Determination of the Dermal Penetration of Esterom Components Using Microdialysis Sampling

Sarah McDonald[1] and Craig Lunte[1,2]

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Purpose. Esterom® Solution, an investigational pharmaceutical product, is derived from the esterification of benzoylmethylecgonine (cocaine) in 1,2 propanediol. The resulting solution contains a mixture of components. Esterom Solution is intended to be a topical analgesic to relieve pain and increase the range of motion in patients suffering from acute inflammation of the shoulder or back. Although the components of Esterom are known, the components that are responsible for analgesia have only recently been identified. The purpose of this research is to evaluate which components have the ability to penetrate the skin, how much actually penetrates, and if and/or how each component is metabolized and distributed locally.

Methods. Linear microdialysis probes were implanted into rat dermis. The individual components present in the Esterom Solution were applied separately to the dermis directly over a probe. Dermal dialysis samples were collected to evaluate the dermal penetration of each compound following topical application.

Results. Following a 10 mg/50 μL application, 1.8 ± 0.6 mM benzoic acid was detected at the plateau after approximately 220 min. Following hydroxypropyl benzoic acid application, complete hydrolysis to benzoic acid was observed with a plateau concentration of 137 ± 19 μM (150 min plateau). When applied separately, hydroxypropyl benzoylecgonine and ecgonine penetrate the skin with plateau concentrations of 32 ± 9 μM (15 h plateau) and 36 ± 5 μM (150 min plateau) respectively. Benzoylecgonine, the hydrolytic product of HP-BE, was also detected with a plateau concentration of 3.9 ± 0.1 μM (16 h plateau) Applied topically, ecgonidine, methylecgonidine, benzoylecgonine, and hydroxypropyl ecgonidine were not detected.

Conclusions. Of the components with analgesic activity, the only compound that penetrates the skin is hydroxypropyl benzoylecgonine. Dermal microdialysis was shown to be an effective technique to monitor the skin penetration of topically applied compounds.

KEY WORDS: microdialysis sampling; transdermal; topical; cocaine; drug delivery.

INTRODUCTION

Esterom® Solution, an investigational pharmaceutical product, is derived from the esterification of benzoylmethylecgonine (cocaine) in 1,2 propanediol. Propylene glycol is a known skin penetration enhancer (1). The resulting solution contains a mixture of components. Esterom Solution is intended to be a topical analgesic to relieve pain and increase the range of motion in patients suffering from acute inflammation of the shoulder or back. Cocaine has been known to have anesthetic properties for many years. Cocaine produces anesthesia by preventing impulse generation and conduction in sensory nerves and blocking presynaptic reuptake of norepinephrine and other catecholamines (2). This causes vasoconstriction when cocaine is topically applied. Cocaine seems to cause vasoconstriction primary through adrenergic nerves and receptors coupled with activation of intracellular Ca^{2+} (3). After topical application, cocaine undergoes both enzymatic and nonenzymatic hydrolysis to form metabolites. Cocaine also undergoes spontaneous hydrolysis of the methylester group to form benzoylecgonine. It has been shown that following topical application of cocaine, significant urinary benzoylecgonine concentrations are detected for prolonged periods of time (4).

Although the components of Esterom are known, the components that are responsible for analgesia have only recently been identified (5). Initial clinical studies yielded promising results that Esterom may have analgesic properties. However, following topical administration of Esterom, 17.2% of patients in Phase 2 studies had detectable Esterom components (benzoylecgonine and ecgonine) in the urine; and 5.2% of the patients tested and who received Esterom had detectable Esterom components (benzoylecgonine and ecgonine) in the blood (6). Because Esterom is topically applied, information is needed on which components of Esterom have the ability to penetrate the skin. The skin acts as a barrier, restricting many polar compounds from entry into the body. Typically, information on transdermal drug transport is usually obtained from *in vitro* studies and *ex vivo* measurements using skin biopsies. However, these methods are unable to directly characterize time vs. concentration profiles of *in vivo* drug release at the site of interest and do not allow for an evaluation of the effects the complex living structure of the skin has on the drug and its metabolism. In order to determine which components of Esterom are able to penetrate the dermis, microdialysis sampling in the dermis was used which can determine the time vs. concentration profiles of each component. Esterom Solution itself was not evaluated for dermal penetration because several of the components can interconvert, using the mixture it would be impossible to tell the difference between penetration of components of the mixture as opposed to conversion.

A better understanding of the mechanism of action of Esterom can be achieved using microdialysis sampling. There are many reviews on the advantages of microdialysis sampling for evaluating drug delivery (7,8,9). Microdialysis has shown to be an effective tool in characterizing transdermal drug transport (10,11). Not only can microdialysis sampling determine qualitatively which components penetrate the skin, but it can also give insight into the kinetics of drug transport and metabolism in the skin.

MATERIALS AND METHODS

Materials

Benzoic acid and propylene glycol were purchased from Sigma (St. Louis, MO). Hydroxypropyl benzoate, benzoylecgonine, hydroxypropyl benzoylecgonine, ecgonidine, methy-

[1] Department of Chemistry, University of Kansas, Lawrence, Kansas 66045.

[2] To whom correspondence should be addressed. (e-mail: clunte@ku.edu)

ABBREVIATIONS: BA, Benzoic Acid; HP-BA, Hydroxypropyl Benzoate; BE, Benzoylecgonine; HP-BE, Hydroxypropyl Benzoylecgonine; EGDN, Ecgonidine; MEGDN, Methylecgonidine; HP-EGDN, Hydroxypropyl Ecgonidine; EGN, Ecgonine.

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Table I. Esterom Solution Composition

Compound	MW g/mol	w/w% in 10% Esterom
BE	289.13	0.04
BA	122.1	0.89
EGN	185.2	0
MEGDN	181.1	0.61
EGDN	167.2	<0.02
HP-BE	347.17	6.06
HP-BA	180.1	0.94
HP-EGDN	225.14	1.59
Cocaine	303.15	0.87

lecgonidine, hydroxypropyl ecgonidine, and ecgonine were provided by Research Triangle Institute (RTI) (Research Triangle Park, NC). HPLC grade acetonitrile, methanol, laboratory grade formic acid (90%) and o-phosphoric acid (85%) were purchased from Fisher Scientific. Ketamine was purchased from Fort Dodge Animal Health (Fort Dodge, IA) and xylazine was purchased from Lloyd Laboratories (Shenandoah, IA). Sterile Ringer's solution (0.9% sodium chloride) used for post-surgical administration was purchased from Abbott Laboratories (North Chicago, IL). Ringer's solution, used as the perfusate, consisted of 145 mM NaCl, 2.8 mM KCl, 1.3 mM $CaCl_2$, and 1.2 mM $MgCl_2$. Roccal D was purchased from Pharmacia and Upjohn (Kalamazoo, MI). Linear microdialysis probes were purchased from Bioanalytical Systems Inc. (BAS) (West Layfayette, IN). Heating pads were purchased from Braintree Scientific (Braintree, MA). All other components were reagent grade or better and used as received. The concentrations of each component in 10% Esterom and molecular weights are provided in Table I. Structures of Esterom components are provided in Fig. 1.

Surgical Procedure

Female Fuzzy rats weighing 275–325 g were housed in temperature-controlled rooms with free access to food and water. All experiments were in accordance with the Principles of Laboratory Animal Care (NIH Publication no. 85-23, revised 1985). The rats were initially anesthetized by inhalation of isoflurane followed by i.m. injection of a ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture. The animal's body temperature was maintained by placing the animal on a heating pad. All surgical instruments were sterilized in a stock solution of Roccal D, which was diluted 1:100 in water. A linear microdialysis probe was implanted in the dermis by first inserting a 21-gauge needle through the dermis. One end of the probe tubing was threaded through the needle. The needle was then withdrawn leaving the dialysis membrane imbedded in the dermis. The dialysis membrane was 10 mm long. The probe was fixed on the skin with tissue glue (Vetbond). The inlet of the microdialysis probe was connected with fluorinated ethylenepropylene (FEP) tubing connectors to a Hamilton 1-mL syringe mounted on a CMA 100 microinjection pump (BAS). The microdialysis probe outlet was connected to a CMA 160 injection valve (BAS) with FEP tubing. The microinjection pump delivered the perfusion medium at a flow rate of 1 μl min^{-1}. Ringer's solution was perfused through the probe for 30–60 min.

The tip of a 3 mL syringe was clipped off and fixed to the skin directly above the dialysis membrane and fixed with tissue glue. This served as a "containment system" for the topical application. This containment system helped prevent the topically applied compounds from seeping through the probe entrance and outlet in the skin.

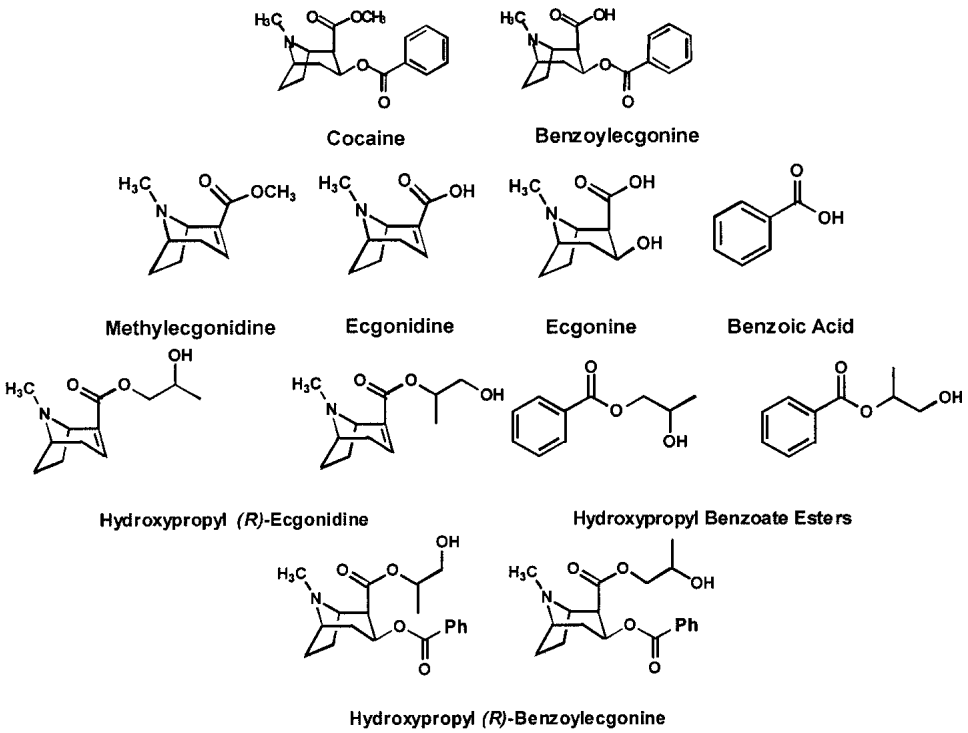

Fig. 1. Structures of Esterom Solution components.

Chromatographic System

The LC-UV system consisted of an ISCO 2350 pump and a Shimadzu SPD-6AV UV-Vis spectrophotometric detector. The LC-MS system consisted of a Micromass Platform LC mass spectrometer with an electrospray interface. Chromatographic data were acquired using TurboChrom software (PerkinElmer) or Mass Lynx (Micromass) software. The analytical methods were as follows:

(1) Separation of BA and HP-BA (LC-UV): Agilent Zorbax RP column (2.1 × 50 mm), 50 mM phosphate buffer, pH 2.6/acetonitrile (80:20 v/v), 215 nm, flow rate = 400 μl/min.

(2) Separation of BE, HP-BE, and BA (LC-UV): Agilent Zorbax RP column (2.1 × 50 mm), 50 mM phosphate buffer, pH 3.5/acetonitrile (85:15 v/v), 232 nm, flow rate = 400 μl/min.

(3) Separation of EGDN and HP-EGDN (LC-UV): Agilent Zorbax RP column (2.1 × 50 mm), 50 mM phosphate buffer, 2 mM SOS, pH 3.0/acetonitrile (89:11 v/v), 215 nm, flow rate = 400 μl/min.

(4) Detection of MEGDN (LC-UV): Phenomenex Synergi Hydro RP (250 × 2.0mm), 50mM phosphate buffer, pH 2.0/methanol (95:5 v/v), 215nm, flow rate = 300 μl/min.

(5) Detection of EGN (LC-MS): Agilent Zorbax RP column (2.1 × 50mm), pH 2.5 methanol buffer (95:5 v/v) with 0.1% formic acid. The MS was operated in positive ion mode. The capillary and cone voltages were set at 3.5 kV and 25 V respectively. The source heater was set at 100°C. The gas flow was set at maximum flow of 250 L/h. It was noted that the high salt content of the Ringer's solution suppressed the ionization of ecgonine. Therefore all standards were formulated in water and the perfusate contained water instead of Ringer's solution. To circumvent deviations in the ionization of the target compound, methylecgonidine was used as an internal standard. Five microliter dialysate samples spiked with methylecgonidine were injected offline every 15 min. Single ion monitoring was performed at 186.2 m/z for ecgonine and 182.1 m/z for methylecgonidine.

The limits of detection of each Esterom component are as follows: BA-0.3 μM, BE-0.3 μM, HP-BE-1 μM, EGDN-0.5 μM, HP-EGDN-1.5 μM, MEGDN-0.5 μM, EGN-0.5 μM.

Calibration of Microdialysis Probes

Microdialysis probe extraction efficiencies (EE) were determined *in vivo* by estimation of the delivery of each of the components of Esterom to the dermis after surgical implantation of the probe. The delivery continued until at least three consistent samples were obtained. Following the delivery, the probe was again perfused with Ringer's solution at a flow rate of 1 μl/min. The (EE) was calculated using:

$$EE_D = (Cp\text{-}Cd)/Cp$$

Table II. *In Vivo* Probe Calibration

Compound	*In vivo* delivery (%)
BA (n = 3)	29 ± 2
BE (n = 4)	22 ± 3
HP-BE (n = 4)	26 ± 6
EGDN (n = 2)	23 ± 7
HP-EGDN (n = 2)	27 ± 10
EGN (n = 3)	51 ± 4

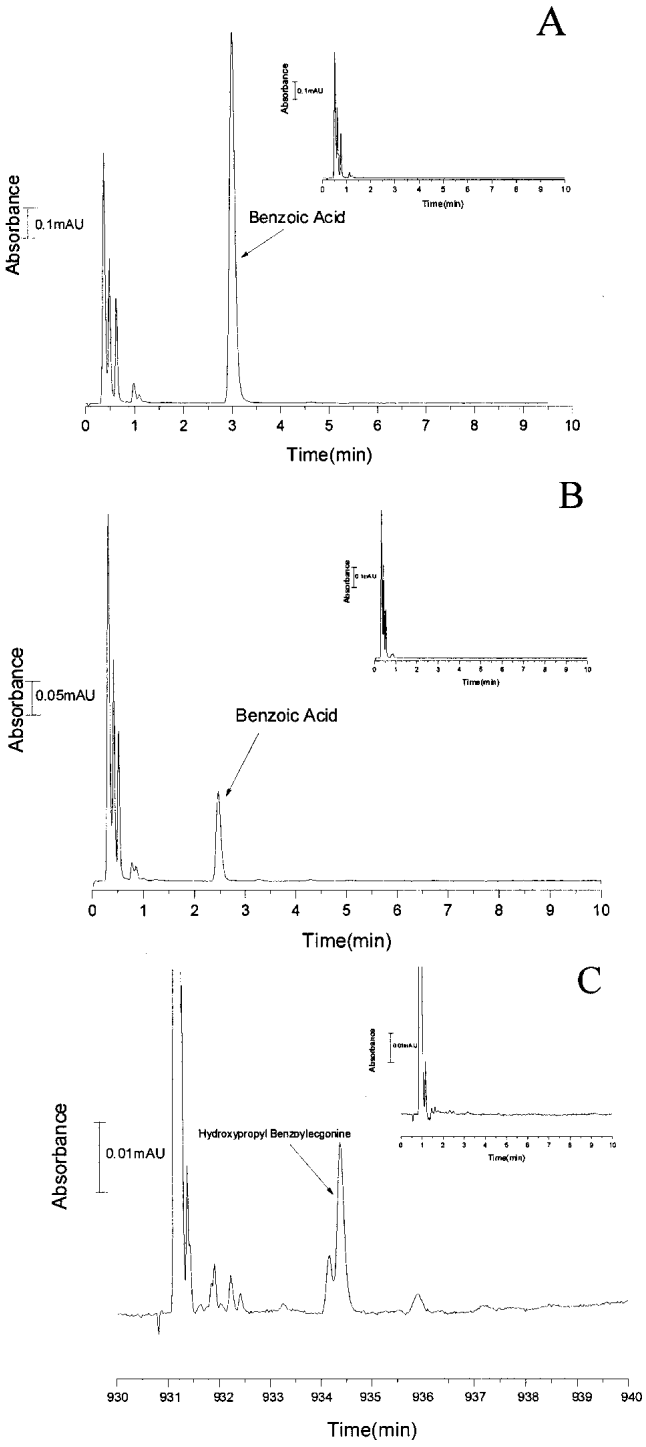

Fig. 2. (A) Representative chromatogram of dermal dialysate obtained 210 min after the administration of benzoic acid. (B) Representative chromatogram of dermal dialysate obtained 130 min after the administration of hydroxypropyl benzoate. (C) Representative chromatogram of dermal dialysate obtained 930 min after the administration of hydroxypropyl benzoylecgonine. Insets represent chromatograms of before the application of the Esterom component.

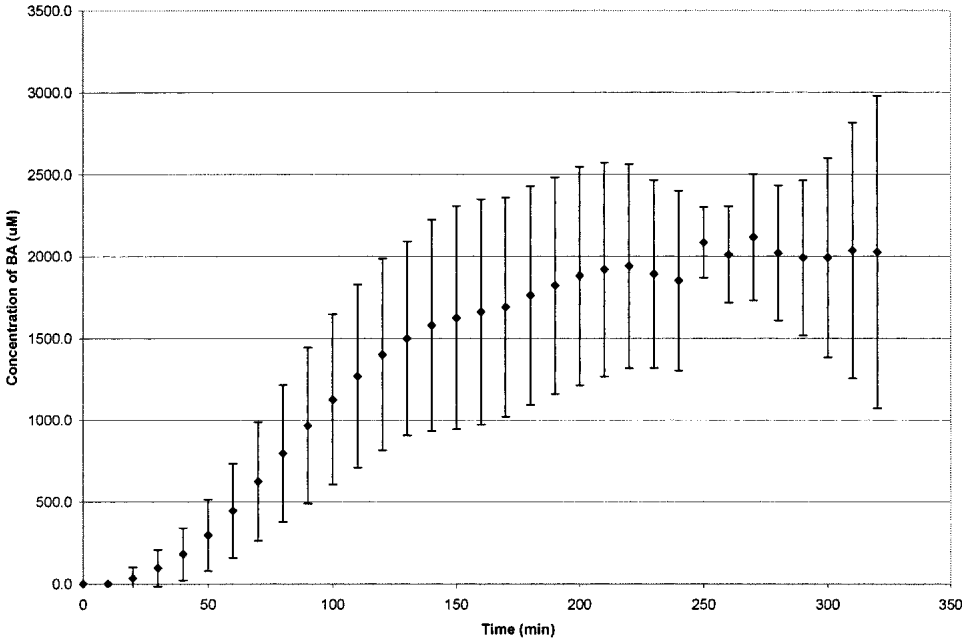

Fig. 3. Average timecourse of detected benzoic acid in the dermis following topical benzoic acid application (n = 4).

Where EE_D is the extraction efficiency of the probe using delivery, Cp is the initial concentration of analyte in the perfusate, and Cd is the concentration of analyte in the dialysate. It has been shown that the EE values determined by delivery are equal to the EE values by recovery (12). Therefore the delivery value obtained for each compound was used as the calibration factor for each component of Esterom in the dermis. Delivery values for each compound are given in Table II.

Hydroxypropyl Benzoylecgonine Hydrolysis Rate Evaluation

Because HP-BE hydrolyzes to BE in Ringer's solution, analysis of this hydrolytic rate had to be determined in order to determine the recovery of HP-BE. A known concentration of HP-BE was injected every 10 min for about 60 min. The same solution of HP-BE was then perfused through a probe that had been previously implanted in the rat dermis. The delivery of HP-BE to rat skin was allowed to reach a steady state. The same standard solution was then injected for a further 60 min. The peak heights of the dialysate were then fit to the degradation rate of HP-BE and the recovery of HP-BE determined.

Study Design

After surgical implantation of the probe, Ringer's solution was perfused through the probe for at least 60 min before

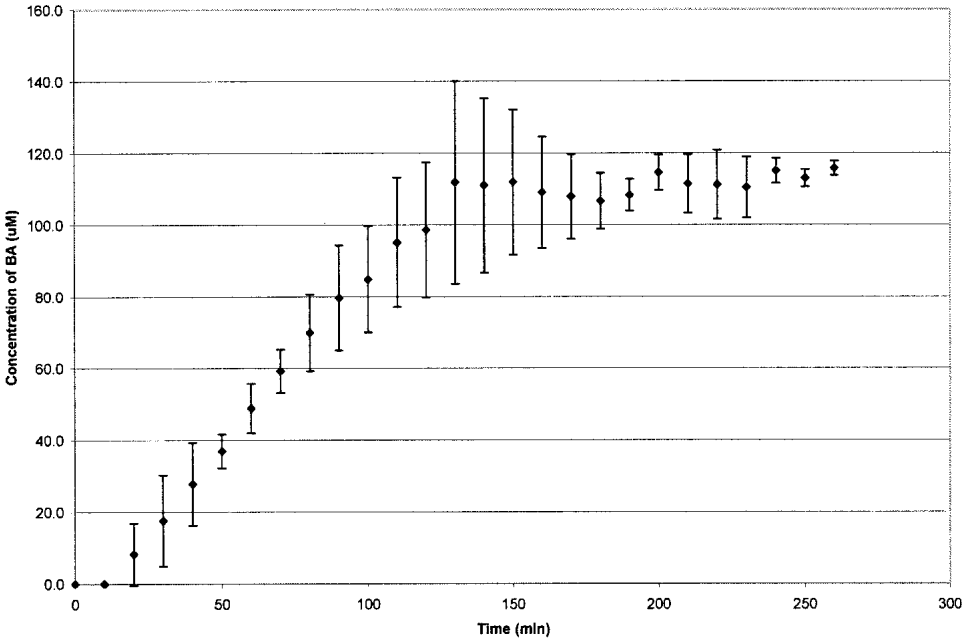

Fig. 4. Average timecourse of detected benzoic acid in the dermis following topical hydroxypropyl benzoic acid application (n = 3).

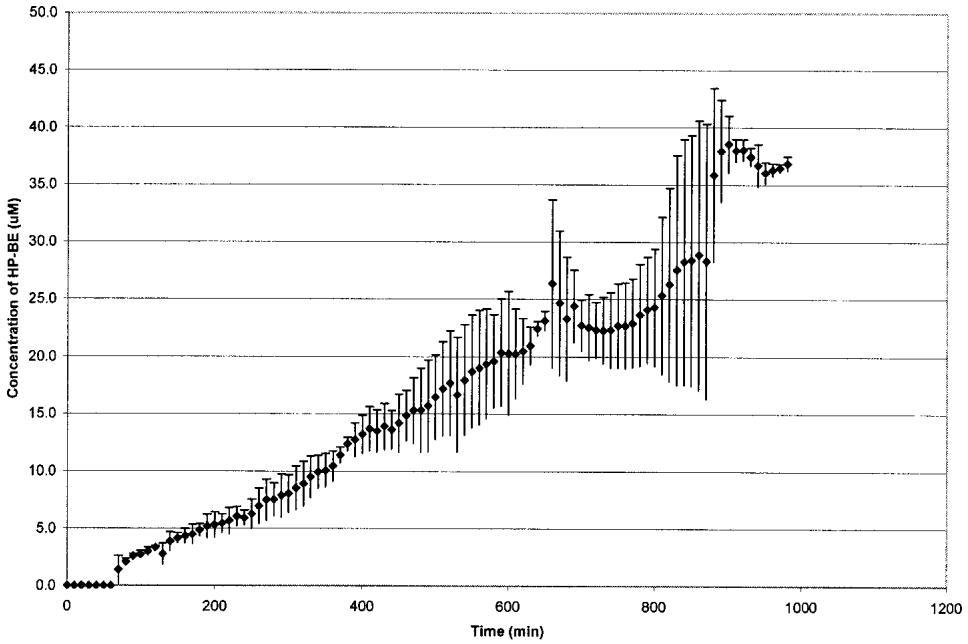

Fig. 5. Average timecourse of detected hydroxypropyl benzoylecgonine in the dermis following topical hydroxypropyl benzoylecgonine application (n = 3).

each experiment. A single component of Esterom dissolved in propylene glycol in a concentration range of 6.67 mg/50 μl to 10 mg/50 μl was applied to the skin in a 50 μl aliquot. Each component of Esterom was formulated in propylene glycol because Esterom solution was formulated in propylene glycol. Samples were collected until a steady-state concentration of the test compound in the dermis was reached. Injections were made in 10–15 min intervals. At the conclusion of the experiment, the skin was excised for histologic analysis of the implantation. This was done in order to ensure that the probe was implanted in the dermis.

RESULTS AND DISCUSSION

The skin penetration of each of the components of Esterom was evaluated and the possible formation of their hydrolysis products. BA was evaluated first because its ability to penetrate the skin has been well documented (13). BA was first detected in the dermis about 30–40 min after topical application. A steady state was reached after approximately 220 min. The plateau concentration of BA detected in the skin was 1.8 ± 0.6 mM when 10mg BA in 50 μl of propylene glycol was applied. A representative chromatogram of a der-

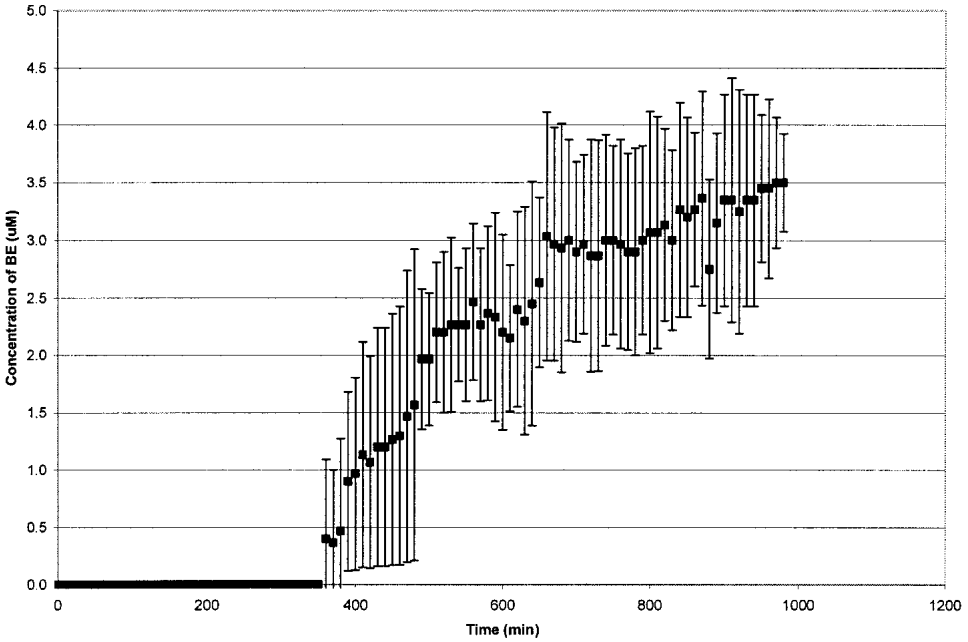

Fig. 6. Average time course of detected benzoylecgonine in the dermis following topical hydroxypropyl benzoylecgonine application (n = 3).

mal dialysate obtained after administration of each component is shown in Fig. 2A. An average time-course of the dermal concentration of benzoic acid is shown in Fig. 3.

When HP-BA (10 mg in 50 μl propylene glycol) was applied to the skin, complete hydrolysis was observed, with only BA detected in the dermal dialysate. BA was first detected about 20–30 min after application. A steady state was reached after approximately 150 min. A concentration of 137 ± 19 μM of BA in the skin was detected on the plateau. A representative chromatogram of a dermal dialysate after administration of HP-BA is shown in Fig. 2B. An average time course is shown in Fig. 4.

The next components evaluated were benzoylecgonine and hydroxypropyl benzoylecgonine. HP-BE can hydrolyze to BE which can further hydrolyze to BA and EGN. When BE (10 mg/50 μL propylene glycol) was applied to the skin, it was not detected in the dermal dialysate. Further degradation of BE was not detected.

Upon application of HP-BE to the skin, both HP-BE and its hydrolysis product, BE, were observed in the dermis. No further BE degradation was detected. HP-BE was first detected approximately 80 min after application. A steady state was reached approximately 15 h after application. A concentration of 32 ± 9 μM of HP-BE was detected in the skin at steady state. A representative chromatogram is shown in Fig. 2C. An average time course following the application of HP-BE is shown in Fig. 5. The hydrolysis product, BE was first detected after 6 h. A steady state was reached approximately 16 h after application with a steady-state plateau concentration of 3.9 ± 0.1 μM. An average time course is shown in Fig. 6.

The skin penetration of 10 mg of ecgonine formulated in 50 μl of propylene glycol was evaluated. It should be noted that this solution was only partially soluble. EGN was first detected in the dermis 15–30 min after topical application. A steady state was reached approximately 2.5 h after application with a plateau concentration of 36 ± 5 μM. A sample chromatogram of the separation of ecgonine from the internal standard methylecgonidine is given in Fig. 7. An average time course is shown in Fig. 8.

Ecgonidine, hydroxypropyl ecgonidine, and methylecgonidine were also evaluated. Attempts to dissolve 10 mg of EGDN in 50 μl of propylene glycol were unsuccessful. Therefore a concentration of 6.67 mg/50 μl of propylene glycol was used. EGDN was not detected in the dermis. On application of HP-EGDN to the skin, neither HP-EGDN nor its hydrolysis product EGDN was detected. When 10 mg of MEGDN formulated in 50 μl propylene glycol was applied to the skin, it was not detected in the dermis. However, it should be noted that this solution was only partially dissolved. A 1.00 mg/50ul concentration was found to be soluble, however again no MEGDN was detected in the dermis. Results for all compounds are summarized in Table III.

The elimination half-lives available in the literature for benzoic acid and benzoylmethylecgonine are 5.9 h (in catfish) (14) and 11 h (15) respectively. This explains why an elimination phase is not observed in anesthetized animals, which limits the experiment to hours rather than days. This experiment would need to be carried out for days in order to observe the elimination phase.

The dermal barrier should restrict the entry of the polar components of Esterom resulting in low systemic exposure. This helps to explain why benzoylecgonine, methylecgonidine, and ecgonidine are not detected in the dermis following topical application whereas ecgonine does penetrate the skin. Ecgonine lacks the double bond of ecgonidine and is therefore less polar than ecgonidine thus allowing its entry into the dermis. The electron withdrawing phenyl ring of benzoic acid most likely helps to bring it through the skin. The esters most likely penetrate through the intercellular route (8). It is cur-

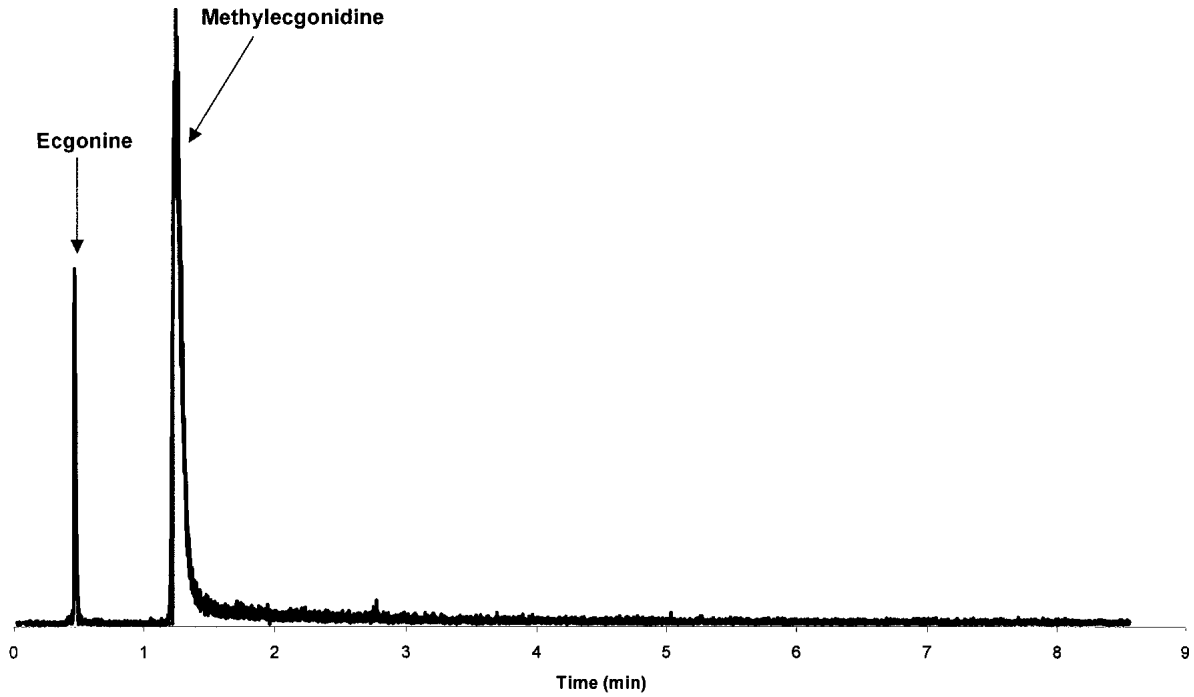

Fig. 7. Chromatogram displaying the separation of ecgonine from the internal standard methylecgonidine.

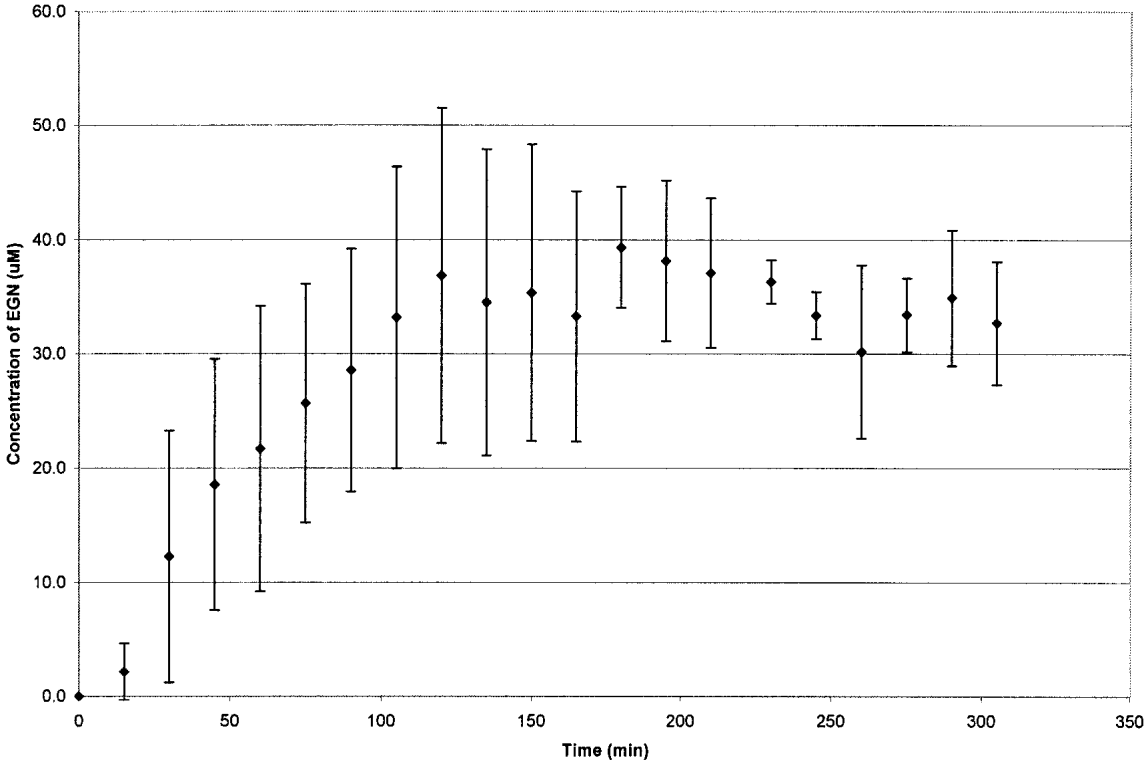

Fig. 8. Average timecourse of detected ecgonine in the dermis following topical ecgonine application (n = 4).

rently unclear as to why the ester of ecgonidine was not detected in the dermis. Perhaps the ester is completely hydrolyzed to ecgonidine on the surface of the skin and ecgonidine is unable to be detected in the dermis.

CONCLUSIONS

A better understanding of the mechanism of action of Esterom can be achieved using microdialysis sampling. Determination of the transdermal kinetics of each component of Esterom® was achieved. The components of Esterom, which penetrate the skin, have been identified. As expected, the polar components did not penetrate whereas the non-polar components did. Benzoic acid readily penetrated the skin. The ester of benzoic acid completely hydrolyzes in the skin. Only the hydrolysis product, benzoic acid is detected in the skin. The HP-BE is detected in the dermis and its hydrolysis product, BE, however it seemed to do so at a slow rate as the peak was slow to appear and represented only a small percentage (0.0006%) of the amount of HP-BE applied. Ecgonine penetrates the skin. Ecgonidine, methylecgonidine, benzoylecgonine, and the ester of ecgonidine were not detected in the dermis.

Of the components that penetrate the skin, the only compound that has been previously shown to have analgesic activity is HP-BE (5). However, the plateau concentration of HP-BE observed in these transdermal studies is below the effective concentration observed in the functional pharmacology assay. Approximately 32 μM of HP-BE is detected in the dermis following topical application of pure HP-BE, a concentration lower than that shown to be pharmacologically active in preclinical animal models. Therefore, the future work for this project involves investigations into possible reformulations to enhance the skin penetration of HP-BE and further investigations into possible synergism/antagonism in the topical formulation of Esterom to help explain the anal-

Table III. Concentration Levels of Cocaine Metabolites Detected in the Skin

Component applied	Component detected	Concentration (mM)	Metabolite detected	Concentration (mM)
BA (n = 4)	BA	1.8 ± 0.6	ND[a]	
HP-BA (n = 3)	BA	0.14 ± 0.02	ND	
BE (n = 6)	ND	ND	ND	
HP-BE (n = 3)	HP-BE	0.032 ± 0.009	BE	0.0039 ± 0.0001
EGN (n = 4)	EGN	0.036 ± 0.005	ND	
EGDN (n = 3)	ND		ND	
HP-EGDN (n = 3)	ND		ND	
MEGDN (n = 3)	ND		ND	

[a] ND = not detected

gesic effect observed in patients. Greater dermal penetration of the components of Esterom may be observed when Esterom solution is applied compared to the individual components.

Microdialysis is an effective tool for monitoring skin penetration. Cutaneous microdialysis is minimally invasive and allows for qualitative and quantitative data involving skin penetration to be determined. In this case, microdialysis has provided valuable information on the absorption and skin penetration of the multiple components found in Esterom Solution. Future studies should allow for formulation development leading to enhanced skin penetration and pharmacologic effect. The possibility of using cutaneous microdialysis to evaluate penetration kinetics of topically applied agents has been clearly demonstrated.

ACKNOWLEDGMENTS

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