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Rapid quantification of histamine in human psoriatic plaques using microdialysis and ultrahigh performance liquid chromatography with fluorescence detection

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ABSTRACT

Psoriasis is a chronic skin disease resulting from abnormal immune function and is characterized by the presence of scaly psoriatic plaques which are areas of inflammation and excessive skin production [1]. The psoriatic plaques contain mast cells which are increased in number in the uppermost dermis of the psoriatic lesion and which may play a role in the initiation and maintenance of the lesion. These processes are thought to be mediated via the local release of histamine along with other mediators from the mast cells; however their precise role still remains a mystery [2]. Our study involved the development of a rapid and ultra-sensitive liquid chromatographic method for the separation and detection of histamine. To this end a state-of-the-art ultra high pressure liquid chromatography (UHPLC) system incorporating the latest technology in fluorescence detection system was employed which allowed for the rapid and reliable trace level detection of histamine in human derived microdialysate samples. This new reverse phase method utilized a sub-two-micron packed C_{18} stationary phase (50 mm × 4.6 mm, 1.8 μm particle size) and a polar mobile phase of ACN:H_2O:acetic acid (70:30:0.05) (v/v). The column temperature was maintained at (30 ± 2 °C), the injection volume was (8 μl), with a flow rate of (1.1 ml/min). Dermal microdialysis was used to collect (20 μl) samples from healthy, peri-lesional and lesional skin regions, in the forearms of a small cohort of subjects (n = 6), and the ultra sensitive liquid chromatographic method allowed for nanomolar quantitation of histamine in 6.7 min. To date this represents one of the fastest reported separations of histamine using fluorescence detection with very high chromatographic efficiency (258,000/m) and peak symmetry of (0.88). Prior to sample analysis being performed method linearity, precision and limit of detection (LOD) were investigated. The results showed that intracutaneous histamine measured at 70 min after catheter implantation was (3.44 ± .52 nmol) (mean ± SEM) in non-lesional (control) skin and was not dissimilar to that observed in either lesional (3.10 ± .76 nmol) or peri-lesional skin (2.24 ± .20 nmol). A second fraction collected 190 min after implantation also revealed similar levels with no difference in intracutaneous histamine observed between control (2.41 ± .56 nmol), lesional (2.69 ± .54 nmol), or peri-lesional skin (2.25 ± .50 nmol).

1. Introduction

Human skin encounters local external stress in its role as the outermost barrier from the environment. Often systemic, physical and psychological stresses manifest themselves in the skin resulting in severe effects. It is already known that psychological stress can exasperate chronic skin diseases such as psoriasis, atopic dermatitis and chronic urticaria [3]. Psoriasis is a chronic skin disease which results in abnormal activity of a patient's immune system [1]. The disease is characterized by hyperprofileration of epidermal keratinocytes as well as lymphocyte infiltration, mainly consisting of T lymphocytes [4]. Psoriasis commonly presents itself as sharply demarcated scaly erythematous red plaques which localize on the skin surfaces of the body [5]. Psoriatic symptoms can present themselves as mild itching and soreness, to painful debilitating and disfiguring lesions [6]. The disease is strongly influenced by one's genetic predisposition and is associated with many different kinds of co-morbidities. In addition to the physical symptoms, a patient has to cope with social and physiological challenges [7], as this disorder can affect sleep, sexual activity, use of hands,

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walking, sitting and ability to stand for long periods, and performance of occupational tasks [8]. The disease is not yet fully understood, so no cure exists at present and the disorder is managed over a patient's lifetime. However, many studies are ongoing using array based techniques for gene expression studies on both psoriatic and control skin samples. For instance, Gudjonsson et al. recently performed a gene expression study of 58 paired lesional and uninvolved psoriatic skin samples and 64 control skin samples, which revealed a group of genes that are dysregulated in lesional psoriatic skin, a large proportion of which had not been described previously. These results provide novel insights into the pathogenic mechanisms involved in psoriasis, and more accurately define the biochemical changes and pathways that underpin this disorder [9]. Treatment for widespread psoriasis includes UV therapy. In addition to standard broadband ultraviolet radiation B (BUVB) (280–315 nm). Narrowband phototherapy (NBUVB) (monochromatic UV 311–312 nm) and heliotherapy (treatment with natural light) have become important treatment methods for psoriasis [10]. Also, pharmaceutical preparations can include: systemic or topical steroids, cyclosporine, psoralen plus UVA (PUVA) (315–380 nm) retinoids, methotrexate, along with vitamin D_3 analogs [11,12]. To better understand the biochemical processes taking place in normal skin, as opposed to inflammatory skin, analytical methods for determining the concentrations of inflammatory mediators, such as histamine are of utmost importance in dermatological research. Of significance is the characterization of the magnitude and chronology of mediator levels during skin provocation [13]. It is known that the dermis of psoriatic plaques contains an increased number of mast cells [14,15]. The mechanisms that underpin activation and degranulation of these mast cells in psoriatic lesions still remain a mystery. Krogstad et al. reported that histamine concentration and release are increased in lesional skin; however the underlying mechanisms for this are unclear. One hypothesis proposes that neuropeptides transmitted from thin sensory cutaneous nerves continuously stimulate mast cells release of histamine [2,16]. Peterson et al. investigated histamine, mast cells and basophils in psoriasis during treatment with high-dose ranitidine [17]. The authors concluded that dialysate histamine levels in psoriatic skin were higher than in the skin of healthy controls. The diffusion capacity was similar in patients and healthy controls, so this observation seems to reflect increased extracellular histamine in psoriatic skin. However the authors duly point out that non-involved skin in psoriasis can display signs of altered biochemical functions and cellular composition. Their study did not probe this and they highlighted that additional studies may further reveal the pathophysiological roles of histamine in evolving psoriasis, and also explore the therapeutic use of H_2-antihistamines in controlled clinical trials.

Histamine, 2-(4-imidazole)-ethylamine, was chemically synthesized by Windaus and Vogt in 1907 for the first time. It is one of the most widely studied biological amines in medicine, with three pivotal roles in the body: smooth muscle contraction, increased vascular permeability, and stimulation of gastric acid secretion. In addition to this, histamine is involved in immunomodulation, inflammation, regulation of cell proliferation and differentiation, hematopoiesis, embryonic development, regeneration and wound healing. Histamine also acts as a neurotransmitter and plays a pivotal role in the pathogenesis of allergic inflammation. It is formed by the decarboxylation of the amino acid L-histidine in a reaction catalyzed by the enzyme histidine decarboxylase (HDC) [18]. It is essential to have state-of-the-art analytical methods for ultra-sensitive detection and trace level quantification of histamine in a clinical setting. From a literature search it appears that many different analytical separation techniques can be used for histamine determination. These include gas chromatography (GC), high performance liquid chromatography (HPLC), capillary electrophoresis (CE), microchip electrophoresis (MCE), capillary electro-chromatography (CEC), radio enzymatic assay (REA) and radioimmunoassay (RIA) or enzyme immunoassay (EIA) which are based on enzyme–co-substrate binding and antigen-antibody specific immunogenic coagulation, which are frequently used for histamine determination. However the relative standard deviation (RSD) values associated with these assays can sometimes be high (RSD > 5%) and sufficient sensitivity and specificity can be difficult to achieve. Separation techniques and assay methods for histamine and other biogenic amines have previously been reviewed [19,20] and the need for reliable and reproducible quantification methods was highlighted, with LC being the preferred separation method of choice [21]. The most common approach using LC is pre-on-column or post-column derivatization, followed by laser induced fluorescence detection (LIF) of the histamine tagged fluorescent derivative, and there are many derivatizing agents mentioned in the literature each with their own advantages and disadvantages. Yoshitake et al. [22] used 4-(1-pyrene)butyric acid *N*-hydroxysuccinimide ester (PSE) for fluorescence derivatization of histamine which had a retention time of approximately 20 min. In the same year Yoshitake et al. used a PSE derivatizing agent to determine histamine in microdialysis samples from rat brain, separation was achieved within 25 min with detection limits of 0.3 fmol histamine in a 20 μl injection volume [23]. More recently the same group conjugated two PSE molecules via their *N*-hydroxysuccinimide groups to the primary and secondary amino moieties of histamine, thus allowing a more highly sensitive and selective method in comparison to the use of an *ortho*-phthaldialdehyde (OPA) as a fluorescence derivatization reagent. The PSE–histamine derivative gave a single peak with a retention time of 18 min and a detection limit of 0.183 nmol [24]. Yoshida et al. [25] developed a sensitive and selective method for histamine and histidine determination using PSE, the derivatization time was 30 min and the best separation was achieved within 40 min with a 2.3 fmol detection limit (per 5 μl injection volume). von Vietinghoff et al. developed a fluorescence based detection method for histamine and 1-methylhistamine (1-MH) using OPA with a separation time of 15 min with limits of quantification of 50 nmol [26]. More recently Peng et al. developed an automated on-line pre-column derivatization method for determination of histamine using OPA. A limit of detection of 10 ng/ml was obtained for standard histamine with a signal to noise (s/n) ratio of 5 and a separation time of 15 min [27]. It would appear from the literature that histamine is frequently determined in skin studies using the microdialysis sampling technique coupled with either radio or enzymatic immunoassay or LC fluorescence based detection methods [2,13,16,17,28,29]. Therefore there is a lot of scope and requirement to develop a very rapid, ultra-sensitive and highly efficient method for histamine detection using state-of-the-art ultra high performance liquid chromatography (UHPLC). With this aim in mind, we employed the very latest LC separation technology and fluorescence detection to develop a reliable, rapid, sensitive and highly efficient chromatographic method for trace histamine detection in human microdialysis skin samples from the intracutaneous layer of psoriatic plaques. The optimized method allowed for quantification of histamine at the nanomolar level with excellent chromatographic resolution and efficiency. In comparison to the other methods outlined here, this method represents one of the fastest reported separations to date of histamine (6.7 min) using fluorescence detection with efficiency of (258,000/m) and peak symmetry of (0.88). Microdialysis usually results in the collection of minute sample volumes; the order of a few microliters. In our study, UHPLC has demonstrated that it is capable of handling sample volumes of 1 μl or less, which is ideal for coupling with microdialysate sampling, whereas the more conventional LC systems usually require greater than 5 μl sample volumes [30].

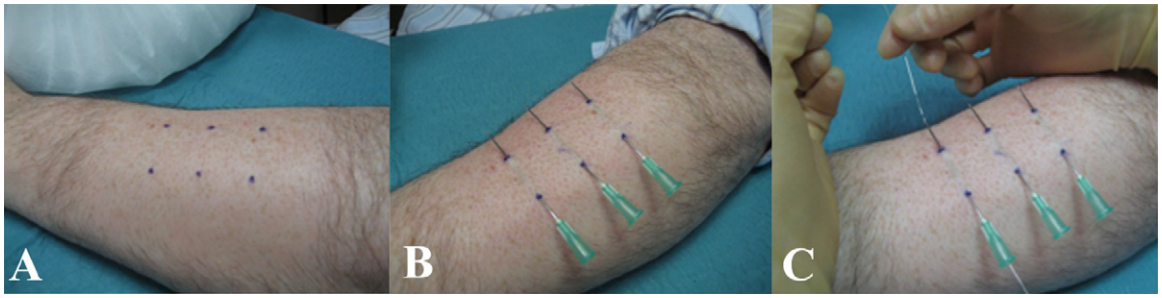

Fig. 1. From left to right: photographs showing the procedure for dermal microdialysis catheter implantation in a healthy control subject.

2. Experimental

2.1. Patients

We investigated 4 age- and sex-matched psoriasis patients, along with 2 age and sex matched healthy controls aged 47 ± 7 years (mean ± SD, range 35–52 years). The patients were being treated for psoriasis for 6–10 years, 3 patients finished treatment during the study, while one patient remained on treatment during the study. The patients had psoriasis for 24 ± 8 years (mean ± SD, range 15–30 years). Psoriatic lesions were carefully selected for dermal microdialysis catheter implantation by a senior dermatologist. For the duration of the study all patients were resting in a comfortable reclining bed position and the room temperature was approximately 25 °C, patients only left the room to visit the bathroom. All patients gave their informed consent for the study which was approved by the ethics research committee at the Mid-Western Regional Hospital, Dooradoyle, Limerick, Ireland.

2.2. Microdialysis – materials procedure and calibration

A CMA 66 linear catheter (10 mm, 20 kDa), pump syringe, and 107 variable flow rate (0.1–5 μl/min) microdialysis pump, and perfusion fluid T1 (chloride Ringer buffer solution) (Na^+ 147 mmol, K^+ 4 mmol, Ca^{2+} 2.3 mmol, Cl^- 156 mmol, pH ∼ 6, osmolality: 290 mosm/kg) were all obtained from Prospect Diagnostics LTD, Dronfield, Derbyshire, UK. Prior to operation, the pump syringe was filled with (2.5 ml) of sterile perfusion fluid from the perfusion vial, and tapped gently to ensure that all air bubbles were removed. The catheter membrane was wetted with perfusion fluid aseptically. The syringe was then connected to the catheter via a luer lock connection. The pump syringe was carefully placed in the syringe housing of the pump, the lid was closed and the tabs were squeezed. This pushed the syringe in place and activated a 5 min flush sequence (15 μl/min) followed by a return to the pre-selected flow rate. Three sets of two dots were marked on the skin using a permanent marker, aligned at equal distances apart to indicate catheter entry and exit points. The area was then shaved and pre-treated with ice packs to provide a localized anesthetic effect. After preliminary optical coherence tomography (OCT) imaging, three sterile needles were inserted through the entry and exit points denoted by the dots at each forearm site as shown in Fig. 1. Three catheters were fed in through each of the three needles, thereby implanted intracutaneously and the needles removed. The catheter membrane must be fully embedded in the skin in the plaque region of interest. The first catheter was inserted into healthy skin, while a second and third were inserted into peri-lesional and lesional regions respectively. The outlet end of each of the three microdialysis catheters were carefully placed into three labeled (0.3 ml) eppendorf vials; a hole was pre-punched in each of the eppendorf lids using a sterile needle and each catheter were fed in through the hole to collect (20 μl) volumes of dialysates. The eppendorf dialysate collection vials were placed on an ice pack to minimize degradation of histamine and taped down to avoid disturbance. Dermal microdialysis commenced after insertion of the three linear catheters which were perfused with Ringer perfusion fluid at a constant flow rate (0.5 μl/min), and dialysate was collected for (10 min) in each of the eppendorf vials. The vials were transported using an ice box to a (−20 °C) freezer where they were stored for chemical analysis. In a preliminary study in healthy controls a concentration curve was established at (0.3, 1 and 2 μl/min) flow rates. On the basis of recovery data a (0.5 μl/min) flow rate was chosen for the study. Catheter position in the intracutaneous layer was confirmed using a swept source, interferometric-based OCT imaging system (Thorlabs, Newton, NJ, USA) operating at a central wavelength of 1325 nm with a bandwidth of >100 nm, resolution of (15 μm and 12 μm) transverse and axial respectively and a maximum imaging depth of (3 mm). Images were recorded by placing a hand held probe above the approximate midpoint of catheter entry and exit points of each implantation site.

2.3. Chemicals and solutions

All chemicals and solvents were of the highest purity available. Histamine (±97%), potassium carbonate (99.99%), and 1-pyrenebutyric acid *N*-hydroxysuccinimide ester (PSE) (95%) were purchased from Sigma–Aldrich, Arklow, Ireland. Acetonitrile (ACN) super gradient grade (B&J) brand for LC (99.95%) and acetic acid were purchased from Reagecon, Shannon, Ireland. Distilled water purified with a Milli-QII system (Millipore, Milford, MA, USA) was used for mobile phase, buffer and solution preparation. A stock solution (1 mmol) of histamine was prepared using distilled water and subsequently diluted for method development and validation studies using distilled water. This solution was stored in a fridge (4 °C) and was freshly prepared every 7 days. A (2.5 mmol) PSE solution was prepared using acetonitrile and was stored at (−20 °C) in a freezer and freshly prepared every 3 days. A (1 mmol) potassium carbonate solution was prepared in distilled water and stored in the fridge (4 °C) and was stable for a one week.

2.4. Sample preparation and derivatization procedure

Following sample collection, all microdialysate samples were immediately stored in a (−20 °C) freezer to minimize degradation of histamine and analyzed within seven weeks of collection. All (20 μl) samples were derivatized employing a variation of a pre-column derivatization procedure as described elsewhere [25] as displayed in Fig. 2. Here a (20 μl) sample and (20 μl) potassium carbonate buffer were combined with PSE (200 μl), vortexed for a few seconds and then heated to (60 °C for 30 min) in amber (2.5 ml) HPLC vials. A thermometer was placed in a test tube heater (Stuart Scientific SHT 2D) to ensure a constant temperature control and this procedure was carried out in a fumehood whereby the amber HPLC vials were tightly sealed. All samples vials were then immediately

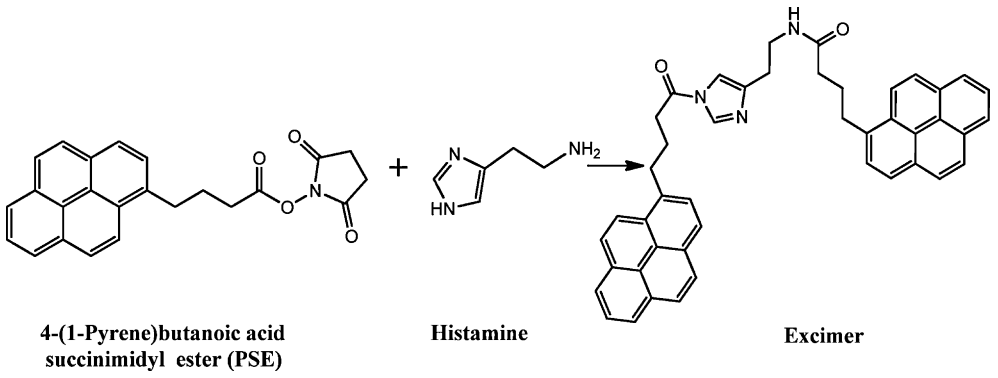

Fig. 2. Derivatization reaction of histamine and 1-pyrenebutyric acid *N*-hydroxysuccinimide ester and formation of an intramolecular excimer complex [25].

cooled in an ice water beaker for a few seconds before transferring to amber HPLC vials which contained (250 μl Agilent) deactivated glass vial inserts. These sample vials were then placed in a refrigerated autosampler (10 °C) along with a series of standard solutions and a blank containing (20 μl) of water.

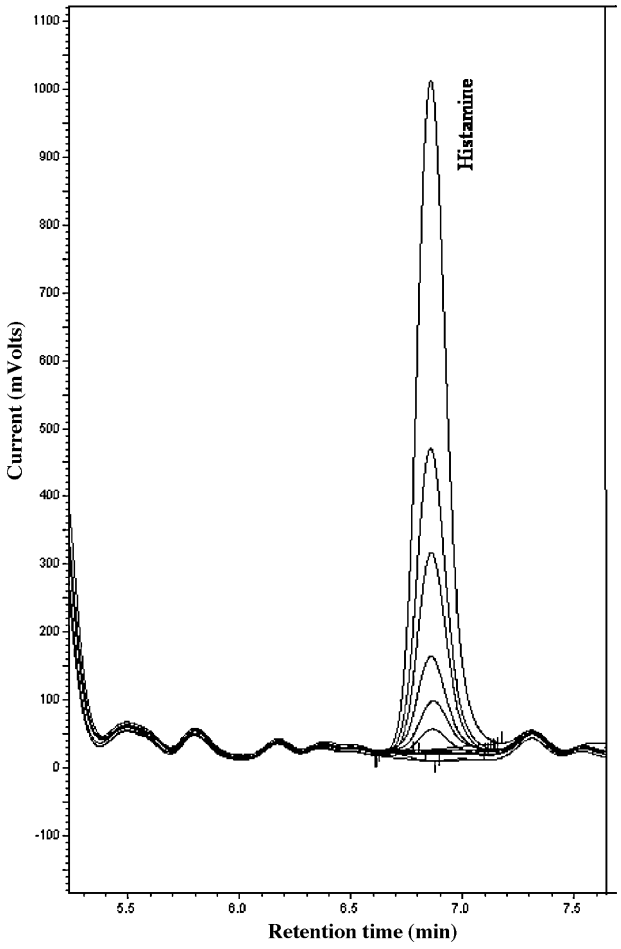

Fig. 3. Chromatographic signal overlay showing histamine standard concentration range (0.4–83 nmol) Separation conditions: (70:30:0.05) (v/v) ACN:H_2O:acetic acid, injection volume (8 μl), fluorescence detection: (ex. 345 nm, em. 475 nm). Column: Agilent Zorbax Eclipse XDB-C_{18} stationary phase (50 mm × 4.6 mm, 1.8 μm particle size), column temperature: (30 ± 2 °C), flow rate: (1.1 ml/min), histamine standard preparation as outlined in Section 2.

2.5. UHPLC separation conditions and mobile phase preparation

All separations were performed using a Shimadzu UFLCxr system (Shimadzu Corporation, Kyoto, Japan), incorporating a Prominence (RF-20AXS) fluorescence detector (ex. 345 nm, em. 475 nm) (gain: 16, sensitivity: medium, response: 0.5 s). The system was controlled by Shimadzu "LC solution" version class – VP 7.4 SP 2 software (Shimadzu Corporation, Kyoto, Japan).

The column was an Agilent Zorbax Eclipse reversed phased XDB-C_{18} stationary phase (50 mm × 4.6 mm, 1.8 μm particle size). The column was temperature controlled to (30 ± 2 °C), the injection volume was (8 μl) and the flow rate was (1.1 ml/min) with a 20 min run time. Sample separation was performed employing a mobile phase containing ACN:H_2O:acetic acid (70:30:0.05) (v/v). The mobile phase was filtered through a Millipore vacuum filtration system equipped with a (0.45 μm) filter, degassed by ultrasonication, for 10 min using an ULTRAsonik NEY. Statistical analysis was performed using Microsoft Office Excel 1997–2003.

2.6. Safety considerations

All chemicals and reagents should be handled and disposed of according to guidelines on the relevant material safety data sheets (MSDS), as provided by the suppliers. During the derivatization step the heating block must not be left unattended and this step should be conducted in a chemical fumehood. All clinical microdialysis samples should be handled with care and treated as a biohazard.

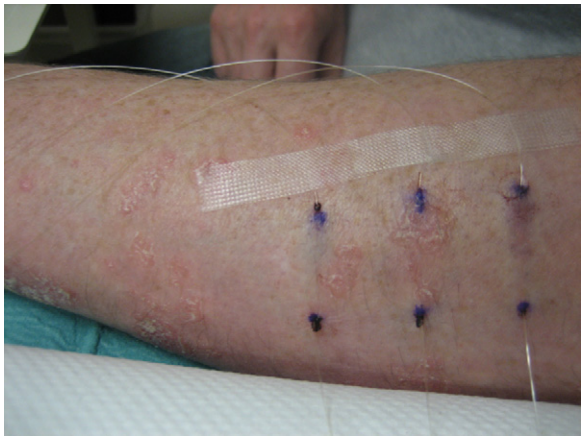

Fig. 4. Photograph showing three catheters implanted into psoriatic plaques in the forearm of a psoriasis patient for microdiaysate collection for subsequent histamine quantification. Microdialysis procedure as outlined in Section 2.

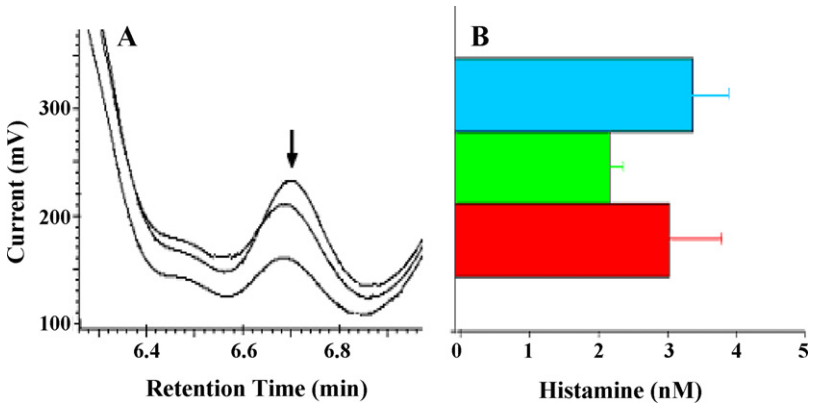

Fig. 5. (A) Chromatogram showing signal overlay for three histamine peaks (arrow) detected in microdialysate samples collected 190 min after catheter implantation in the intracutaneous layer of healthy (top), lesional (middle) and peri-lesional (bottom) skin of a psoriasis patient. (B) Basal dialysate intracutaneous histamine levels (nmol) collected 70 min after catheter implantation in healthy (blue), peri-lesional (green) and lesional (red) skin. Histograms represent the (mean ± SEM) from four psoriasis patients per group. All method conditions as given in Fig. 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results and discussion

3.1. UHPLC method development

The approach that was taken here to develop a rapid, ultra-sensitive and reliable new method for histamine quantification was to employ a reversed phase method using an octadecyl silica (ODS) stationary phase with a bed of 1.8 μm size silica packing coupled with an acidic mobile phase. Histamine is quite a polar analyte so it was difficult to retain chromatographically. Also there were many peaks present from the derivatization reaction due to some unreacted and side product reactions, so a UHPLC method with a sub-two-micron stationary phase packing allowed for extra peak capacity, a high number of theoretical plates, and a much faster separation. This separation efficiency and speed would not have been possible on using the traditional larger stationary phase bed packing of (3 and 5 μm silica sized particles) used for conventional HPLC methods. The microdialysate samples contained a wide range of extracellular molecular components up to 20 kDa in size, however the advantage of this collection method is that the samples were easy to handle and protein free, therefore no sample clean-up step was necessary. All of the proteinaceous material was filtered out through the catheter semi-permeable membrane, making it ideal for derivatization, direct injection and subsequent chromatographic separation using.

3.2. Limit of detection, linearity, precision, and specificity study

A unique feature of this newly designed fluorescence optical system, is that the detector settings (gain, sensitivity and response) can be varied to achieve ultra-sensitive detection. This feature is exclusive to this state-of-the-art new detection system and could prove invaluable for trace histamine detection in clinical samples. On using a sensitivity setting of: medium, with a gain setting of: 16 and detector response of: 0.5 s a limit of detection of (0.4 nmol) was possible for histamine. This was suitable for our study but the limit of detection can be further lowered by variation of the detector sensitivity settings as required. Limit of detection was determined by visual examination of the s/n ratio being (3:1) with serial dilution of a PSE–histamine derivative standard solution. This new detection system can operate at speeds of 100 Hz with wavelength switching for multi-component analysis and is designed for high speed detection and analysis, which is ideally suited for analysis of low volume and low concentration microdialysates.

The emission wavelength was varied (465, 475, 485, and 495 nm) while keeping the excitation wavelength constant, the signal response was highest on using (345 nm and 475 nm), therefore these values were chosen for the rest of the study.

The method was linear over the investigated concentration range of (0.4–83 nmol) with ($n = 6$) and a correlation coefficient of (0.998) as displayed in Fig. 3. Method precision was investigated over the linear range (0.4–10 nmol) ($n = 6$) where the RSD for PSE–histamine derivative peak area was (1%) and the RSD for PSE–histamine derivative retention time was (0.07%) respectively. Six replicate injections of six different standard solutions of histamine–PSE derivative were used to determine linearity and precision in the concentration range of interest highlighted in Fig. 2. Method specificity was evaluated by overlaying PSE–histamine derivative standard samples with PSE-blank samples and no interfering peaks were observed in the histamine region of interest. Also the run time was extended to two and a half times that of the chosen separation time and no extra peaks were detected.

3.3. Determination of histamine in human psoriatic plaques and control skin microdialysates

In order to determine the levels of histamine in human microdialysate samples from the intracutaneous layer of control, peri-lesional and lesional skin regions, the microdialysis catheter was first validated by doing a recovery study at selected perfusion solution flow rates. In our preliminary study, in healthy control skin a concentration curve for histamine was established at (0.3, 1 and 2 μl/min) flow rates and on the basis of the recovery data the (0.5 μl/min) flow rate was selected for the subsequent studies in psoriatic plaques. As mentioned earlier, three catheters were inserted intracutaneously at three forearm sites in each of four sex- and age-matched psoriasis patients as shown in Fig. 4. The first catheter was inserted into healthy skin, while a second and third were inserted into peri-lesional and lesional regions respectively. The catheter position in the intracutaneous layer was confirmed using an OCT imaging system as already detailed in the experimental section.

Using the newly developed UHPLC method, it was possible to measure intracutaneous histamine 70 min after catheter implantation which was (3.44 ± .52 nmol) (mean ± SEM) in non-lesional (control) skin, and was not dissimilar to that observed in either lesional (3.10 ± .76 nmol) or peri-lesional skin (2.24 ± .20 nmol). A second fraction collected 190 min after implantation also revealed similar levels with no difference in intracutaneous histamine observed in control (2.41 ± .56 nmol), lesional (2.69 ± .54 nmol), or peri-lesional skin (2.25 ± .50 nmol). These values are shown in Fig. 5. The present finding provides evidence that when combined

with a novel rapid and sensitive chromatographic method for detecting histamine, intracutaneous microdialysis may be used successfully to explore the role of mast cells in psoriatic skin. Based on the successful detection of nanomolar levels of histamine in the microdialysate samples here, further studies are underway involving a larger cohort of psoriasis patients to investigate the role of histamine in psoriasis.

4. Conclusion

A rapid UHPLC isocratic method was successfully developed for histamine detection in human derived microdialysate samples following derivatization with PSE. The nanomolar levels of histamine present in control, peri-lesional and lesional skin microdialysate samples were in 6.7 min with high chromatographic efficiency and excellent peak symmetry. The RSD value for histamine–PSE derivative peak area was (1%) and the RSD for histamine–PSE retention time was (0.07%) respectively. The study highlights the importance of advancements in analytical chemical separation techniques and their application in clinical analysis in the quest to understand complex skin disorders such as psoriasis. Here microdialysis has shown its potential in skin research and is ideally suited for coupling with miniaturized new analytical separation techniques. Our success in this study can enable us to pursue a further investigation into psoriasis using a larger cohort of patients and research work is ongoing at present.

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