



Development and validation of a reversed-phase high-performance liquid chromatographic method for quantification of peptide dendrimers in human skin permeation experiments

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ABSTRACT

The aim of the present work was to develop and validate a simple RP-HPLC method with UV detection to quantify peptide dendrimers in skin permeation experiments. Six dendrimers of varying positive charges (4⁺, 8⁺ and 16⁺) containing either histidine or arginine as terminal aminoacids were prepared by solid phase peptide synthesis. Mobile phase containing 0.02% (v/v) heptafluorobutyric acid in 90% acetonitrile–water was capable of separating all dendrimers from interfering peaks of receptor fluid. For the calibration of each dendrimer, a different dendrimer from the same class was selected as the internal standard. The results of preliminary human skin permeation studies showed that the developed analytical method can be successfully used for the quantification of cationic poly(aminoacid)-based dendrimers in skin permeation experiments.

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1. Introduction

Dendrimers form a new class of artificial macromolecules with several attractive properties such as extremely low polydispersity, regular and high degree of branching, multi-valency, nano-sized scale, globular architecture and well-defined molecular weight. They are promising new scaffolds for drug/gene delivery along with other applications such as protein mimics, antiviral and anticancer agents and vaccines [1,2].

Studies using non-peptidic poly(amidoamine) (PAMAM) dendrimers in transdermal delivery are present within the literature. Although they exhibited promising results in the delivery of drugs such as tamsulosin [3], indomethacin [4], ketoprofen and diflunisal [5] and 5-fluorouracil [6], their biodegradation and inherent cytotoxicity is an issue that has to a large extent been overlooked [7].

Transdermal drug delivery offers many advantages over the more conventional routes, i.e. reduced side effects, non-invasiveness, improved patient compliance, circumvents the first-pass effect while also enabling sustained drug delivery. The approach also allows the almost immediate interruption or with-

drawal of treatment should the need arise [8]. To this end very few reports have been published on the application of peptide dendrimers in transdermal drug delivery. Peptide-based dendrimers are radial or wedge-like macromolecules comprising of various basic aminoacids linked via peptide/amide bonds which are present both within the branching core and on their outer surface [9,10]. They possess many advantages over the already reported PAMAM dendrimers including lower toxicity, being broken down to harmless aminoacids by endogenous peptidases (whereas PAMAM dendrimers break down into toxic 'acrylates'), cost-effective to synthesize in bulk, easy to purify by RP-HPLC and monodisperse when synthesised by solid phase peptide synthesis.

Hence our goal was to investigate the permeation/deposition of peptide dendrimers across/within human skin and more importantly to assess the effect of iontophoresis on the transdermal permeation of peptide dendrimers. A simple, robust and validated HPLC method was necessary to quantify the dendrimers in and across the skin. Although HPLC methods are available for PAMAM dendrimers, there are no reports on methods for cationic poly(aminoacid) dendrimers [11]. Development of a suitable HPLC analytical method for such compounds is a great challenge as they are not directly amenable to retention and separation under standard conditions using reversed phase (RP) columns because of their high charge/polarity. In such cases, a number of strategies may be followed. Retention may be obtained by employing different types of stationary phases such as hydrophilic interaction chromatogra-

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phy (HILIC) or various ion-pairing reagents may be used as mobile phase additives to achieve retention on RP columns [12].

Ion pair RP-HPLC is a well-known approach and different anionic ion-pairing agents such as hexanesulfonic acid, octanesulfonic acid and perfluoroalkanoic acids (trifluoroacetic acid, pentafluoroacetic acid and heptafluorobutyric acid) have been successfully used to retain peptides and like compounds on RP columns [13–16]. Hence the aim of the present study was to develop and validate a simple, accurate, precise and reliable RP-HPLC analytical method that would enable us to quantify peptide dendrimers synthesized in our laboratory and used in human skin permeation studies.

2. Experimental

2.1. Materials

Acetonitrile, dichloromethane (DCM) and *N,N*-dimethylformamide (DMF) were purchased from Labscan, Thailand. Trifluoroacetic acid (TFA) was purchased from SDS, France. *N,N*-diisopropylethylamine (DIEA), triisopropyl silane (TIPS) and piperidine were procured from Sigma–Aldrich Inc., USA. Fmoc-Gly-OH, Fmoc-Lys(Fmoc)-OH, Fmoc-His(Trt)-OH, Fmoc-Arg(Pbf)-OH, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate (HBTU), Rink amide resin (100–200 mesh; 0.70 mmol/g) were purchased from Novabiochem-Merck, Germany. HEPES was procured from Amresco, USA. Heptafluorobutyric acid (HFBA) was obtained from Fluka, Switzerland. Sodium chloride was purchased from Biolab, Australia. All the chemicals and reagents used were of HPLC/analytical/synthesis grade.

Columns used: C4 Protein column (GraceVydac; size: 4.6 mm × 250 mm; particle size: 5 μm; pore size: 300 Å), C18 Protein column (GraceVydac; size: 4.6 mm × 250 mm; particle size: 5 μm; pore size: 300 Å) and HILIC column (Cosmosil HILIC column; Phenomenex; size: 2 × 150 mm; pore size: 12 nm; particle size: 5 μm).

2.2. Dendrimer synthesis, purification and characterization

Different peptide dendrimers of varying positive charge were synthesized by solid phase peptide synthesis using standard Fmoc chemistry protocols [17] (Table 1; Fig. 1). Briefly, Rink amide resin was swollen with dimethylformamide (DMF) and activated with 20% (v/v) piperidine in DMF, this was followed by flow washing with DMF. Initially, Fmoc-Gly-OH (3 equiv.) activated with HBTU (3 equiv.) and DIEA (3 equiv.), was coupled to the resin. The product was then treated with 20% (v/v) piperidine in DMF and the next amino acid was coupled in the same manner as outlined above. This process was continued until the desired dendrimer was formed. At each amino acid coupling step, its efficiency was confirmed by the Ninhydrin test, with the next amino acid only coupled after achieving at least 99% coupling of the previous amino acid. Once the desired dendrimer was synthesized, it was cleaved off resin by:

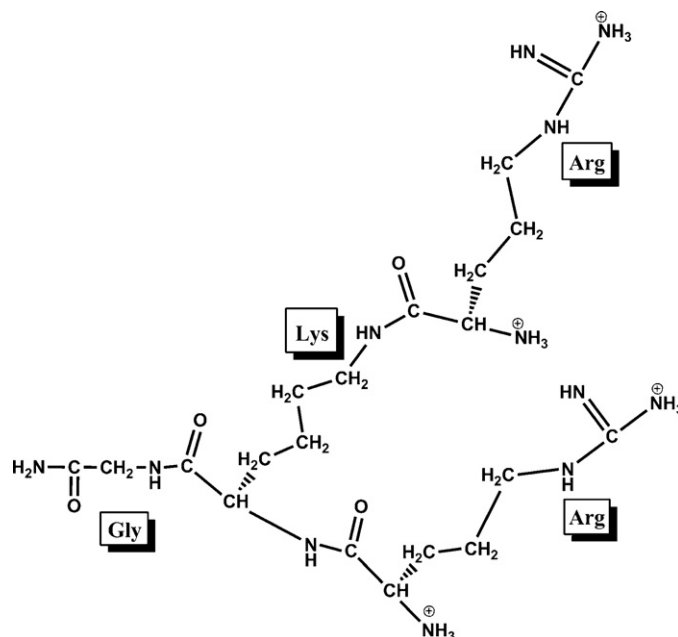


Fig. 1. Structure of 4⁺ arginine (R4) dendrimer: Gly-Lys-(Arg)₂; 515.8 [M+H]⁺.

1. Treating the final product with 20% (v/v) piperidine in DMF (2 × 10 min washes), followed by flow washing with DMF and finally with DCM. The resin was then dried *in vacuo* overnight.
2. Gentle stirring of the dry resin in a mixture of TFA, DCM, H₂O and TIPS (20 mL; 90:5:2.5:2.5) for 3 h and then removal of the TFA mixture *in vacuo*.
3. Azeotroping the residue with toluene (3 × 10 mL) was followed by triturating with ice-cold diethyl ether (3 × 10 mL).
4. The residue obtained was dissolved in deionised (DI) water, lyophilized and stored at 2–8 °C until required.

Dendrimers were purified using a preparative HPLC system employing the following chromatographic conditions:

HPLC: Waters (USA) HPLC system equipped with Waters 600 pump, Waters 600 controller, Waters 2996 PDA detector, Elite All-tech Degassing system and MassLynx software; Column: C4 Protein Preparative column (GraceVydac; size: 22 mm × 250 mm; particle size: 10 μm); Mobile phase: Solvent A: H₂O; Solvent B: 90% acetonitrile_(aq); flow rate: 10 mL/min; run time: 35 min. Crude dendrimer (100 mg) was dissolved in DI water and injected into the column and fractions were collected up to 20 min.

Synthesized dendrimers were characterized by ESI⁺-MS (QTRAP LC/MS/MS system) for [M+H]⁺ peak and analytical RP-HPLC for single peak purity.

2.3. Chromatographic conditions for analytical RP-HPLC

HPLC system (Shimadzu, Japan) was equipped with a pump (LC-10 AD), a system controller (CBM-20A Prominence), a variable wavelength UV/vis detector (SPD-10A), a degasser (DGLU 20As Prominence), an auto-injector (SIL-10A_{XL}) and LC Solution software. The compounds were separated on an RP C4 Protein column (GraceVydac; size: 4.6 mm × 250 mm; particle size: 5 μm; pore size: 300 Å) using a mobile phase composed of- Solvent A: 0.02% (v/v) HFBA in H₂O; Solvent B: 0.02% (v/v) HFBA in 90% acetonitrile_(aq). Following preparation of the solvents, they were all passed through a 0.45 μm filter. The HPLC system was operated in gradient mode as given in following table at a flow rate of 1 mL/min. Injection volume was 100 μL and detection wavelength was 218 nm.

Table 1
Synthesized peptide dendrimers and ESI⁺-MS data.

Dendrimer	Structure and [M+H] ⁺ (calculated)	[M+H] ⁺ (found)
4 ⁺ Histidine (H4)	Gly-Lys-(His) ₂ ; 476.5	477.6
4 ⁺ Arginine (R4)	Gly-Lys-(Arg) ₂ ; 514.6	515.8
8 ⁺ Histidine (H8)	Gly-Lys-(Lys) ₂ -(His) ₄ ; 1007.2	1008.2
8 ⁺ Arginine (R8)	Gly-Lys-(Lys) ₂ -(Arg) ₄ ; 1083.3	1084.4
16 ⁺ Histidine (H16)	Gly-Lys-(Lys) ₂ -(Lys) ₄ -(His) ₈ ; 2068.4	2069.4
16 ⁺ Arginine (R16)	Gly-Lys-(Lys) ₂ -(Lys) ₄ -(Arg) ₈ ; 2220.8	2222.5

Time (min)	0	20	25	30	50
Solvent B (%)	0	100	100	0	0

2.4. Effect of HFBA concentrations and selection of internal standard

The chromatograms for a mixture of H4+H8+H16 and R4+R8+R16 dendrimers in water as well as in receptor fluid were recorded at different concentrations of HFBA (0.01, 0.02 and 0.03% v/v) in Solvents A and B. The effect of different HFBA concentrations on the retention time of each dendrimer was noted. From the obtained chromatograms, an internal standard (IS) was selected which consisted of another dendrimer from the same class (i.e. one comprising the same head group).

2.5. Determination of the limit of quantification (LOQ) and limit of detection (LOD)

The limit of detection (LOD) and limit of quantification (LOQ) for the proposed method was established by signal to noise ratio (S/N ratio). Different standard concentrations ranging from 0.02 to 10 µg/mL of each dendrimer were injected in a decreasing order and peak height was measured. The analyte concentration that produced a S/N ratio >3 was accepted as the LOD and that >10 was accepted as the LOQ [18,19].

2.6. Calibration curves

The linearity of the detector response for the test compounds was evaluated by injecting a total of nine calibration (working) standard solutions of concentrations 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 µg/mL of each dendrimer, covering the full working range of the assay. An internal standard (20 µL; 50 µg/mL) was added to standard solutions to obtain a final internal standard (IS) concentration of 2.5 µg/mL. The calibration curves were constructed by plotting peak area ratios of dendrimers to (IS) against the corresponding concentrations outlined above. The linearity (R^2) of the calibration curves was tested and evaluated using linear regression analysis and coefficients of calibration equation were also expressed.

2.7. Stability of dendrimers

The stability of dendrimers was studied in water and in receptor fluid by determining their concentrations at different time intervals (0, 24 and 48 h).

2.8. Area precision and accuracy

The precision of the assay was determined by injecting 4 standard concentrations of dendrimer (0.1 or 0.2, 1.0, 2.5 and 5.0 µg/mL; 6 times each). The intraday repeatability was assessed by injecting different standard concentrations (1.0, 2.5 and 5.0 µg/mL) 6 times at different times over the course of a day and the interday repeatability was determined by injecting 6 times on two different days. The intra- and interday repeatabilities were quoted as the relative standard deviation (RSD) [18,19].

Accuracy of the analytical method was determined for *in vitro* skin permeation studies as follows: separate samples of receptor fluid were spiked with each dendrimer separately to give final concentrations of 0.1 or 0.2, 2.5 and 5.0 µg/mL, respectively (samples). At the same time, similar concentrations were prepared in water (standards). Six samples and standards (0.1 or 0.2, 2.5 and 5 µg/mL concentrations) were injected into the HPLC and the percentage between each standard and sample (corresponding spiked sample) was calculated.

2.9. *In vitro* skin permeation studies [20]

2.9.1. Passive diffusion

A circular piece of heat separated human epidermis was fixed between two halves of a side-by-side diffusion cell with the epidermis facing the donor compartment. The donor and receptor compartments were filled with 1.7 mL of receptor solution (20 mM HEPES + 150 mM NaCl, pH 7.4) and allowed to equilibrate for 30 min. Membrane integrity was determined using electrical resistance using a digital multimeter and the epidermal membranes showing a resistance of >20 kΩ were used in the study. The donor and receptor compartments were then emptied and the donor compartment was filled with 1.7 mL of donor solution (50 mM HEPES + 75 mM NaCl, pH 4.5) containing 10 mg/mL of dendrimer. The receptor solution was filled with 1.7 mL of receptor solution. The donor and receptor solutions were constantly stirred using stirrer bars. Aliquots of 0.2 mL were removed from the receptor compartment at different time intervals up to 6 h (0.5, 1, 2, 3, 4, 5 and 6 h) and fresh receptor solution added each time. The samples were stored at -80 °C until required for analysis. Internal standards (2.5 µg/mL) were added to the samples just prior to HPLC analysis. The cells were maintained at 32 °C throughout the experiment. After completion of the diffusion study, the membrane integrity was re-measured to confirm that it had retained its resistance (>20 kΩ) after the permeation study.

2.9.2. Iontophoresis

The permeation experiment using iontophoresis was conducted in a similar way to passive diffusion studies except a current of 0.38 mA/cm² was applied using an iontophoresis cell with electrodes being silver (anode; donor compartment) and silver chloride (cathode; receptor compartment).

3. Results and discussion

3.1. Dendrimer synthesis and characterization

The objective of the present study was to develop a simple and validated HPLC method to quantify synthesized cationic poly(aminoacid) dendrimers in skin permeation studies. Dendrimers containing arginine and histidine as their terminal aminoacids and having varying positive charges (arginine group: 4⁺, 8⁺ and 16⁺; histidine group: 4⁺, 8⁺ and 16⁺) were synthesized by solid phase peptide synthesis. The dendrimers synthesized are outlined in Table 1 with the structure of 4⁺ arginine dendrimer (R4) as an example shown in Fig. 1. The yield of all dendrimers after purification was >70%.

All the dendrimers were successfully purified by semi-automated preparative HPLC and were present in the fractions collected within first 10 min of the total run time. In the characterization using ESI⁺-MS, all dendrimers showed the desired molecular ion, [M+H]⁺ with good intensity and the observed molecular ion peaks were in accordance with theoretically calculated molecular weights, confirming formation of the desired product (Table 1). The purity of all dendrimers was also confirmed via analytical RP-HPLC.

3.2. Method development process

For separation of peptides using reversed phase procedures, acetonitrile is generally the organic solvent of choice. The reagent has a low viscosity, allowing high flow rates without causing high back pressures, low absorbance at peptide/amide bond wavelength (e.g. 200–220 nm) and relatively high volatility [21]. All the dendrimers showed no retention when a RP C4 (Protein) stationary phase was employed (retention time ≈ 3 min for all the dendrimers). Retention times were not increased even with the more hydrophobic

RP C18 (Protein) column (retention time \approx 3–5 min for all the dendrimers). Polar stationary phases (e.g. Cosmosil® HILIC column) were also trialled although were unsuccessful in retaining any of the dendrimers. This was surprising given that our dendrimers are fully ionised (positively charged) under the RP-HPLC conditions employed and would be expected to have an increased affinity for the HILIC column. This highlighted that an alternative strategy was needed and we turned our attention to the numerous ion-pairing agents to see if they could offer greater retention.

Ion-pair HPLC is the separation technique of choice for most peptides and like compounds because of its general applicability and high separation efficiency. Initially we selected TFA as the ion-pairing agent, as it is by far the most commonly used ion-pairing reagent. It is generally accepted that TFA, at a final concentration of 0.12% (v/v) in the aqueous solvent and 0.10% (v/v) in organic solvent, provides better retention times for peptides [21]. Initially, TFA (0.10% v/v) was used in both mobile phases with a C4 column. There was an increase in the retention times of 8⁺ and 16⁺ charge dendrimers in comparison with no TFA (retention times of 7.24, 10.36, 7.88 and 10.82 min, respectively for H8, H16, R8 and R16 dendrimers); however the 4⁺ charged dendrimers were not retained (2.82 and 2.88 min of retention times for H4 and R4, respectively). When TFA was used with the more hydrophobic C18 column, the retention times of all the dendrimers increased (c.f. no TFA); but here the retention times of our 4⁺ charged dendrimers was still low and the peaks interfered with peaks of the receptor fluid (retention times of 6.64, 9.14, 10.23, 7.44, 9.55, and 10.50 min for H4, H8, H16, R4, R8 and R16, respectively). Moreover, irrespective of whether C4 or C18 stationary phases were employed (\pm TFA) the chromatograms generated for each group (histidine and arginine dendrimers) were not well resolved. These results confirmed that TFA was not a suitable ion-pairing agent with either C4 or C18 columns for our synthesized peptide dendrimers in human skin permeation experiments. In contrast, it has been reported that TFA (0.14%) increased retention of the G5 PAMAM dendrimer (128⁺ charge), satisfactorily [11]. The favourable retention obtained with G5 PAMAM dendrimers can be attributed to not only the presence of significantly more terminal amine groups but also the larger hydrophobic network which forms the inner core of these commercial dendrimers. Moreover, it has been reported that TFA did not show good extension in retention times or good separation when a mixture of peptides were present [16]. Our aim was to use one of the dendrimers in a class as the internal standard for other dendrimers within the same class. So, to prolong the retention time as well as achieving good separation we trialled HFBA as an alternative ion-pairing agent to TFA. Although the optimum concentration of HFBA has been previously reported to be 0.05–0.10% (v/v) [16,21,22], we began the optimization process with 0.01% (v/v). The effect of various HFBA concentrations on the retention time of dendrimers is presented in Table 2.

At the initial concentration (0.01% v/v) all dendrimers showed satisfactory retention (Table 2). Different gradients were studied at this stage (e.g. 0% solvent B to 100% solvent B linearly in first 15, 20, 25 or 30 min). A gradient of 0% solvent B to 100% solvent B linearly in first 20 min was selected for further studies based

on the peak resolution obtained. HFBA, a more hydrophobic ion-pairing agent than TFA, has been reported to increase the retention time of peptides when TFA had failed to do so [16]. It forms an ion pair with the amino groups of peptide dendrimers more efficiently than TFA thereby increasing their retention on RP stationary media. Moreover it is considered to be the ion-pairing agent of choice where resolving of two peptides is required but has proven to be troublesome [21]. The ion-pairing effect with peptides depends on the hydrophobicity of the reagent used and since HFBA is more hydrophobic than TFA, it exhibited longer retention times in comparison to TFA. In an earlier report, HFBA greatly increased the retention time of peptides in comparison with TFA and pentafluorobutyric acid [23].

The effects of HFBA as an ion-pairing agent are likely to be attributed to interaction with the basic residues of the peptide. However, ion-pairing may also involve a complex multiplicity of phenomena according to both the stoichiometric and thermodynamic retention models [13]. In general, each positive charge, whether originating from a lysine, arginine or histidine side chain, or from any other *N*-terminal α -amino group, exerts an equal effect on peptide retention [24]. A similar mechanism may therefore be responsible for the increased retention seen with our synthesized dendrimers which also possess positive charges arising from histidine and arginine residues. However, electrostatic interaction is not the only driving force that leads to ion pairing; the hydrophobic nature of both the analyte and the ion-pairing reagent are also crucial [13].

Further experiments were carried out using two different HFBA concentrations *viz.*, 0.02 and 0.03% v/v to elucidate the effect of HFBA concentration on retention times. The retention times of all dendrimers were further increased upon raising HFBA concentrations (e.g. H4 dendrimer – 0.02%: 12.06 min; 0.03%: 12.55 min – see Table 2). These results are in accordance with an earlier report where increasing the concentration of HFBA resulted in increased peptide retention times [24]. It was also noted that the retention time of dendrimers increased with an increase in positive charge and molecular weight within the set of dendrimers, and in the presence of HFBA. These results are also in agreement with previous reports where peptides were separated by charged groups (retention times: +1 < +3 < +5) [15]. This indicates the increased electrostatic interaction with the ion-pairing reagent with an increase in charge on dendrimers. Dendrimers with the same charge (whether in the histidine or arginine class) showed almost similar retention times at all the HFBA concentrations tested, indicating that density of positive charge is an important factor in separation of these cationic poly(aminoacids).

Optimum separation of both the histidine- and arginine-dendrimer sets was achieved using a final HFBA concentration of 0.02% (v/v). Our intention was then to use the optimized method to analyze the dendrimers in skin permeation studies and hence we determined whether any peaks arising from substituents in human skin were likely to interfere with the dendrimer peaks. The chromatogram of blank receptor fluid which was in contact with human skin shows that there are no peaks from skin at or near the retention times of any of the dendrimers (Fig. 2A). It was found that when utilising 0.02% (v/v) HFBA all three dendrimer peaks in both classes were devoid of the effect of interfering peaks of human skin constituents (Fig. 2B and C).

3.3. Selection of internal standard (IS)

One dendrimer from each class was selected as the IS for other dendrimers within the same class. The main dendrimer peak and its corresponding IS peaks were well separated from each other. H4 (12.06 min) was selected as the IS for H8 (13.55 min) and H16 (14.31 min). Likewise, R4 (12.16 min) was selected as the IS for R8

Table 2
Effect of HFBA concentration on retention time of dendrimers.

HFBA (% v/v)	Retention time (min) of dendrimers					
	H4	H8	H16	R4	R8	R16
0.01	11.11	12.95	13.73	11.08	13.02	13.94
0.02	12.06	13.55	14.31	12.16	13.57	14.48
0.03	12.55	14.02	14.90	12.63	14.07	15.11

HFBA = Heptafluorobutyric acid.

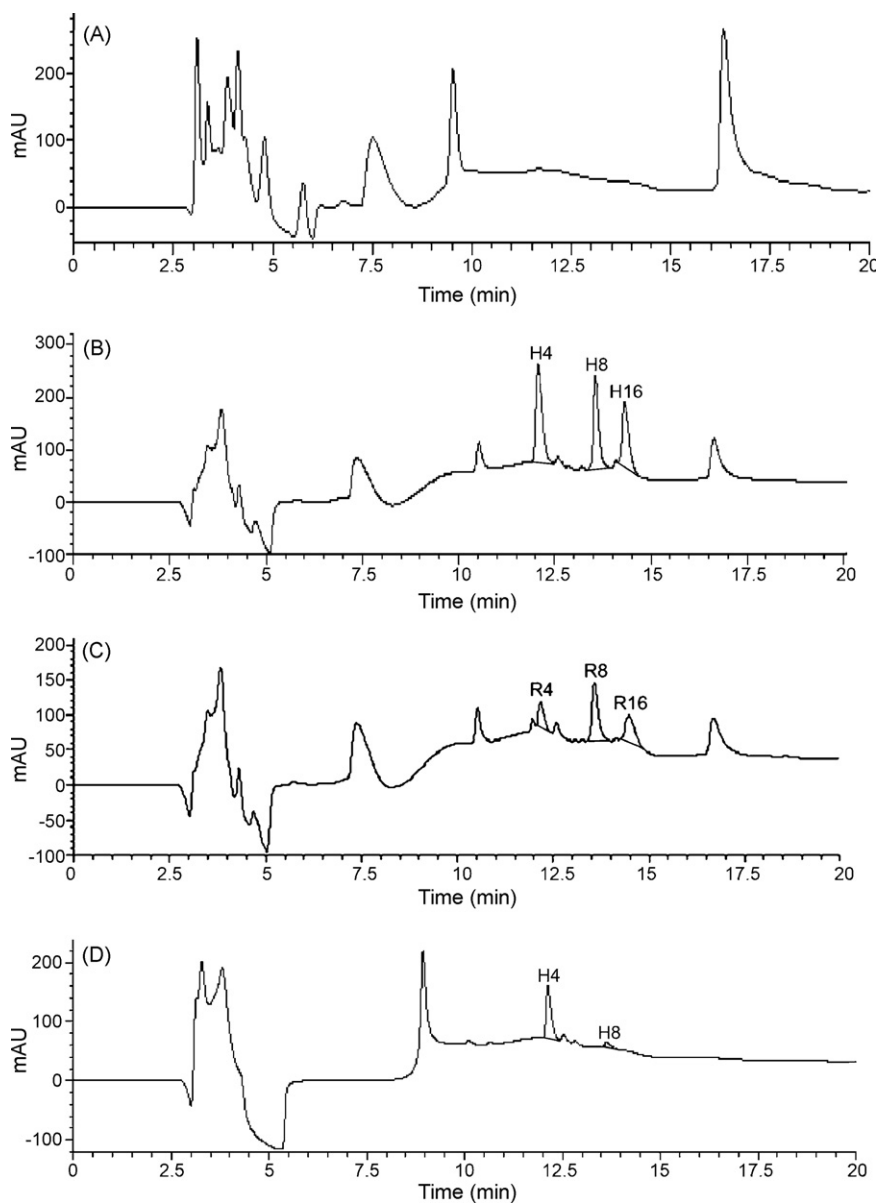


Fig. 2. Representative HPLC chromatograms of blank receptor fluid (A); H4, H8 and H16 dendrimers in receptor fluid which was in contact with human skin (B); R4, R8 and R16 dendrimers in receptor fluid which was in contact with human skin (C); H4 dendrimer in receptor fluid after permeating across the human epidermis using iontophoresis along with its internal standard, H8 (D). Concentration of all dendrimers in Fig. 2B and C is 10 $\mu\text{g/mL}$; conditions: Column-RP C4 Protein column; Mobile phase-Solvent A (0.02% v/v HFBA in H_2O) and Solvent B (0.02% v/v HFBA in 90% acetonitrile_(aq)); gradient: 100% A to 100% B linearly over first 20 min, 100% B for 20–25 min, 100% B to 100% A linearly over 25–30 min, 100% A for 30–50 min; flow rate: 1 mL/min; injection volume: 100 μL ; detection wavelength: 218 nm.

(13.57 min) and R16 (14.48 min). H4 and R4 were used as internal standards for H8 and R8, respectively.

3.4. LOQ, LOD, calibration curves and stability studies

The results of LOQ and LOD are presented in Table 3. The detection and quantification limits were found at very low concentrations which would enable us to analyse the dendrimers in skin permeation studies where usually very low permeation of compounds is expected. LOQ and LOD of all dendrimers were found to be in the range of 22–42 and 7–12 ng/mL, respectively.

Parameters pertaining to the calibration curves are presented in Table 3. The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations of the analyte in the sample. With every synthesized dendrimer, the calibration curve was found to be linear with R^2 values >0.9990. The range employed for the calibration curves was

0.025–10 $\mu\text{g/mL}$ (for H4, R4, H8 and R8 dendrimers; 9 concentration points) and 0.05–10 $\mu\text{g/mL}$ (for H16 and R16 dendrimers; 8 concentration points).

In stability studies, all the dendrimers exhibited $\geq 98\%$ stability at the end of 48 h at room temperature both in water as well as in receptor fluid suggesting their excellent stability in the tested media under ambient conditions.

3.5. Assay precision and accuracy

The results of validation parameters such as precision and accuracy are also presented in Table 3. The area of precision for all the dendrimers was excellent as indicated by very low RSD values ranging between 0.05 and 1.51% at all the different standard concentrations tested (0.1 or 0.2, 1.0, 2.5 and 5.0 $\mu\text{g/mL}$). The intra- and interday precision was also determined at 1.0, 2.5 and 5.0 $\mu\text{g/mL}$ concentration levels and RSD values were again found

Table 3
Precision, accuracy, LOQ, LOD and calibration curve parameters.

Dendrimer	Precision						Accuracy (%)			LOQ and LOD ($\mu\text{g/mL}$)		Calibration curves			
	5 $\mu\text{g/mL}$		2.5 $\mu\text{g/mL}$		1 $\mu\text{g/mL}$		0.1 ^a or 0.2 ^b $\mu\text{g/mL}$		5 $\mu\text{g/mL}$	2.5 $\mu\text{g/mL}$	0.1 ^a or 0.2 ^b $\mu\text{g/mL}$	LOQ	LOD	R ²	Linear range ($\mu\text{g/mL}$)
	Area ratio	RSD (%)	Area ratio	RSD (%)	Area ratio	RSD (%)	Area ratio	RSD (%)	Area ratio	RSD (%)	Area ratio	RSD (%)			
H4	2.10 ± 0.007	0.33	1.07 ± 0.005	0.48	0.40 ± 0.003	0.90	0.037 ± 0.0004	1.21	99.9 ± 0.11	100.0 ± 0.51	100.1 ± 0.29	0.022	0.007	1.000	0.025–10
H8	1.84 ± 0.003	0.17	0.92 ± 0.001	0.19	0.37 ± 0.004	1.08	0.032 ± 0.0004	1.28	99.9 ± 0.20	100.1 ± 0.39	100.3 ± 0.61	0.024	0.007	1.000	0.025–10
H16	1.53 ± 0.001	0.05	0.73 ± 0.002	0.40	0.26 ± 0.002	0.95	0.042 ± 0.0003	0.78	99.8 ± 0.19	100.1 ± 0.43	99.8 ± 0.59	0.041	0.012	0.9997	0.050–10
R4	2.19 ± 0.004	0.17	1.03 ± 0.007	0.75	0.41 ± 0.004	1.19	0.042 ± 0.0004	0.88	99.9 ± 0.25	100.1 ± 0.74	100.5 ± 0.35	0.024	0.007	0.9998	0.025–10
R8	1.96 ± 0.002	0.11	0.96 ± 0.011	1.15	0.36 ± 0.004	1.33	0.040 ± 0.0002	0.59	99.8 ± 0.26	100.2 ± 0.70	99.7 ± 0.25	0.023	0.007	0.9999	0.025–10
R16	1.10 ± 0.003	0.31	0.49 ± 0.001	0.33	0.17 ± 0.001	0.68	0.033 ± 0.0005	1.51	100 ± 0.44	100.4 ± 0.45	100.3 ± 0.39	0.042	0.012	0.9991	0.050–10

Area ratio and accuracy values are expressed as mean ± SD, n = 6.

^a For H4, H8, R4 and R8 dendrimers.^b For H16 and R16 dendrimers.

to be favourable for all dendrimers (0.37–1.73% and 0.33–1.71%, respectively for intra- and interday precision; detailed data not shown). The precision results are well within the acceptable criteria for intra- and interday repeatability of RSD at <5% [19]. The accuracy data indicated that the developed method permitted the detection of all dendrimers at 0.1 or 0.2, 2.5 and 5 $\mu\text{g/mL}$ concentrations with an accuracy of 99.7–100.5% (Table 3).

3.6. In vitro skin permeation studies

To test whether the developed and validated RP-HPLC method could be successfully employed to quantify the cationic poly(aminoacids) in skin permeation experiments, a series of passive diffusion and iontophoresis studies were conducted using human skin. Iontophoresis is a physical skin permeation enhancement technique generally employed for charged molecules. We intended to increase the permeation of peptide dendrimer using iontophoresis if negligible permeation was observed with passive diffusion.

Using passive diffusion, we were not able to quantify any of the dendrimers at various time intervals up to 6 h. No peak corresponding to any of the dendrimers (except internal standards) was found in passive diffusion studies up to 6 h. Another set of passive diffusion experiments were then conducted up to 24 h with similar sampling intervals up to 6 h as in the previous set of diffusion experiments, and one additional sampling performed 24 h after the 6 h sampling. At the end of 24 h, very low concentrations of H4 and R4 dendrimers only were found (0.15 ± 0.06 and $0.34 \pm 0.06 \mu\text{g/mL}$, respectively; mean ± SD, n = 3). The remaining four dendrimers did not show any permeation at 24 h.

Iontophoresis was then employed and this significantly ($p < 0.05$) increased the permeation of all dendrimers. Intense peaks were found for each dendrimer in the chromatograms at different time intervals so that they were easily quantified. The extent of penetration varied between the dendrimers in a manner consistent with penetration being dendrimer size dependent. A typical chromatogram showing the easily identifiable peak of H4 dendrimer along with internal standard (H8) is shown in Fig. 2D. These results confirm that the synthesized peptide dendrimers permeate via passive diffusion (only H4 and R4 dendrimers) albeit to a much lesser extent than when iontophoresis is employed. The physicochemical properties of peptide dendrimers (charged; high MW) render them inappropriate for passive transdermal delivery and it is widely accepted that molecules >500 Da are not suitable candidates for skin permeation by passive diffusion [8,25]. In contrast, iontophoresis, an electrically assisted drug delivery technology, offers a controlled and non-invasive route of administration for charged molecules even with high molecular weight [25,26]. Our findings with peptide dendrimers indicated that all the synthesized dendrimers permeated across the skin by iontophoresis and only the lower molecular weight dendrimers permeated in passive diffusion. Since the primary purpose of this part of the study was to determine whether this method can be successfully used in skin permeation studies of cationic poly(aminoacid) dendrimers, the detailed results pertaining to transdermal permeation and skin deposition are ongoing and will be published shortly.

Given our findings it seems feasible that the same protocol outlined above can be used with mass spectrometric detection (LC-ESI-MS) in the analysis of peptide dendrimers with HFBA. As a result of the relatively high volatility of HFBA [27] and the very low concentrations needed to impart a good ion-pairing effect, it will very likely contribute a negligible ion-suppression effect. Another application of this method would be in the purification of peptide dendrimers. If used in the purification of peptide-based macromolecules such as dendrimers, volatile HFBA can be removed upon lyophilization and thus should not interfere with most pep-

tide assays compatible with trifluoroacetate peptide salts [16]. We are also currently working on using this method with HFBA to purify a different series of peptide dendrimers.

4. Conclusions

A simple, precise and accurate RP-HPLC method has been developed and validated using HFBA as the ion-pairing agent to quantify synthesized, highly charged peptide dendrimers. HFBA (0.02% v/v) increased the retention time of all the synthesized peptide dendrimers satisfactorily and the peaks were devoid from the effect of interfering peaks of skin constituents. This method was successfully used to quantify cationic poly(aminoacids), i.e. peptide dendrimers in skin permeation studies where passive diffusion and iontophoresis were employed. The volatile nature and low concentration of HFBA employed in this study (0.02% v/v) demonstrates its possible adaptability to an LC-ESI-MS method of analysis.

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