Cutaneous Iontophoresis of Treprostinil in Systemic Sclerosis: A Proof-of-Concept Study

M Roustit[1,2], F Gaillard-Bigot[1], S Blaise[1,3], F Stanke-Labesque[1,4], C Cracowski[2], C Seinturier[3], J-F Jourdil[4], B Imbert[3], PH Carpentier[3] and J-L Cracowski[1,2]

Ischemic digital ulcer (DU) is a serious complication of systemic sclerosis (SSc). Intravenous prostanoids are the only approved treatment for active DUs, but they induce dose-limiting side effects and require hospitalization. Our objective was to evaluate the effect of iontophoresis (a noninvasive drug delivery method) of treprostinil in SSc patients. Three studies were conducted: a pharmacokinetic study in 12 healthy volunteers showed that peak dermal concentration was reached at 2 hours, whereas plasma treprostinil was undetected. Then, a placebo-controlled, double-blind incremental dose study assessed the effect of treprostinil on digital skin blood flow in 22 healthy subjects. The effect of the highest dose was then compared with that of placebo in 12 SSc patients. Treprostinil significantly increased skin blood flow in healthy subjects ($P = 0.006$) and in SSc patients ($P = 0.023$). In conclusion, digital iontophoresis of treprostinil is feasible, is well tolerated, and increases digital skin perfusion. It could be tested as a treatment for SSc-related DUs.

Systemic sclerosis (SSc) is a rare disease characterized by extensive fibrosis and vascular alterations predominantly affecting the extremities. Microvascular dysfunction is a key feature of the pathophysiology of SSc that occurs early in the progression of the disease.[1] One of the main complications of SSc microvasculopathy is the development of digital ulcers (DUs), which are painful, cause functional impairment, and have a major negative impact on the quality of life.[2] Chronic ulcers can become infected, resulting in gangrene, osteomyelitis, and amputation, leading to permanent disability and self-image problems.[2]

Intravenous prostacyclin analogues (iloprost) are the only approved treatment for active SSc-related DUs.[3] However, their therapeutic effect is counterbalanced by potentially serious vasodilation-induced, dose-limiting side effects (e.g., severe headaches, flushing, tachycardia, and hypotension). Their use usually requires hospitalization and close monitoring while infusing the drug, which are associated with increased costs. The paradox is that elevated concentrations of prostanoids are needed in the extremities (where ulcers develop), but decreased functional capillary density prevents the drug from diffusing properly to the digits. Elevated doses of drugs are therefore needed, leading to systemic adverse drug reactions. Therefore, topical administration of these drugs may be a way of avoiding the toxicity of systemic treatments.

Iontophoresis is a noninvasive, current-driven drug delivery method. It has been suggested as an interesting alternative to systemic delivery of vasodilators in the treatment of SSc-related DUs.[4,5] Previous studies by our group have focused on the screening of vasodilators (including prostacyclin analogues and endothelin receptor antagonists) that can be iontophoretically administered.[6,7] The 20-minute cathodal iontophoresis of treprostinil, a prostacyclin analogue, induced sustained vasodilation and was well tolerated in a rat model[6] and on the forearm of healthy subjects.[8] However, key issues remain to establish the proof of concept of treprostinil iontophoresis in the treatment of DUs: systemic and dermal pharmacokinetics of iontophoretically delivered treprostinil are unknown; this procedure has never been performed on the digits where the skin is glabrous (with a high density of arteriovenous anastomoses) and therefore presents distinct characteristics from nonglabrous (i.e., hairy) skin; and finally, the safety of treprostinil iontophoresis and its effect on skin blood flow have never been tested in SSc patients, in whom sclerodactyly may affect intradermal drug delivery.

The primary objective of this work was to evaluate the effect of iontophoretically delivered treprostinil on digital skin blood

[1]UMR 1042–HP2, INSERM and University Grenoble-Alpes, Grenoble, France; [2]Clinical Pharmacology Unit, INSERM CIC1406, Grenoble University Hospital, Grenoble, France; [3]Vascular Medicine Department, Grenoble University Hospital, Grenoble, France; [4]Laboratory of Pharmacology, Grenoble University Hospital, Grenoble, France. Correspondence: M Roustit (MRoustit@chu-grenoble.fr)

Received 22 October 2013; accepted 17 December 2013; advance online publication 12 February 2014. doi:10.1038/clpt.2013.255

flow in patients with SSc. We also assessed the safety of the procedure. First, we performed pharmacokinetic and incremental dose studies in healthy subjects.

RESULTS

Intradermal vs. plasma concentrations of treprostinil after iontophoresis on the forearm of healthy subjects

Twelve healthy volunteers (six men and six women) were enrolled in this study. Their mean age was 20.8 ± 2 and mean body mass index was 22.2 ± 2.6 kg/m^2. Systolic and diastolic arterial blood pressures were 114.6 ± 11.3 and 70.2 ± 10.4 mm Hg, respectively. Four women were taking oral contraceptives.

Baseline cutaneous vascular conductances were 0.48 ± 0.1 and 0.47 ± 0.09 perfusion units/mm Hg at the placebo and the treprostinil skin sites, respectively. The insertion of microdialysis catheters induced inflammation, which increased mean cutaneous vascular conductances despite apparent return to baseline during the resting period in individual tracings (0.45 ± 0.08 and 0.53 ± 0.06 perfusion units/mm Hg, respectively; $P = 0.006$). Because the data are expressed as percentage of baseline (%BL), we used cutaneous vascular conductances after fiber insertion as the BL to avoid exaggeration of the treatment effect. Following iontophoresis, skin blood flow was significantly higher at the treprostinil site than at the placebo site (values of area under the concentration–time curve (AUC) up to 8 hours after the end of iontophoresis ($AUC_{0\text{-}8h}$) were 23,066.1 ± 15,718 and 11,187.1 ± 10,092 %BL·minute, respectively; $P = 0.02$; **Figure 1**).

The intradermal concentration of treprostinil was measurable from the first to the seventh hour after the end of iontophoresis. Peak concentration was reached during the second hour and was <1.8 pg/ml in all samples at hour 8 (**Figure 1**). By contrast, the treprostinil concentration was lower than the quantification threshold in all plasma samples.

Petechiae ($n = 4$) and a small hematoma ($n = 1$) were observed after the insertion of the microdialysis fibers. They spontaneously resolved within 24–72 hours. Iontophoresis induced erythema at both sites (placebo: $n = 3$; and treprostinil: $n = 1$) without any itching. It spontaneously resolved within 30 minutes to 5 hours.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ DUs related to SSc are associated with substantial morbidity. Intravenous prostacyclin analogues comprise the only approved treatment for active DUs, but they induce dose-limiting side effects. They also require hospitalization, which is associated with increased costs.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ Our objective was to establish the proof of concept of the use of treprostinil (a prostacyclin analogue) through iontophoresis, a noninvasive, current-driven drug delivery method.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✓ Iontophoresis of treprostinil delivers elevated dermal concentration, although it is undetectable in the plasma. The different protocols tested showed a correlation between the quantity of current and the pharmacological effect. A single dose at 240 mC/cm^2 significantly increases digital skin perfusion in both healthy subjects and SSc patients, and the procedure is safe.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY AND THERAPEUTICS

✓ This original approach may improve the safety of prostacyclin analogues used to treat SSc-related DUs while decreasing associated costs. The efficacy of treprostinil iontophoresis on ulcer healing should now be tested on a larger scale.

Safety and pharmacodynamic effect of incremental doses of treprostinil delivered by digital iontophoresis in healthy subjects

Twenty-two healthy subjects were included in this study. All but one subject included in Study 1 participated in Study 2 (**Supplementary Table S1** online). The mean age of subjects was 21.4 ± 2.3 and their mean body mass index was 21.5 ± 2.2 kg/m^2. Systolic and diastolic arterial blood pressures were 115.3 ± 11.2 and 68.1 ± 10.4 mm Hg, respectively. Ten of 13 women were taking oral contraceptives.

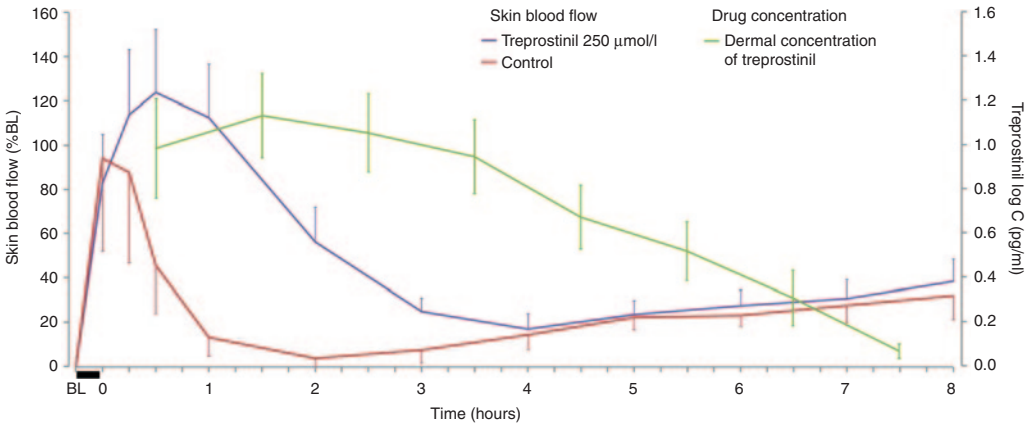


Figure 1 Skin blood flow recorded on the forearm after a 40 mC/cm^2 cathodal iontophoresis (black bar) of treprostinil and placebo, expressed as the percentage increase from baseline flow. Treprostinil was quantified in the dermis with microdialysis samples collected every hour and analyzed by liquid chromatography–tandem mass spectrometry. Treprostinil was detected in the dermis for up to 8 hours after the end of iontophoresis.

All three continuous iontophoresis protocols were well tolerated. Desquamation was observed on the fingertips of one hand in a young male. Because all the fingertips were concerned, the involvement of iontophoresis was excluded. Discontinuous currents were stopped after the 40 mC/cm^2 protocol due to uncomfortable tingling (reported by all subjects) and the absence of pharmacodynamic effect.

Skin blood flow was significantly higher at the treprostinil site than at the placebo site only for the highest dose (240 mC/cm^2). Values of $AUC_{0\text{-}4h}$ were 29,703 ± 23,460 and 18,426 ± 18,365 %BL·minute, respectively ($P = 0.006$; detailed data for all iontophoresis protocols are available in **Supplementary Table S2** online).

Treprostinil concentration was below the quantification threshold in all plasma samples for all doses. Following the intermediate analysis, in the confirmatory study a complete pharmacokinetic study was not performed as originally planned (**Supplementary Methods** online). However, one blood sample was collected from each subject 30 minutes after the end of iontophoresis (240 mC/cm^2, $n = 6$). The plasma concentration of treprostinil was 783 and 933 pg/ml in two volunteers and <1.8 pg/ml in the four other volunteers.

Safety and pharmacodynamic effect of treprostinil delivered by digital iontophoresis in SSc patients

Twelve SSc patients were enrolled in this study. The characteristics of the population are summarized in **Table 1**. The most commonly prescribed drugs were proton-pump inhibitors ($n = 8$), calcium channel blockers ($n = 6$; stopped 1 week prior to inclusion), statins ($n = 3$), and angiotensin-converting enzyme inhibitors ($n = 3$).

Skin blood flow was significantly higher at the treprostinil site than at the placebo site. Values of $AUC_{0\text{-}4h}$ were 47,826 ± 43,941 and 30,000 ± 27,543 %BL·minute, respectively ($P = 0.023$; **Figure 2**). In responding patients, vasodilation did not spread beyond the application site (**Figure 3**). We ran *post hoc* analyses to identify parameters associated with nonresponse. We found that the two patients with a "late" capillaroscopy pattern were nonresponders.

Iontophoresis at 240 mC/cm^2 was well tolerated. We observed one episode of Raynaud's phenomenon at the end of iontophoresis at the placebo site but not at the treprostinil site. Two patients experienced mild headaches. However, all plasma samples collected from each patient 30 minutes after the end of iontophoresis showed treprostinil concentration <1.8 pg/ml.

DISCUSSION

The treatment of active SSc-related DUs is challenging. The dose-limiting adverse effects of i.v. iloprost and the costs associated with the hospitalization required for its administration are currently limitations. Treprostinil, a related compound, has been tested in a pilot trial[9] with encouraging results. However, s.c. treprostinil induces severe injection site pain, leading to a high rate (5 of 12 patients) of drug discontinuation.[9] Alternative routes of administration are therefore needed. Recently, a sustained release oral formulation of treprostinil has been shown to be effectively absorbed in SSc patients but, logically, prostanoid-related adverse drug events were observed in the vast majority of patients.[10]

The current work was aimed at establishing the proof-of-concept for the local, noninvasive delivery of treprostinil using iontophoresis. We show that treprostinil remains detectable in the dermis of healthy skin for up to 8 hours, although systemic concentrations are extremely low. On the finger pad, iontophoresis at 240 mC/cm^2 was feasible, was well tolerated, and induced vasodilation in most healthy participants and SSc patients. We also observed a transient increase in skin blood flow at the placebo site, which is attributed to a nonspecific, current-induced vasodilation.[11] This tends to reduce the difference in absolute values of skin blood flow between placebo and treprostinil. However, it is not possible to predict how this difference can translate into a clinical effect. Although the only drug approved in DU healing (i.e., iloprost) is a potent vasodilator, and vascular dysfunction is the hallmark of SSc, the efficacy of prostaglandin I_2 analogues is not expected to be solely due to their vascular properties. Indeed, the efficacy of i.v. therapy with iloprost is prolonged over several weeks, whereas vasodilation-induced effects are observed only during drug infusion or shortly after. This prolonged effect is attributed to other properties,

Table 1 Demographic and clinical characteristics of SSc patients ($N = 12$)

Age (years)	59.9 ± 11.4
Female	12 (100%)
Postmenopausal	11 (92%)
BMI (kg/m^2)	26.6 ± 3.6
Arterial blood pressure	
Systolic (mm Hg)	138 ± 19
Diastolic (mm Hg)	70 ± 13
Raynaud's phenomenon	12 (100%)
Duration (years)	11.8 ± 7.4
Number of fingers involved	7.7 ± 1.7
Thumb involved	10 (83%)
Feet involved	2 (17%)
Scleroderma (lcSSc)	12 (100%)
Duration (years)	10 ± 6.9
Sclerodactyly	12 (100%)
Rodnan score	8.7 ± 4.9
Previous digital ulcer	1 (8%)
Capillaroscopy pattern[22]	
Early	4 (33%)
Active	6 (50%)
Late	2 (17%)

Quantitative data are expressed as mean ± SD. Qualitative data are expressed as number (percentage). BMI, body mass index; lcSSc, limited cutaneous SSc; SSc, systemic sclerosis.

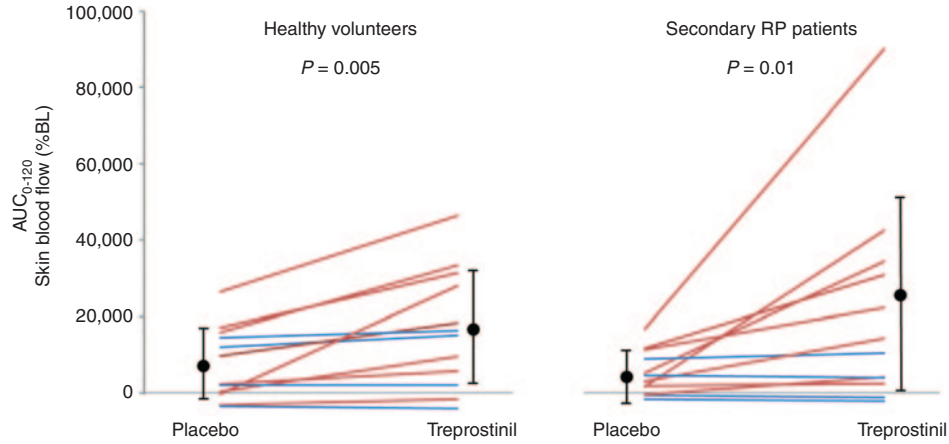


Figure 2 Skin blood flow expressed as area under the curve (AUC) of cutaneous vascular conductances after iontophoresis (240 mC/cm^2) of treprostinil and placebo in 12 healthy subjects and 12 systemic sclerosis patients. Black dots and bars represent mean (SD) area under the curve of cutaneous vascular conductances. Eight subjects (four healthy volunteers and four patients) did not respond to treatment (blue lines).

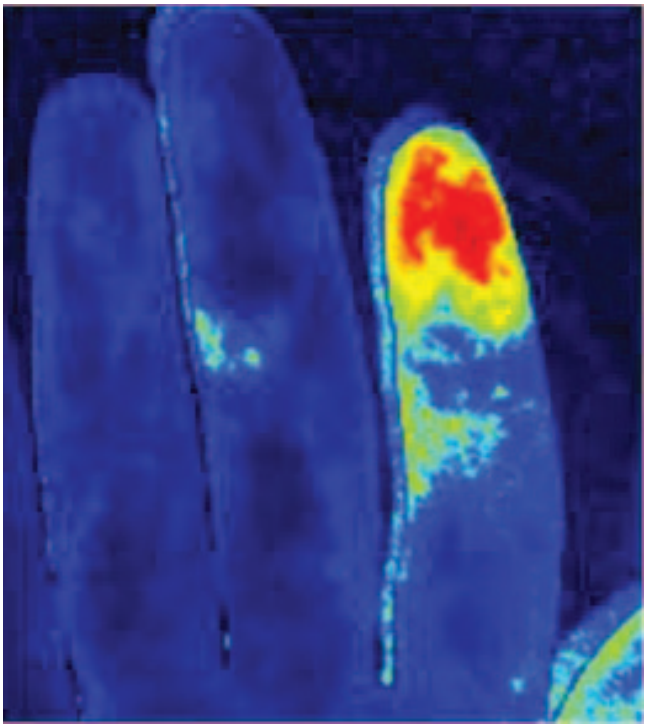


Figure 3 Skin blood flow recorded with laser speckle contrast imaging after removal of electrodes. Treprostinil was applied on the index finger and placebo on the middle finger. Colors range from dark blue (no perfusion) to red (high perfusion).

such as downregulation of lymphocyte adhesion to endothelial cells and inhibition of the production of tumor necrosis factor-α. Furthermore, iloprost has been recently shown to exert prostaglandin I2 receptor–dependent immunomodulatory activity in SSc patients.[12]

The sustained vasodilation that we observed on the forearm of healthy subjects is consistent with our previous results using comparable iontophoretic parameters (i.e., quantity of charge of 40 mC/cm^2).[8] The current study shows that on the finger pad, however, this quantity of current is not sufficient to increase skin blood flow, as has been reported in studies with other vasodilators such as sodium nitroprusside, both in patients with SSc and in healthy participants.[13] This may be due to anatomical differences in the skin's microcirculation between the forearm and the finger pad, with a higher density of vessels and arteriovenous anastomoses in the latter, leading to increased dermal clearance. Another explanation could be the greater thickness of the epidermis on the finger pad as compared with that of the forearm.

Many parameters are involved in iontophoretic transport. The parameters that can be controlled by investigators include the treated skin area, the drug concentration in the iontophoresis reservoir, the intensity of the current, and the duration of current application.[14] We decided to gradually step up the dose in Study 2 by adjusting the duration of the iontophoresis protocol. Indeed, increased current intensity may be responsible for tingling and itching, in addition to discomfort from the procedure, leading in some cases to the discontinuation of iontophoresis before the complete dose had been delivered.[15] On the other hand, using higher concentrations did not show any further vasodilation in previous experimental studies.[6]

Discontinuous current was also tested because iontophoretic transport might be enhanced when intermittent current sequences are used rather than a continuous sequence, with the same quantity of charge. Experimental animal studies in our laboratory had suggested a potential benefit of such a protocol, with a greater increase in skin blood flow. However, we observed no effect when discontinuous sequences at 40 mC/cm^2 were used in humans. Moreover, intermittent short, high-intensity sequences generated discomfort in all subjects. Thus, we maintained only continuous administration in the incremental study. We finally observed that a six-times-higher dose was required to achieve vasodilation on the finger pad as compared with the dose on the forearm.

Our preliminary pharmacokinetic study in healthy participants suggests that when delivered by cutaneous iontophoresis, treprostinil remains in the dermis for up to 8 hours.

Simultaneous plasma monitoring shows that the systemic diffusion of the drug is below the detection threshold. Thus, the dermal vs. systemic concentration ratio in the forearm suggests that iontophoresis of treprostinil could allow sufficient doses to be delivered locally while limiting systemic exposure. However, this assumption could not be confirmed on the finger pad. A limitation of this study is that for technical and ethical reasons, the dermal pharmacokinetics of treprostinil was not explored in the finger pads of either healthy subjects or patients. Indeed, as fiber insertion in the finger pad is traumatic and leads to bleeding, we decided not to perform microdialysis to avoid any trauma to patients with possible healing impairment. Tape stripping, another technique used to assess dermal pharmacokinetics *in vivo,* could not be used in this protocol because continuous quantification over the same skin area is not possible.[15] However, the pharmacodynamic effect observed in patients despite the absence of positive plasma concentration assessment suggests a favorable pharmacokinetic profile in the finger pad.

Treprostinil concentration from all blood samples in all series of the incremental dose study (Study 2) was below the quantification threshold. As planned in the protocol, the collection of complete pharmacokinetic data was stopped at the interim analysis, and only one sample was collected in the confirmatory study, 30 minutes after the end of iontophoresis (corresponding to the maximum pharmacodynamic effect). Nevertheless, although lower than that observed after i.v. or s.c. infusion, significant treprostinil concentrations were found in two healthy volunteers.[16] One of these two subjects was a responder, whereas the other was not, and neither experienced adverse drug events.

On the finger pad, we observed vasodilation in two-thirds of the healthy subjects and patients. The absence of vasodilation was not related to the Rodnan skin score. When looking at the capillaroscopy pattern, the two patients with a "late" pattern did not respond, which could be explained by the reduced capillary density in these patients. Nonetheless, further investigations are needed to explore in depth the parameters that determine the response. Indeed, several properties of the skin barrier that were not explored in the current study may influence iontophoresis, e.g., transepidermal water loss, skin hydration, epidermal and dermal thickness, and skin elasticity. Future studies should also determine the optimal concentration of treprostinil needed to enhance the pharmacodynamic effect on the finger pad, as well as determine the appropriate rate of repeated administrations.

The current study did not aim at directly comparing the effect of treprostinil iontophoresis on skin blood flow between healthy subjects and SSc patients, and the two populations are not comparable. Rather, we aimed at establishing the feasibility of the local administration of treprostinil, its pharmacodynamic effect on skin blood flow, and the safety of the procedure. This establishes its proof-of-concept in patients without ulcers, suggesting that the procedure can now be tested in patients with DUs. However, our objective was not to assess efficacy in ulcer healing, and to date, skin blood flow has not been established as a surrogate for DUs. In the current study, it was used as a marker of the pharmacodynamic effect of treprostinil to objectify the dermal delivery of the drug. Nonetheless, recent work has demonstrated that differences in skin blood flow, assessed with laser speckle contrast imaging, are correlated with the nailfold capillaroscopy pattern of microangiopathy.[17] Importantly, the capillaroscopy pattern is a predictive marker of severe peripheral vascular involvement assessed using the Medsger severity scale[18] (i.e., the occurrence of fingertip ulceration). The odds ratios for the future occurrence of DU were 2.49, 6.18, and 15.35 for the early, the active, and the late pattern, respectively.[19] Although the surrogacy of skin blood flow remains to be directly established, these recent data suggest a potential benefit of laser speckle contrast imaging as a surrogate for the occurrence of DU.

In conclusion, the iontophoresis of treprostinil is feasible and well tolerated. Moreover, it increases digital skin blood flow in both healthy subjects and in SSc patients. Taken together, our data suggest that the iontophoresis of treprostinil could provide an efficient, safe, noninvasive, and cost-saving treatment for SSc-related DUs. However, controlled studies are needed both to assess the efficacy of iontophoretically administered treprostinil in DU healing and to compare this strategy with the systemic administration of iloprost.

METHODS

Study population. Healthy volunteers were recruited through local newspaper advertisements, and patients were recruited through our Vascular Medicine department. All patients had limited cutaneous SSc, according to the criteria of LeRoy and Medsger,[20] with sclerodactyly. All subjects were included between January 2012 and January 2013.

All subjects were 18 years of age or older. Noninclusion criteria included pregnancy (urine pregnancy tests were performed at the beginning of each visit) and cigarette smoking. None of the healthy subjects had any chronic disease or ongoing treatment (other than oral contraception for women). For patients, the presence of active DUs was a noninclusion criterion. Patients taking calcium channel blockers were instructed to stop medication 1 week before inclusion. Patients taking endothelin receptor antagonists or phosphodiesterase-5 inhibitors or those who had received i.v. iloprost in the previous 15 days were not included.

The investigation conforms to the principles outlined in the Declaration of Helsinki. Grenoble Institutional Review Board CPP Sud-Est V (Institutional Review Board 6705) approval was obtained on 21 November 2011, and each subject gave written informed consent before participation.

Study design

Study 1: intradermal vs. plasma concentration of treprostinil after iontophoresis on the forearm of healthy subjects. This was an open-label pharmacokinetic study enrolling healthy subjects. On arrival at the laboratory, subjects were placed in a temperature-controlled room (23 ± 1 °C); they remained supine during all measurements. After 30-minute acclimatization, two ellipsoid skin sites (~12 cm^2) were chosen on the ventral side of the right forearm, avoiding visible veins. One of these two sites was randomly selected as the treprostinil site, and the other one as the placebo site. Baseline skin blood flow was recorded for 10 minutes with laser speckle contrast imaging (**Supplementary Methods** online). Both sites were then treated with 5 g of lidocaine/prilocaine cream (Anesderm; Pierre Fabre, Boulogne, France). One hour later, the lidocaine/prilocaine cream was removed, and two linear microdialysis fibers were inserted at

the dermis–hypodermis junction at the treprostinil site (**Supplementary Methods** online and **Supplementary Figure S1** online) and an i.v. catheter was inserted controlaterally. Skin blood flow was continuously recorded at the two skin sites on the forearm until the vasodilation induced by the trauma returned to baseline (at least 45 minutes).

Iontophoresis at 40 mC/cm^2 (**Supplementary Methods** online) was then performed at both sites; treprostinil was delivered at the site equipped with the microdialysis fibers, whereas NaCl was used at the placebo site. Skin blood flow measurements, microdialysis samples, and venous blood samples were collected until 8 hours after the end of iontophoresis (**Supplementary Figure S1** online). Blood pressure was recorded continuously (Nexfin monitor, Bmeye BV, Amsterdam, The Netherlands) on the left hand during skin blood flow measurements. The design of Study 1 is schematized in **Supplementary Figure S2** online.

Study 2: safety and pharmacodynamic effect of incremental doses of treprostinil delivered by digital iontophoresis in healthy subjects. The primary objective of this double-blind study was to assess the safety of different iontophoresis protocols of treprostinil and placebo (NaCl) on the finger pads of healthy subjects. Digital skin blood flow was also assessed. Subjects enrolled in Study 1 could participate in one or two visits of Study 2. The iontophoresis sites were the finger pads of two fingers randomly chosen from the index, the middle, and the ring fingers. The operator and the volunteer were blinded as to whether the volunteer received treatment or placebo.

Four protocols were sequentially tested, with a go/no-go decision based on safety and tolerability: 40 mC/cm^2 (continuous current), 40 mC/cm^2 (discontinuous current), 120 mC/cm^2 (continuous current), and 240 mC/cm^2 (continuous current), where the dose of treprostinil delivered is considered as being proportional to the current used (**Supplementary Methods** online). Six participants were included in each iontophoresis protocol. A confirmatory study with six other participants was subsequently performed at the highest dose. Data were pooled for the 12 participants who were tested with the 240 mC/cm^2 protocol.

Skin blood flow and blood pressure were continuously recorded for at least 2 hours after the end of iontophoresis, until return to baseline skin blood flow. Blood samples from the controlateral arm were also collected (**Supplementary Methods** online).

Study 3: safety and pharmacodynamic effect of treprostinil delivered by digital iontophoresis in SSc patients. This double-blind study aimed at evaluating safety and comparing skin blood flow after digital iontophoresis of treprostinil or placebo (240 mC/cm^2) in SSc patients. The skin sites were the finger pads of two fingers chosen as described above. The operator and the volunteer were blinded as to whether the volunteer received treatment or placebo.

At the end of iontophoresis, skin blood flow and blood pressure were measured as described in Study 1 and continuously recorded for up to 4 hours after the end of iontophoresis. Blood samples from the contralateral arm were also collected (**Supplementary Methods** online).

In all studies, photographs of all skin sites were taken before and immediately after iontophoresis, and at the end of each visit. A clinical visit to monitor safety was planned 10 ± 6 days after the final visit.

Data analysis

Data were analyzed with signal processing software (PIMSoft 1.1.1; Perimed, Järfälla, Sweden). Skin blood flow was expressed as cutaneous vascular conductance, which is the flow in arbitrary perfusion units divided by the mean arterial pressure in mm Hg.[21] We then calculated the percentage change from baseline flow (%BL) and subsequently performed a minute-by-minute analysis to calculate AUC until return to baseline flux, with a minimum recording time of 2 hours ($AUC_{0\text{-}2h}$) and up to 8 hours ($AUC_{0\text{-}8h}$) after the end of iontophoresis.

Statistical analysis

Categorical data were reported as frequency and percentage, and continuous data were reported as mean and SD. Skin blood flow after treprostinil and placebo iontophoresis simultaneously recorded in the same subject were compared using paired *t*-tests or nonparametric tests (Wilcoxon rank test), the latter when the conditions of application of parametric tests were not respected. We considered *P* values of ≤0.05 as significant. Statistical analysis was performed with SPSS 13.0 for Windows (SPSS, Chicago, IL).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

ACKNOWLEDGMENTS

We thank Dominique Abry and Anne Tournier for blood sampling; Adeline Paris, Sylvain Kotzki, and Constance Minebois for preparing the drugs and blinding; Manon Gabin and Benoît Sulpis for helping with data collection; and Alison Foote for critically reading and editing the manuscript.

AUTHOR CONTRIBUTIONS

M.R., F.G.B., S.B., F.S.-L., C.C., C.S., and J.-L.C. wrote the manuscript. M.R., S.B., F.S.-L., P.H.C., and J.-L.C. designed the research. M.R., F.G.B., S.B., C.C., C.S., B.I., P.H.C., and J.-L.C. performed the research. M.R., F.S.-L., J.-F.J., and J.-L.C. analyzed the data. F.S.-L. and J.-F.J. contributed new reagents/analytical tools.

CONFLICT OF INTEREST

This study was funded by grants from the French government ("Programme Hospitalier de Recherche Clinique Inter-régional 2011") and from the "Association des Sclérodermiques de France." Bioprojet Pharma supplied treprostinil. None of the sponsors had any involvement in the design and conduct of the study, nor in the preparation, review, or approval of the current article. M.R., S.B., and J.-L.C. hold a patent on treprostinil iontophoresis for the treatment of ulcers. The other authors declared no conflict of interest.

1. Gabrielli, A., Avvedimento, E.V. & Krieg, T. Scleroderma. *N. Engl. J. Med.* **360**, 1989–2003 (2009).
2. Steen, V., Denton, C.P., Pope, J.E. & Matucci-Cerinic, M. Digital ulcers: overt vascular disease in systemic sclerosis. *Rheumatology (Oxford).* **48** (suppl. 3), iii19–iii24 (2009).
3. Kowal-Bielecka, O. *et al.*; EUSTAR Co-Authors. EULAR recommendations for the treatment of systemic sclerosis: a report from the EULAR Scleroderma Trials and Research group (EUSTAR). *Ann. Rheum. Dis.* **68**, 620–628 (2009).
4. Murray, A.K., Herrick, A.L., Gorodkin, R.E., Moore, T.L. & King, T.A. Possible therapeutic use of vasodilator iontophoresis. *Microvasc. Res.* **69**, 89–94 (2005).
5. Murray, A.K., Moore, T.L., King, T.A. & Herrick, A.L. Vasodilator iontophoresis a possible new therapy for digital ischaemia in systemic sclerosis? *Rheumatology (Oxford).* **47**, 76–79 (2008).
6. Blaise, S., Roustit, M., Millet, C., Ribuot, C., Boutonnat, J. & Cracowski, J.L. Cathodal iontophoresis of treprostinil and iloprost induces a sustained increase in cutaneous flux in rats. *Br. J. Pharmacol.* **162**, 557–565 (2011).
7. Roustit, M. *et al.* Iontophoresis of endothelin receptor antagonists in rats and men. *PLoS ONE* **7**, e40792 (2012).
8. Blaise, S., Roustit, M., Hellmann, M., Millet, C. & Cracowski, J.L. Cathodal iontophoresis of treprostinil induces a sustained increase in cutaneous blood flux in healthy volunteers. *J. Clin. Pharmacol.* **53**, 58–66 (2013).
9. Chung, L. & Fiorentino, D. A pilot trial of treprostinil for the treatment and prevention of digital ulcers in patients with systemic sclerosis. *J. Am. Acad. Dermatol.* **54**, 880–882 (2006).
10. Shah, A.A. *et al.* Open label study of escalating doses of oral treprostinil diethanolamine in patients with systemic sclerosis and digital ischemia: pharmacokinetics and correlation with digital perfusion. *Arthritis Res. Ther.* **15**, R54 (2013).
11. Roustit, M. & Cracowski, J.L. Assessment of endothelial and neurovascular function in human skin microcirculation. *Trends Pharmacol. Sci.* **34**, 373–384 (2013).

12. Truchetet, M.E., Allanore, Y., Montanari, E., Chizzolini, C. & Brembilla, N.C. Prostaglandin I(2) analogues enhance already exuberant Th17 cell responses in systemic sclerosis. *Ann. Rheum. Dis*. **71**, 2044–2050 (2012).
13. Roustit, M., Blaise, S. & Cracowski, J.L. Sodium nitroprusside iontophoresis on the finger pad does not consistently increase skin blood flow in healthy controls and patients with systemic sclerosis. *Microvasc. Res*. **77**, 260–264 (2009).
14. Kalia, Y.N., Naik, A., Garrison, J. & Guy, R.H. Iontophoretic drug delivery. *Adv. Drug Deliv. Rev*. **56**, 619–658 (2004).
15. Roustit, M., Blaise, S. & Cracowski, J.L. Trials and tribulations of skin iontophoresis in therapeutics. *Br. J. Clin. Pharmacol*. (2013); e-pub ahead of print 16 April 2013.
16. Laliberte, K., Arneson, C., Jeffs, R., Hunt, T. & Wade, M. Pharmacokinetics and steady-state bioequivalence of treprostinil sodium (Remodulin) administered by the intravenous and subcutaneous route to normal volunteers. *J. Cardiovasc. Pharmacol*. **44**, 209–214 (2004).
17. Ruaro, B., Sulli, A., Alessandri, E., Pizzorni, C., Ferrari, G. & Cutolo, M. Laser speckle contrast analysis: a new method to evaluate peripheral blood perfusion in systemic sclerosis patients. *Ann. Rheum. Dis*. (2013); e-pub ahead of print 16 August 2013.
18. Medsger, T.A. Jr *et al*. A disease severity scale for systemic sclerosis: development and testing. *J. Rheumatol*. **26**, 2159–2167 (1999).
19. Smith, V. *et al*. Do worsening scleroderma capillaroscopic patterns predict future severe organ involvement? a pilot study. *Ann. Rheum. Dis*. **71**, 1636–1639 (2012).
20. LeRoy, E.C. & Medsger, T.A. Jr. Criteria for the classification of early systemic sclerosis. *J. Rheumatol*. **28**, 1573–1576 (2001).
21. Roustit, M. & Cracowski, J.L. Assessment of endothelial and neurovascular function in human skin microcirculation. *Trends Pharmacol. Sci*. **34**, 373–384 (2013).
22. Cutolo, M. *et al*. Nailfold videocapillaroscopic patterns and serum autoantibodies in systemic sclerosis. *Rheumatology (Oxford)*. **43**, 719–726 (2004).

Iontophoresis of treprostinil as a treatment of ischemic digital ulcers in systemic sclerosis: a proof-of-concept study

SUPPLEMENTARY INFORMATION

SUPPLEMENTAL METHODS

1. Drugs

Treprostinil 1 mg/mL solution (Remodulin®) was supplied by Bioprojet Pharma (Paris, France). The commercially available solution was diluted with isotonic sodium chloride (NaCl 0.9%, Aguettant, Lyon, France) to obtain a 0.1 mg.mL-1 (250 µM) solution, which was previously shown to induce sustained vasodilation in the forearm skin of healthy subjects[1]. NaCl solution was chosen as placebo and diluant as it has been shown to induce less nonspecific axon reflex vasodilation than deionized water[2]. Moreover, we previously showed that iontophoresis of the buffer of the commercially available solution of treprostinil modifies skin blood flow in animals[3]. All solutions were prepared extemporaneously by a pharmacist independent from the study.

2. Dermal sampling procedure (study 1)

After lidocaine/prilocaine cream removal, local asepsia was performed and two parallel microdialysis fibers were inserted less than 1 cm apart on the treprostinil site. A sterile 21 G, 50 mm needle was inserted into the skin over a length of 3 cm to place each fiber at the dermal-subdermal interface. CMA 66® linear microdialysis catheters with a 30 mm

long, 500 µm diameter, 20 kDa cut-off membrane (CMA Microdialysis, Solna, Sweden), previously rinsed with sterile NaCl 0.9 %, were subsequently inserted through the needle. Then the fiber was connected to a portable battery driven syringe pump (CMA 107 Microdialysis Pump®, CMA Microdialysis, Solna, Sweden). The whole procedure has been previously published in detail[4]. The location of the microdialysis fibers was checked at the end of the procedure by ultrasonography echography with 10-5 MHz transducer (Titan®, SonoSite Inc., Bothell, WA, USA). Their mean depth was 0.8 (±0.27) mm.

3. In vitro calibration

In vitro calibration was performed to estimate the relative recovery (RR), i.e. the fraction of drug collected in the dialysate relative to the actual extra-cellular fluid concentration of drug[5]. Although the RR is usually higher *in vitro* than *in vivo*, it provided an indication as to the feasibility of the *in vivo* sampling procedure[6].

Four solutions of treprostinil (0.01, 0.1, 1 and 10 $\mu g.mL^{-1}$) were prepared by diluting the commercially available solution with isotonic sodium chloride. Two microdialysis fibers (CMA66®, CMA Microdialysis, Solna, Sweden) were placed within the solution and connected to a pump (as described above). As the flow rate of the perfusate influences the RR, we tested two flow rates (1 and 2 $\mu L.min^{-1}$) in the two fibers. Microdialysis was run for 50 to 100 min according to the flow rate in order to collect 100 µL samples to assess treprostinil concentration (procedure detailed in the following section). The procedure was repeated four times for each concentration.

Mean RR were 0.3 (±0.04) to 0.73 (0,03), which is higher than that has been observed for lipophilic eicosanoids[7]. We concluded that a flow rate set at 2 µL/min was a good compromise between recovery and sampling frequency.

4. Quantification of treprostinil

Treprostinil was quantified by liquid chromatography – tandem mass spectrometry (LC-MS/MS).Ten µL of sample (plasma or microdialysis samples) were treated with 60 µL of a precipitation reagent ($ZnSO_4$ 60 mM/methanol 30/70 *v/v*) containing the isotope labeled internal standard (6 keto PGF1α-d_4). After vortex-mixing and centrifugation at 21 000 g for 10 min, the supernatant was collected and put in an autosampler vial ready for injection.

A two dimensional liquid chromatography was performed. First, an online sample clean-up step was performed on a Perfusion column POROS R1/10 micron 4.6 × 50 mm (Applied Biosystems, Foster City, CA, USA), with an aqueous mobile phase containing 30% methanol and 0.07% formic acid delivered at 3 mL/min for 1.5 min. After valve switching the compounds of interest trapped on the perfusion column were eluted through the analytical column Vydac Denali C_{18} 2 × 150 mm (Grace, Hesperia, CA, USA) housed in the column oven maintained at 60°C. An elution gradient was performed with a mixture of 0.3% acetic acid in water (eluent A) and acetonitrile (eluent B) with a starting composition of 30% B, increasing to 75% B in 3 min, and with a subsequent clean-up step at 100% for 5 min and a re-equilibration step of 30%B for 1.5 min. Six min after the beginning of the run, the valve was switched back to its initial position in order to re-equilibrate the perfusion column for the next injection. The complete two dimensional chromatographic run lasted 11 min per sample.

Measurements was performed on an API 4000 tandem mass spectrometer (ABSciex, Toronto, Canada) equipped with a turbo V® ion source. Quantification was achieved in the multiple reaction monitoring (MRM) mode, monitoring two ion transitions for treprostinil (m/z 389.2/331.2 for quantization, m/z 389.2/143.1 for confirmation) and one ion transition for 6 keto PGF1α-d_4 (m/z 373.3/167.1) (Figure S3). The ion source was operated in negative mode with an ESI potential of -4500 V and the following parameters: turbo heater and

nebulizer gas respectively set at 40 and 55 psi, ion source temperature at 600°C and curtain gas setting at 10 psi.

Calibration curves were calculated by least-square regression with a 1/x weighting with linear regression. The lower limit of quantification was fixed at the level of the concentration corresponding to the first point of the calibration curve (1.8 pg/mL). The intra-day and inter-day coefficients of variation were <12 %.

5. Iontophoresis protocols

Two 7.2 cm² Ag–AgCl electrodes (Iogel, Iomed Inc., Salt Lake City, UT, USA) were soaked with 1.5 mL of treprostinil or NaCl solution (open-labeled in study 1, double-blinded in studies 2 and 3). Drug-containing electrodes were connected to the cathode, while the anode was connected to dispersive electrodes fixed at 10 cm. Low-voltage, computer-driven generators were used (PF 751 PeriIont USB Power Supply, Perimed, Järfälla, Sweden). Voltage was continuously recorded to estimate skin resistance (which was not different between the two skin sites; data are not detailed for clarity).

The two essential parameters that can be readily controlled by the operator and that determine iontophoretic transport include the intensity of the current and the duration of current application[8]. The parameters used in the three studies are the following:

Current				Location	
Quantity (mC/cm²)	Continuous	Duration (min)	Intensity (mA/cm²)	Forearm	Finger pad
40	Yes	20	0.033	Study 1	Study 2
40	No	20	0.033		Study 2
120	Yes	60	0.033		Study 2
240	Yes	120	0.033		Study 2 & 3

6. Skin blood flow measurements

Cutaneous blood flow was measured with high frame rate laser speckle contrast imaging (LSCI) (PeriCam PSI System, Perimed, Järfälla, Sweden). The wavelength was 785 nm and the laser head was placed 20 cm above the skin. The image size was 12×12 cm and the acquisition rate was 3 s^{-1}. We and others have previously shown the excellent reproducibility of LSCI to assess skin blood flow[2,9].

Considering the influence of skin temperature on blood flow and the variability of digital skin perfusion, we standardized skin temperature in study 2 to improve reproducibility[2]. As SSc patients have lower baseline flux/skin temperature that healthy subjects, we set a lower baseline skin temperature in healthy participants. Refrigerated cold packs (Nexcare, 3M, St. Paul, MN, USA) were placed on the skin over the hand to maintain skin temperature between 26 and 28°C. Skin temperature was recorded continuously.

7. Blood samples collection

Venous blood samples were collected from the catheter inserted in the contralateral forearm. Eight samples were collected after the end of iontophoresis: T0, 15 min, 30 min, 1h, 2h, 4h, 6h and 8h (Figure S2). In studies 1 and 2, interim analyses were planned after 6 volunteers had been included in each series. In case of plasma concentrations being below the quantification threshold at all points for all patients, only one point was collected per patient, 30 min after the end of iontophoresis (which corresponds to the maximal pharmacodynamic effect in a previous work)[1].

8. Sample size calculation

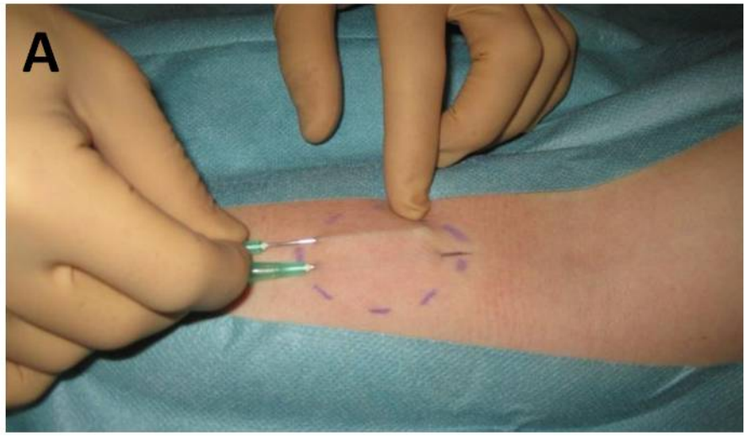
The sample size was calculated such as to be able to show a 0.9 effect size between the AUC of skin blood flow after iontophoresis of treprostinil and of placebo. For a two-sided paired t-test with an alpha risk of 0.05 and a power of 80%, 12 subjects were needed. For the Wilcoxon rank test, this sample size corresponds to a probability of 83% that an observation at the placebo site will be less than one at the treprostinil site when the alternative hypothesis is true (nQuery Advisor 7.0, Statistical Solutions ltd., Cork, Ireland). Response to treatment was arbitrarily defined as a 25% increase or more in skin blood flow between treprostinil and placebo.

SUPPLEMENTAL FIGURE LEGENDS

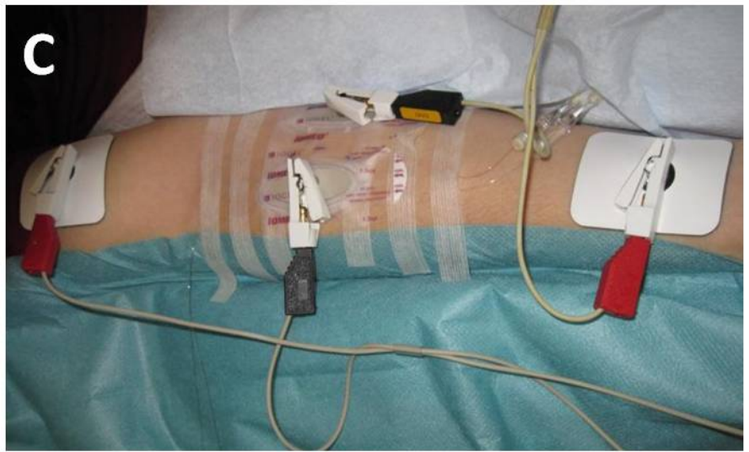
Figure S1. Dermal sampling procedure using microdialysis coupled with iontophoresis on the forearm of healthy subjects (study 1). Catheters are inserted into the skin over a length of 4 cm (A) to place each fiber at the dermal-subdermal interface (B). Active iontophoresis electrodes containing treprostinil are then placed on top of the fibers while the second electrode (placebo) is placed on the other skin site (C). (D) Schematic representation of the experimental setup.

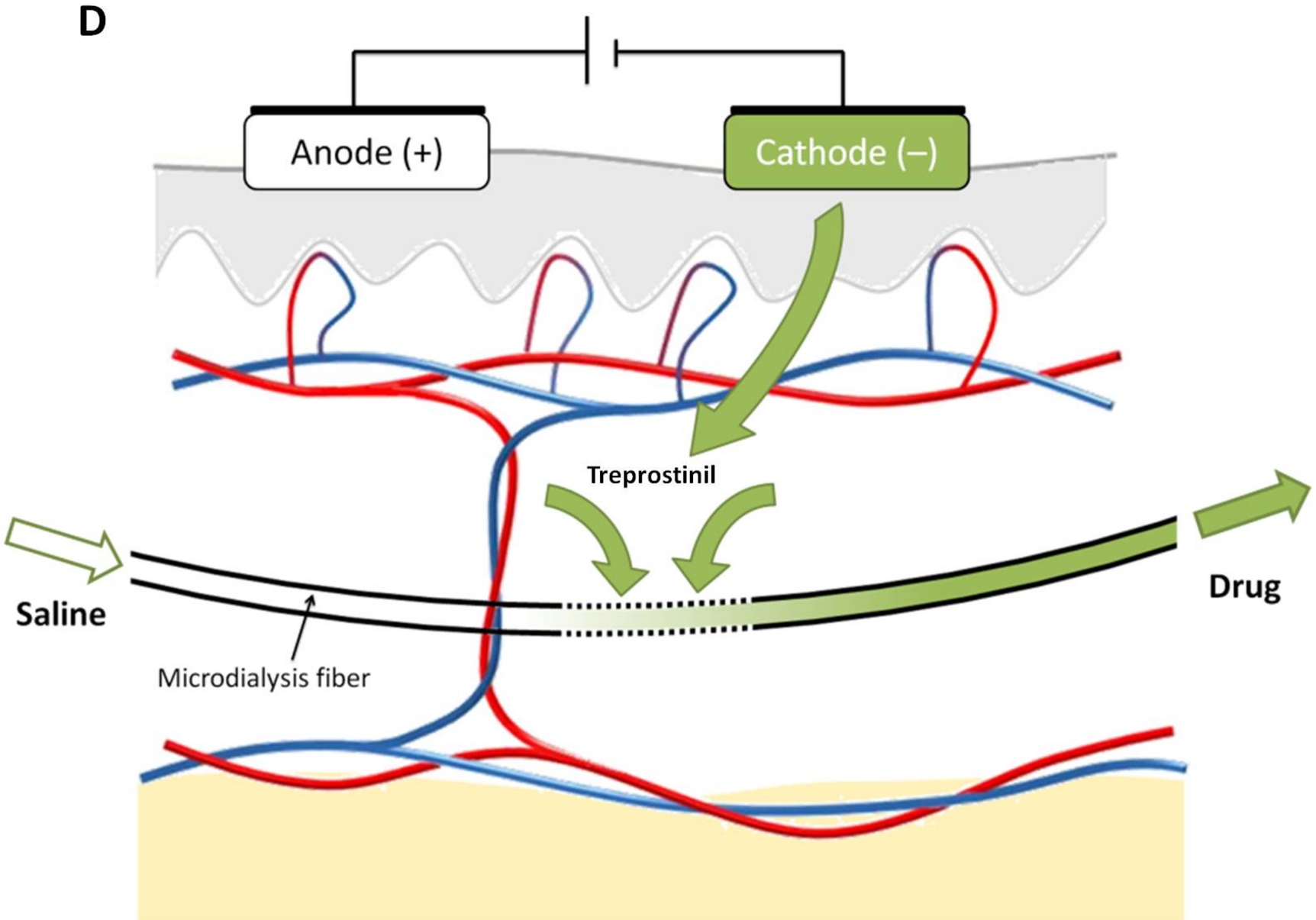
Figure S2. Study 1 design. Dermal samples were collected each hour at the treprostinil site. T0 was defined as the time point immediately after the end of iontophoresis. L/P: local anesthesia with lidocaine/prilocaine. SkBF: Skin blood flow; MAP: mean arterial pressure.

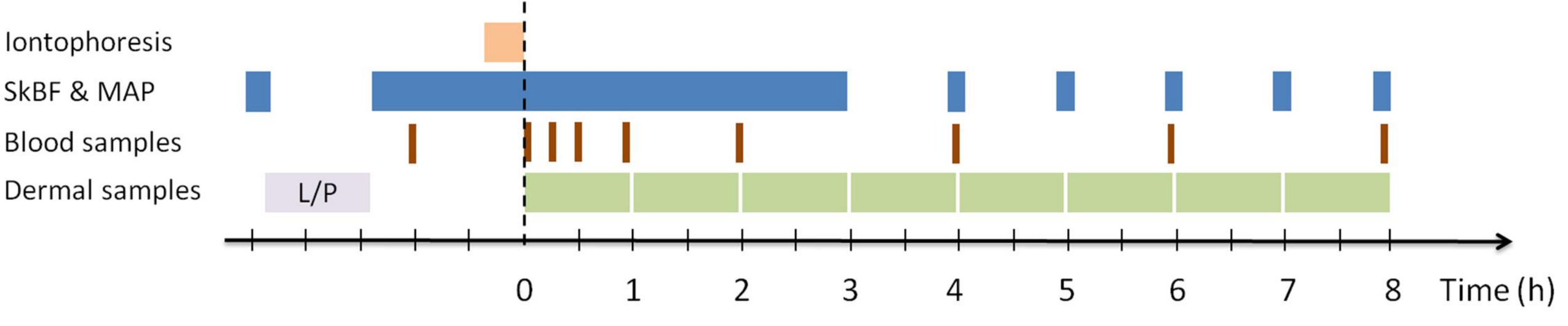
Figure S3. Typical chromatograms of a blank (A), a calibration point for treprostinil (concentration 29.1 pg/ml) (B) and a dialysat from a volunteer who received treprostinil by iontophoresis (C).


A

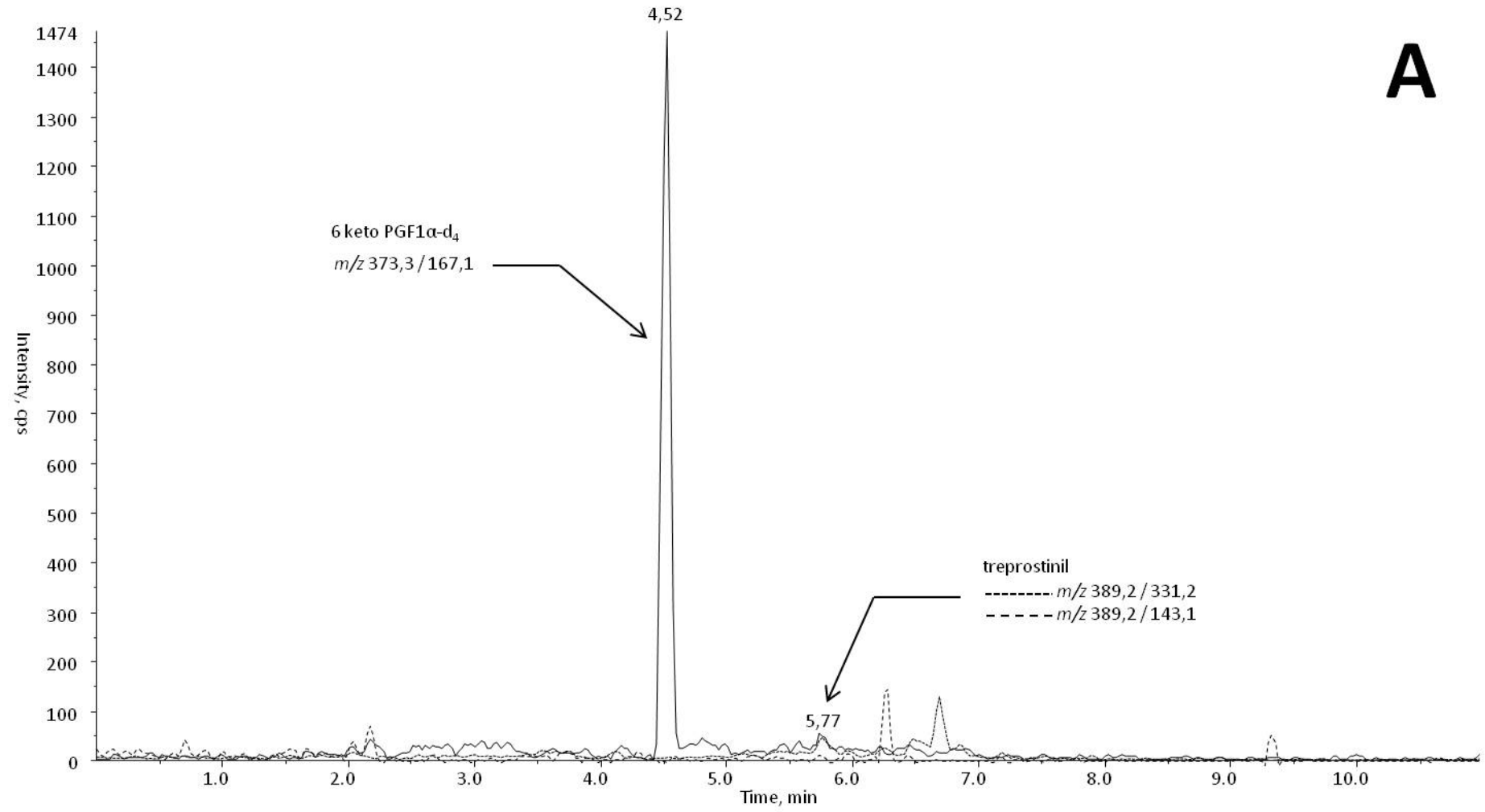

B


C

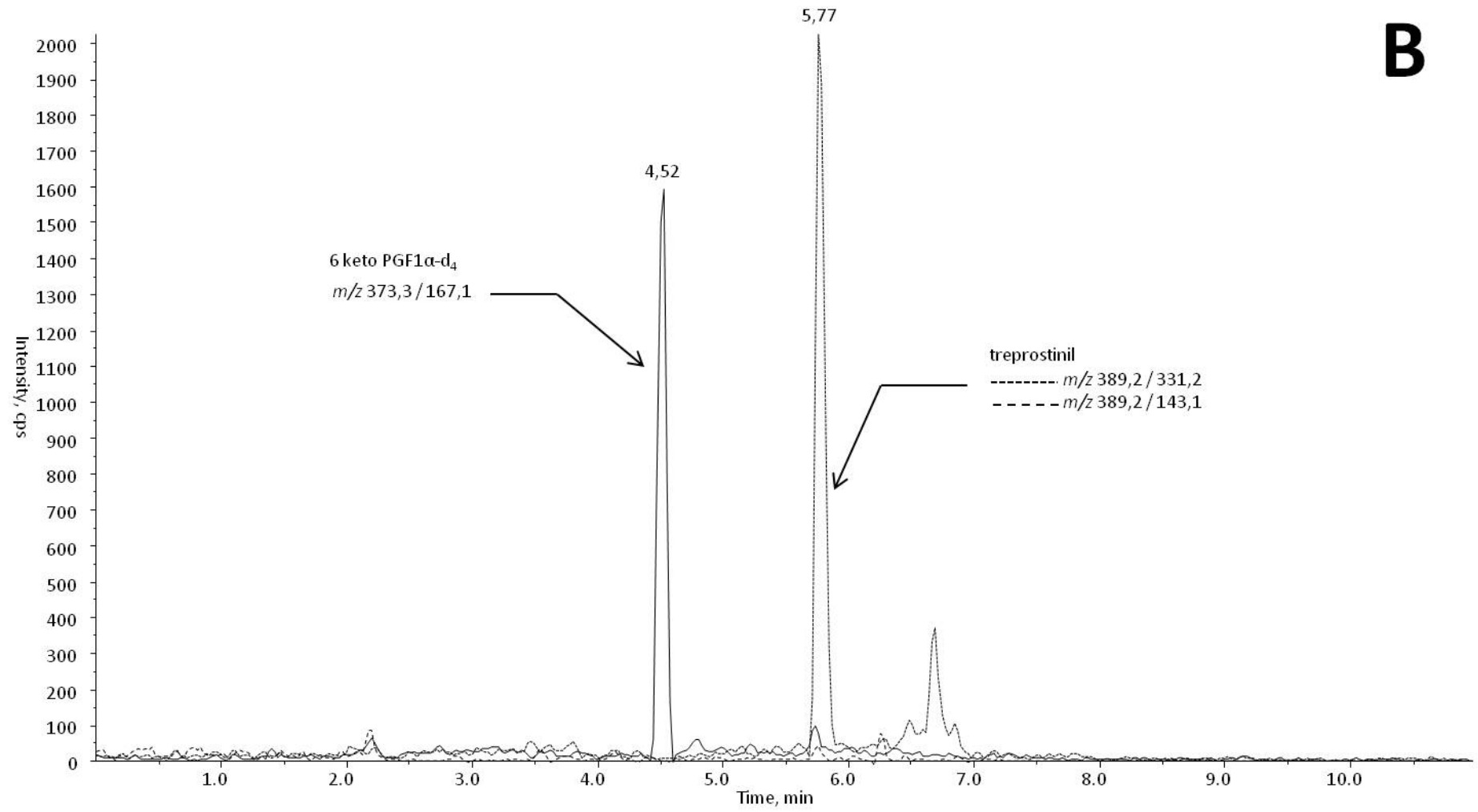

D
Anode (+)
Cathode (–)
Treprostinil
Saline
Drug
Microdialysis fiber


Iontophoresis
SkBF & MAP
Blood samples
Dermal samples
L/P
0
1
2
3
4
5
6
7
8
Time (h)

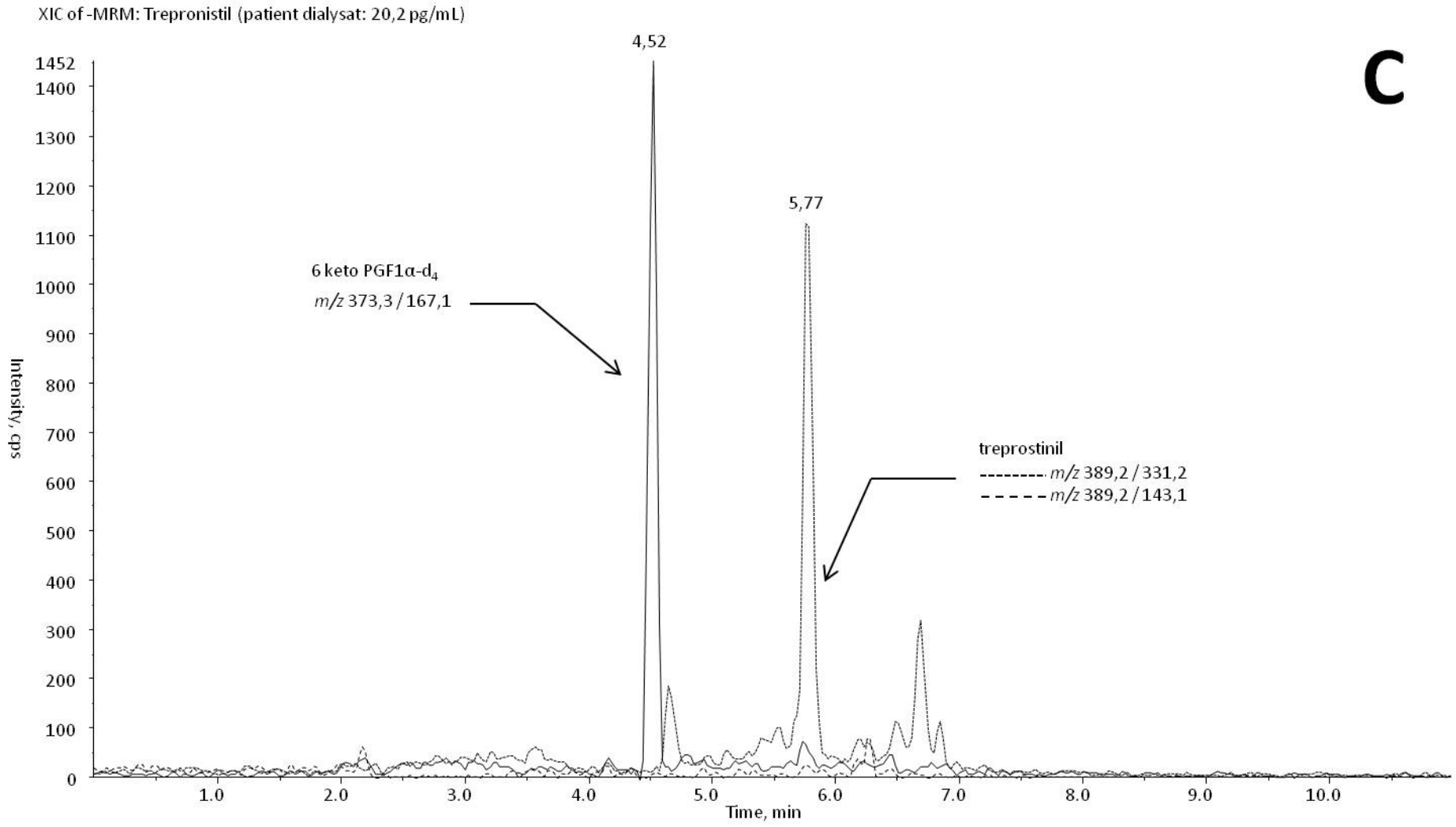
XIC of -MRM: Trepronistil (Blank)



XIC of -MRM: Trepronistil (Calibrator : 29,1 pg/mL)



XIC of -MRM: Trepronistil (patient dialysat: 20,2 pg/mL)



SUPPLEMENTAL REFERENCES

1. Blaise, S., Roustit, M., Hellmann, M., Millet, C. & Cracowski, J. L. Cathodal iontophoresis of treprostinil induces a sustained increase in cutaneous blood flux in healthy volunteers. *J Clin Pharmacol* **53**, 58–66 (2013).
2. Roustit, M. & Cracowski, J. L. Non-invasive assessment of skin microvascular function in humans: an insight into methods. *Microcirculation* **19**, 47–64 (2012).
3. Blaise, S. *et al.* Cathodal iontophoresis of treprostinil and iloprost induces a sustained increase in cutaneous flux in rats. *Br J Pharmacol* **162**, 557–65 (2011).
4. Cracowski, J. L., Gaillard-Bigot, F., Cracowski, C., Roustit, M. & Millet, C. Skin microdialysis coupled with laser speckle contrast imaging to assess microvascular reactivity. *Microvasc Res* **82**, 333–8 (2011).
5. Kreilgaard, M. Assessment of cutaneous drug delivery using microdialysis. *Adv. Drug Deliv. Rev.* **54, Supplement**, S99–S121 (2002).
6. Holmgaard, R., Nielsen, J. B. & Benfeldt, E. Microdialysis sampling for investigations of bioavailability and bioequivalence of topically administered drugs: current state and future perspectives. *Skin Pharmacol Physiol* **23**, 225–43 (2010).
7. Sun, L. & Stenken, J. A. Improving microdialysis extraction efficiency of lipophilic eicosanoids. *J. Pharm. Biomed. Anal.* **33**, 1059–1071 (2003).
8. Kalia, Y. N., Naik, A., Garrison, J. & Guy, R. H. Iontophoretic drug delivery. *Advanced drug delivery reviews* **56**, 619–58 (2004).
9. Roustit, M., Millet, C., Blaise, S., Dufournet, B. & Cracowski, J. L. Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. *Microvasc Res* **80**, 505–11 (2010).