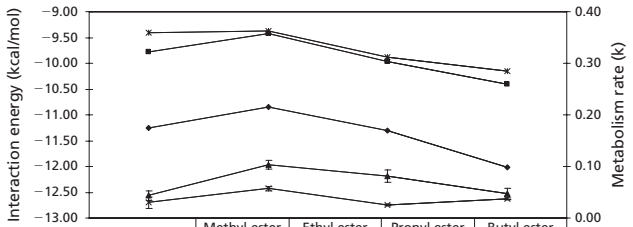
### In-silico metabolism results

Figure 2 shows the visualisation of captopril and ester ligands docked at the position of lowest energy on CYP2C9. This is an indication of the active site 'pocket' on the protein. The space-fill atoms belong to the CYP2C9 protein and the conformation of captopril and the derivative ligands are circled. Similar visualisations were prepared between each of the ligands docked with RNase and acetylcholinesterase in order to verify the location of the docking sites and to confirm similar (overlaying) docking conformations. Figure 3 shows a comparison of the results from the PLE, skin metabolism and in-silico studies, where binding energy is seen to increase with increasing metabolism rate. The relationship between in-silico and in-vitro results (using $r^2$ values) is shown in Table 2.

### Pharmacological characterisation

ACE inhibition curves were produced by plotting the contractile responses of mouse uterus (reduced, due to inhibition of ACE by captopril and the derivatives) against the doses of each inhibitor (Figure 4). The concentration of angiotensin I was kept constant at $2 \times 10^{-7}$ M, which was the concentration determined to elicit 70% of the total contractile response. Non-linear regression analysis (GraphPad Prism version 5) was used to produce lines of best fit for each ACE inhibitor. The concentrations (M) at 50% of total inhibition (IC50 values) were also determined as a comparable measure of inhibition. The IC50 values were: captopril 0.0001876;

**Figure 2** Space-fill image of cytochrome P450 2C9 with captopril and derivatives (circled) overlaid at conformation of lowest mean docked energy ($n$ = 100).




| | Methyl ester | Ethyl ester | Propyl ester | Butyl ester |
|---|---|---|---|---|
| CYP2C9 in-silico energy | −9.78 | −9.42 | −9.97 | −10.41 |
| RNase in-silico energy | −11.25 | −10.85 | −11.31 | −12.01 |
| AChE in-silico energy | −9.41 | −9.38 | −9.88 | −10.15 |
| PLE in-vitro rate | 0.04351 | 0.1028 | 0.08171 | 0.04669 |
| Skin in-vitro rate | 0.03018 | 0.0571 | 0.02587 | 0.03692 |

**Figure 3** Comparison of in-silico interaction energies and porcine liver esterase and skin metabolism rates. CYP2C9, cytochrome P450 2C9; AChE, acetylcholinesterase; PLE, porcine liver esterase. Where (■) CYP2C9; (◆) RNase; (∗) AChE; (▲) PLE; (×) Skin.

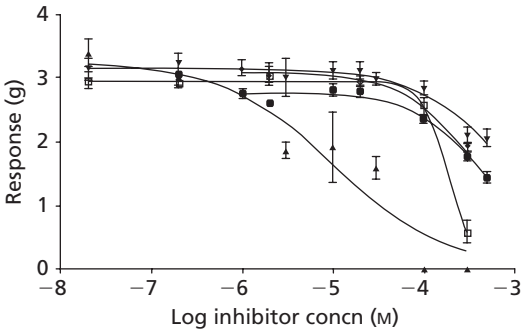



**Figure 4** Competitive angiotensin converting enzyme inhibition by captopril and esters. Where (□) Captopril, (▲) Methyl, (▼) Ethyl, (■) Propyl and (■) Butyl. Data are mean values ± SD ($n$ = 4).

**Table 2** Relationship between in-silico and in-vitro results

| | CYP2C9 in-silico energy | AChE in-silico energy | RNase in-silico energy |
|---|---|---|---|
| PLE in-vitro rate | 0.9309 | 0.6447 | 0.9712 |
| Skin in-vitro rate | 0.8525 | 0.5692 | 0.8152 |

Results are $r^2$ values between in-silico interaction energies and metabolism rates. CYP2C9, cytochrome P450 2C9; AChE, acetylcholinesterase; PLE, porcine liver esterase.

methyl ester $1.018 \times 10^{-5}$; ethyl ester 0.0008631; propyl ester 0.0004255; and butyl ester 0.0005126.

The results show that the lowest concentration needed to inhibit ACE was given by captopril methyl ester. This IC50 value was 10-times less than that of captopril, suggesting that the methyl ester could be a more potent ACE inhibitor than the parent drug. The other esters in the series displayed inhibition values suggesting that the ethyl, propyl and butyl esters of captopril were approximately 8-, 4-, and 5-times less potent than the parent drug, respectively.

## Discussion

Other investigators that have used skin homogenates to investigate the hydrolysis of esters by skin esterases include Doh *et al.*[22] who studied prodrugs of ketorolac, Seki *et al.*[23] who researched prodrugs of zidovuline, and Wang *et al.*[5] who reported the improved transdermal delivery of morphine esters. However, to the best of our knowledge, no other studies have been performed that have drawn comparisons between metabolism rates and in-silico interaction energies between enzymes and prodrugs. In this case, AutoDock was chosen as suitable software to calculate the interaction between any given ligand and macromolecule, as has been demonstrated in the literature,[24] which demonstrates that the docking process can locate a valid active site on the enzymes. Captopril ester prodrugs were used because these had previously been synthesised and had displayed remarkable stability to hydrolysis. Selection of the correct esterases to interact with the prodrugs *in silico* was challenging due to the volume of non-specific esterases that are present in the literature, but CYP had been isolated as a prominent type of esterase.[25] Other enzymes were selected because they are well known to reside in plasma (acetylcholinesterase) or generally in cells (RNase). Previous diffusion studies have led us to believe that contact between the prodrug and an esterase could occur not only in the epidermis but also deeper in the skin and perhaps in the bloodstream. Acetylcholinesterase was chosen as a model enzyme for interaction because it is well known as a component part in plasma. However, the focus of the predictive part of this study was on skin metabolism, not metabolism in plasma, and as such the correlations between acetylcholinesterase in-silico values were omitted at that point. RNase was also chosen for in-silico studies because extracellular RNase is expected to be excreted from squamous cells during differentiation within the stratum corneum, and so would also be abundant for contact/interaction with the ester prodrugs in the uppermost levels of the skin.

Any in-vitro method aims to replicate the in-vivo situation, but this is often difficult to achieve while retaining the level of control needed to isolate a response experimentally. The first in-vitro method reported here used PLE to mimic the esterases that may be found in human skin. PLE may at first seem too dissimilar from human skin, but it is known that it contains the same esterases as are present in human liver, and these are also found (to a reduced extent) in human skin.[8] Other investigators have also used PLE during in-vitro metabolism studies.[26–28] However, the type and amount of non-specific esterases is not well defined, which presents a limitation to this method. Also, it should be remembered that no pharmacodynamic information can be obtained from these experiments, only comparable rates of metabolism.

The use of homogenised full-thickness skin is perhaps more akin to the in-vivo situation, and as such it may be argued that determination of enzyme concentration and type is not strictly necessary since this information would not be available *in vivo*, and enzyme localisation may vary depending on skin thickness.[29,30] Porcine skin has often been identified as a good substitute for human skin[31] for use in transdermal diffusion studies, and is known to contain CYP and other non-specific esterases.[8,9] There are also no

regulatory issues pertaining to the use of porcine tissue, therefore it was the most convenient and applicable tissue to use during these investigations. However, the in-vitro skin method assumes that enzymes of the correct type are present in sufficient quantities to have a measurable effect and remain active after extraction.

Pharmacological experiments were performed to characterise the activity of the prodrugs relative to captopril using a well established technique measuring the response of live mouse uterus in an organ bath. Mouse uterus was chosen because it is known to contain epithelial ACE,[32] and is convenient to use. However, it is acknowledged that other tissues containing ACE (such as guinea-pig ileum) could also have been used, although these would require extra preparation. Mouse uterus is known to produce a contractile response when stimulated by angiotensin II, and, by extension, the conversion of angiotensin I to angiotensin II also produces a contractile response. The ability of the prodrugs to inhibit the conversion of angiotensin I to angiotensin II was measured, whereby an observed reduction in contractions (as a percentage of the optimal contraction induced by angiotensin I) can be taken as a measure of the pharmacological activity of the prodrugs relative to captopril.

In a general sense, consideration of skin metabolism is important due to the potential effects it may exert on the flux, and consequent efficacy, of topically applied therapeutic agents. As discussed earlier, Ngawhirunpat *et al.*[12] considered that cutaneous metabolism had a significant effect on the consistency of drug delivery. The inference from this and the study by Moss *et al.*[7] is that, hypothetically, an increase in metabolic activity may result in the maintenance of a high concentration gradient across the skin. This, in turn, should ensure the maintenance of a high flux across the skin. However, Moss *et al.*[7] used LC-MS to analyse the percutaneous absorption of captopril esters across porcine skin. They found no such reversion to the parent drug and were only able to detect the esters in the receptor phase. This is an interesting result as they found that the ethyl ester had the highest flux of the captopril esters investigated.[7] However, in the absence of any reversion of the prodrugs to the parent drug, it should be concluded that such enhancements are due solely to the physicochemical nature of the prodrugs and not to potential metabolic activity. Therefore, although theoretically an interesting concept, this study would indicate that, in practice, the use of metabolic activity, or responses, to increase percutaneous absorption is an area that requires substantially more research before such conclusions can be drawn.

## Conclusions

PLE studies showed that the ethyl ester was the most rapidly hydrolysed and this was confirmed by in-vitro skin metabolism studies. A similar trend was observed with the in-silico results, suggesting that there may be relationships between ligand–macro interaction energies and rates of hydrolysis, which require further investigation. Pharmacology studies indicate that the methyl ester was the best ACE inhibitor, exhibiting 10-times greater activity than captopril, which appears to be due to the physicochemical properties of that prodrug and how it binds to the receptors. Correlations show that in-vitro PLE metabolism rates inform in-vitro skin rates well, and in-silico interaction energies relate well to both (in particular the CYP2C9 values), so the main implication of this work is that in-silico methods may be developed that include interaction energies to predict metabolism rates. It should be noted that this study was limited to captopril esters only.

### Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

### Funding

Darren Gullick acknowledges funding from the University of Portsmouth (Portsmouth, UK).

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