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# An optimized reverse-phase high performance liquid chromatographic method for evaluating percutaneous absorption of glucosamine hydrochloride

Ismaiel A. Tekko [a], Michael C. Bonner [a], Adrian C. Williams [b,*]

[a] *Drug Delivery Group, School of Pharmacy, University of Bradford, Richmond Road, Bradford, West Yorkshire BD7 1DP, UK*
[b] *School of Pharmacy, University of Reading, P.O. Box 224, Reading, Berkshire RG6 6AD, UK*

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**Abstract**

A relatively simple, selective, precise and accurate high performance liquid chromatography (HPLC) method based on a reaction of phenylisothiocyanate (PITC) with glucosamine (GL) in alkaline media was developed and validated to determine glucosamine hydrochloride permeating through human skin in vitro. It is usually problematic to develop an accurate assay for chemicals traversing skin because the excellent barrier properties of the tissue ensure that only low amounts of the material pass through the membrane and skin components may leach out of the tissue to interfere with the analysis. In addition, in the case of glucosamine hydrochloride, chemical instability adds further complexity to assay development.

The assay, utilising the PITC–GL reaction was refined by optimizing the reaction temperature, reaction time and PITC concentration. The reaction produces a phenylthiocarbamyl–glucosamine (PTC–GL) adduct which was separated on a reverse-phase (RP) column packed with 5 μm ODS ($C_{18}$) Hypersil particles using a diode array detector (DAD) at 245 nm. The mobile phase was methanol–water–glacial acetic acid (10:89.96:0.04 v/v/v, pH 3.5) delivered to the column at 1 ml $min^{-1}$ and the column temperature was maintained at 30 °C. Galactosamine hydrochloride (Gal-HCl) was used as an internal standard.

Using a saturated aqueous solution of glucosamine hydrochloride, in vitro permeation studies were performed at 32 ± 1 °C over 48 h using human epidermal membranes prepared by a heat separation method and mounted in Franz-type diffusion cells with a diffusional area 2.15 ± 0.1 $cm^2$.

The optimum derivatisation reaction conditions for reaction temperature, reaction time and PITC concentration were found to be 80 °C, 30 min and 1% v/v, respectively. PTC–Gal and GL adducts eluted at 8.9 and 9.7 min, respectively. The detector response was found to be linear in the concentration range 0–1000 μg $ml^{-1}$. The assay was robust with intra- and inter-day precisions (described as a percentage of relative standard deviation, %R.S.D.) <12. Intra- and inter-day accuracy (as a percentage of the relative error, %RE) was ≤−5.60 and ≤−8.00, respectively. Using this assay, it was found that GL-HCl permeates through human skin with a flux 1.497 ± 0.42 μg $cm^{-2}$ $h^{-1}$, a permeability coefficient of 5.66 ± 1.6 × $10^{-6}$ cm $h^{-1}$ and with a lag time of 10.9 ± 4.6 h.


*Keywords:* Glucosamine hydrochloride; Percutaneous absorption; Reverse-phase high performance liquid chromatography; Derivatisation; Phenylisothiocyanate; Skin

## 1. Introduction

Glucosamine (GL) is an amino monosaccharide that is classified by the food and drug administration (FDA) as a dietary supplement and which has gained a great deal of public interest as a potential treatment for osteoarthritis [1]. This compound is an intermediate substrate involved in the biosynthesis of proteoglycans and glycosaminoglycan that are found in gastrointestinal mucosal membranes, the articular cartilage matrix and synovial fluid [2,3]. It has been suggested that GL from exogenous sources stimulates the production of proteoglycans and incorporates into the metabolic pathway for glycosaminoglycan synthesis in the articular cartilage, thus inhibiting its degradation [4] and indeed can rebuild damaged cartilage [5].

Typically a salt of glucosamine (either the hydrochloride (GL-HCl) or sulphate) alone or in combination with other

* Corresponding author. Tel.: +44 118 378 6196; fax: +44 118 378 6632.
*E-mail address:* a.c.williams@reading.ac.uk (A.C. Williams).

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ingredients (for example chondroitin sulphate) are formulated into capsules, tablets and liquids for oral administration and have been marketed heavily over the last two decades. The recommended oral dose is 1500 mg day$^{-1}$ and is generally administered in two or three doses. However, pharmacokinetic studies in rats showed that glucosamine undergoes extensive hepatic first-pass metabolism resulting in a relatively low bioavailability (about 19%) [6]. Moreover, the average elimination half-life of unchanged GL after intravenous dosing is about 1 h and is around 2 h after oral dosing [6].

Transdermal delivery route possess several advantages in therapy compared with oral route. These include circumventing first-pass metabolism or other possible problems associated with passage through gastrointestinal tract, producing relatively constant plasma levels of drugs and improving the patient compliance [7]. Considering the extensive first-pass metabolism and rapid elimination of glucosamine, transdermal delivery appears an attractive alternative route to oral dosage forms, though the high doses recommended and high water solubility of the compound could in themselves prove problematic. With increasing sales and marketing of topical preparations of glucosamine, such as creams, gels and patches, for the treatment of osteoarthritis, we have recently investigated transdermal permeation of GL-HCl through human epidermal membranes from a 10% w/v aqueous solution spiked with a tritium labelled isotope of GL-HCl, using scintillation counting for analysis [8]. Radioisotopic determinations of permeants traversing human skin are attractive as typically very low levels of compounds traverse the skin barrier. However, tritium labelled compounds are prone to tritium exchange; though we compensated for exchange with water in our studies, scintillation counting does not allow us to differentiate the parent compound from its metabolites and/or degradation products. Thus, an alternative appropriate analytical technique was sought.

As glucosamine is a relatively fragile permeant, prone to degradation and metabolism, a chromatographic method was desirable to detect the compounds permeating through skin whilst being relatively rapid and easy to use with appropriate limits of detection [9]. However, GL or its derivatives do not contain a chromophore absorbing in a region useful for ultraviolet detection. Thus, several approaches were proposed to overcome the detection difficulty; a HPLC method with a refractive index detector was described to quantify glucosamine in raw materials and dosage forms [10]. Unfortunately, the low sensitivity and selectivity preclude using this method to quantify glucosamine in our biological samples. Pre-column derivatisation using UV tags such as 1-naphthyl isothiocyanate (NITC) [11] or phenylisothiocyanate (PITC) [12] have also been described for glucosamine assay in raw materials, dosage forms, and plasma. Pre-column derivatisation using NITC offers relatively high sensitivity (up to 1 μg ml$^{-1}$) and is selective, yet the reaction is water-sensitive so samples must be dried before adding the UV tag (our biological samples are wet). The procedure also requires extraction of the excess derivatising reagent and its degradation products, which may affect the NITC–GL adduct recovery. Further, the NITC–GL adduct elutes at 29 min in the HPLC analysis which is time and solvent consuming.

In comparison, pre-column derivatisation using PITC offers relatively simple sample preparation and a short analysis time; the reaction produces a phenylthiocarbamyl–glucosamine (PTC–GL) adduct. However, the derivatisation reaction was poorly reproducible with turbid solutions forming after derivatising and reconstituting samples with the HPLC mobile phase [11]. This method was recently modified by other workers and employed to assay glucosamine sulphate [13], yet the method was not fully optimized and was validated within only a narrow concentration range (16–28 μg ml$^{-1}$).

The objective of our study was to optimise and validate a PITC–GL reaction method for determining glucosamine. The factors which may influence the efficiency and reproducibility of the derivatisation process, were identified and optimized. The method was validated following the International Conference of Harmonisation (ICH) guidelines [14] and was successfully employed to quantify GL-HCl after permeation through human epidermal membrane. The permeation parameters determined using this protocol were then compared with data obtained using our previous radiotracer assay method.

## 2. Materials and methods

### 2.1. Materials

D (+) Glucosamine hydrochloride (GL-HCl) and triethylamine were purchased from Sigma Chemical Company (St. Louis, USA). D (+) Galactosamine hydrochloride (Gal-HCl) was obtained from Acros Organics, USA. Phenyl isothiocyanate (PITC) was purchased from Lancaster Synthesis, UK. Methanol (HPLC grade) was from Fisher, UK and glacial acetic acid, di-sodium phosphate and *ortho*-phosphoric acid 85% v/v were obtained from BDH, UK. PTFE syringe filters (0.45 μm pore size, 13 mm diameter) were purchased from BGB analytic AG, Germany. MilliQ water was used to prepare solutions.

### 2.2. Methods

#### 2.2.1. Preparation of glucosamine standard solutions, buffers and reagent solutions

GL-HCl standard solutions were prepared from an aqueous stock solution of GL-HCl (100 mg ml$^{-1}$ calculated as the free base) by dilution to produce solutions of 0.5, 1, 10, 20, 50, 100, 500, and 1000 μg ml$^{-1}$. Standard solutions of GL-HCl at 0.5, 1, 5, 10, 20, 50, 100, 500 and 1000 μg ml$^{-1}$ were also prepared in a biological matrix (see Section 2.2.4).

PITC solutions were freshly prepared in methanol at 0.1, 1, 2.5 and 5% v/v immediately before the derivatisation step.

0.3 M phosphate buffer solution was prepared in water using di-sodium phosphate and adjusted to (pH 8) by ortho phosphoric acid 85% v/v.

Triethylamine (TEA) solution (1% v/v) was prepared in methanol–water (80:20 v/v) and adjusted to pH 8 using glacial acetic acid.

*2.2.2. Derivatisation procedure*

Four hundred microliters of GL-HCl solution along with 100 μl of Gal-HCl (40 μg ml$^{-1}$) were transferred into a 2 ml screw capped glass vial. Then, 250 μl of 0.3 M phosphate buffer (pH 8.0) and 200 μl of methanol were added, shaken and left for 15 min before 250 μl of PITC methanolic solution was added. The solutions were vortexed for 30 s before being placed in an oven at the test reaction temperature for a defined time. The samples were then cooled to 4 °C before evaporation to dryness at 50 °C under either a vacuum or nitrogen. The residue was dissolved in 400 μl of HPLC mobile phase, filtered through a PTFE syringe filter (0.45 μm) before analysis. The reaction efficiency was assessed by comparing the peak areas of PTC–GL after calibration with the internal standard (PTC–Gal) peak area.

*2.2.3. Factors may affect PITC–glucosamine reaction*

In order to obtain a reproducible PITC–GL reaction, the influence of reaction temperature, reaction time and PITC concentration were investigated and optimized. A standard solution containing 20 μg ml$^{-1}$ GL-HCl and 10 μg ml$^{-1}$ Gal-HCl (internal standard) was used to probe the above factors. To investigate reaction temperature effects, derivatisation was performed for 30 min using 5% v/v PITC at room temperature (RT), 30, 60, 80 and 100 °C. When examining the effect of reaction time, derivatisation was conducted at 80 °C using 5% v/v PITC for 10, 20, 30 and 60 min. To investigate PITC concentration effects, the reaction was performed at 80 °C for 30 min using 0.1, 1, 2.5 and 5% v/v PITC methanolic solution. Solutions were assayed according to the method below (see Section 2.2.5).

*2.2.4. Preparation of human skin extract*

Human skin extract was obtained following a method developed by Chun and Chein [15] using Franz-type diffusion cells at 32 °C for 48 h. Human epidermal membranes were prepared using a heat separation method [16] and were mounted between the two compartments of diffusion cells, with stratum corneum facing the donor compartment. Both donor and receptor chambers were filled with 0.002% w/v aqueous sodium azide solution (typical concentration when used as an antimicrobial agent) and extraction of skin components was permitted for 48 h. After 48 h, the receptor solution from each diffusion cell was collected and combined then stored in the freezer until use.

*2.2.5. Instrumentation and chromatographic conditions*

Separation of phenylthiocarbomyl–glucosamine (PTC–GL) and the internal standard phenylthiocarbomyl–galactosamine (PTC–Gal) adducts from other compounds was achieved on a reverse-phase column (250 mm × 4.6 mm, i.d.) packed with 5 μm ODS ($C_{18}$) Hypersil particles (Jones Chromatography, UK) guarded by a column (25 mm × 4.6 mm, i.d.) packed with the same materials using a high performance liquid chromatography (HPLC) system (Hewlett Packard 1100, Palo Alto, California) fitted with UV–vis diode array detector (DAD). Samples (20 μl) were injected using an auto-sampler with a 100 μl loop. The solvent reservoirs were de-gassed by a steam of helium. Analysis was carried out using a methanol–water–glacial acetic acid (10:89.96:0.04 v/v/v, pH 3.5) mobile phase delivered at 1 ml min$^{-1}$. The column was maintained at 30 °C and the UV detector, following a wavelength scan, was operated at 245 nm.

*2.2.6. Instrument calibration*

The calibration curve was constructed using triplicate concentrations of 0, 0.5, 1, 5, 10, 20, 50, 100, 500 and 1000 μg ml$^{-1}$ GL-HCl standard solution along with the internal standard (Gal-HCl, 10 μg ml$^{-1}$). Calibrants were derivatised using the optimized conditions (80 °C for 30 min with 1% v/v PITC methanolic solution) and assayed as above. The PTC–GL/PTC–Gal peak area (milli absorbance units seconds, mAU s) ratio was plotted versus the nominal GL-HCl concentration. The linearity of the method was assessed by the least squares linear regression coefficient ($R^2$).

*2.2.7. Method validation using GL-HCl standard solutions prepared in the biological matrix*

The method was validated in the biological matrix (skin extract obtained as Section 2.2.4) following the ICH guidelines [14].

*2.2.7.1. Method selectivity.* Method selectivity was verified by screening blank samples ($n = 10$), which were taken from the receptor solution prepared as described in Section 2.2.4. Half of the samples were spiked with the internal standard (Gal-HCl). All samples were then derivatised according to the optimized conditions as above and then assayed by HPLC. The retention times of leached and derivatised skin components were then compared with those of PTC–GL and PTC–Gal to ensure no interference.

*2.2.7.2. Calibration curve/linearity.* The calibration curve was constructed in triplicate using nine concentrations (0.5, 1, 5, 10, 20, 50, 100, 500 and 1000 μg ml$^{-1}$) of GL-HCl prepared in the drug-free biological matrix (receptor solution), along with a blank sample of the receptor solution. Internal standard (10 μg ml$^{-1}$) was added and the whole set was derivatised at the optimized condition before HPLC analysis. Linearity was tested between 0 and 1000 μg ml$^{-1}$ by the least square regression coefficient ($R^2$) and a lack-of-fit determination ($P = 0.05$).

*2.2.7.3. Precision and accuracy.* Intra-day precision was evaluated by the relative standard deviation (%R.S.D.) of five measurements ($n = 5$) of four concentrations (1, 10, 100 and 1000 μg ml$^{-1}$) of GL-HCl standard solution prepared in the biological matrix (receptor solution). This protocol was repeated over 5 days to test inter-day precision. %R.S.D. was calculated by:

$$\%\text{R.S.D.} = \frac{\text{S.D.}}{\text{mean}} \times 100 \quad (1)$$

The precision of the method is generally considered to be acceptable if %R.S.D. is lower than 15%, except at the lower limit of quantitation (LLOQ) where it should not deviate by more than 20% [14].

Intra- and inter-day accuracies were tested by determining the standardised agreement between the measured value and

the true value and expressed as a relative error (%RE) of five measurements ($n = 5$). Again, four concentrations (1, 10, 100 and 1000 μg ml$^{-1}$) of GL-HCl standard in the biological fluid were used for these evaluations. %RE was calculated by:

$$\%\text{RE} = \frac{\text{mean exptl. conc. of solution} - \text{true conc.}}{\text{true conc.}} \times 100 \quad (2)$$

The accuracy of the method was considered to be acceptable when %RE is lower than 15% except at LLOQ where it should not deviate by more than 20%.

*2.2.7.4. Limit of detection (LOD) and lower limit of quantitation (LLOQ).* Limit of detection (LOD) can be defined as the sample concentration resulting in a peak area of three times the noise level (signal to noise ratio = 3:1).

The lower limit of quantitation (LLOQ) can be defined as the lowest drug concentration which can be determined, where the signal to noise ratio is 10:1, with an accuracy (%RE) and precision (%R.S.D.) lower than 20%.

*2.2.8. In vitro permeation studies*

For permeation studies, an aqueous saturated solution of GL-HCl was prepared by dissolving an excess amount of GL-HCl in water and warming at 60 ± 1 °C for 30 min before the solution was equilibrated at 32 ± 1 °C for 48 h with stirring. The donor solution was then filtered (PTFE filter, 0.45 μm pore size) producing a saturated solution containing 265.9 mg ml$^{-1}$ of GL-HCl (calculated as the free base).

Human epidermal membranes (HEMs) were prepared from abdominal skin specimens obtained from three female donors (74, 82 and 57 years) by a heat separation technique [16]. Skin specimens were thawed at room temperature and the subcutaneous fat was removed before the skin was immersed in distilled water at 60 ± 1 °C for 60 s, after which the epidermal membrane was gently peeled from the dermis. Epidermal membranes were then hydrated for 24 h by immersion in an aqueous solution of 0.002% w/v sodium azide at room temperature.

Diffusion studies used HEMs sandwiched between the two compartments of Franz-type diffusion cells, with the stratum corneum facing the donor compartment, providing an effective diffusional area of 2.15 ± 0.10 cm$^2$. The receptor compartments (5 ± 0.5 ml) were filled with an aqueous solution of 0.02% w/v sodium azide (a concentration which minimized glucosamine degradation through microbial and enzymatic activity during drug permeation) and was stirred using Teflon coated magnetic stirrers. The diffusion cells were equilibrated at 32 ± 1 °C for 24 h prior to conducting the in vitro diffusion studies. Blank diffusion cells (no donor solution loaded) were also utilized to avoid erroneous results which could arise from possible interference of skin components leaching into the receptor fluid during the permeation studies.

Diffusion studies were performed over 48 h by loading 2 ml (an infinite dose) of the GL-HCl saturated solution into the donor compartment of each diffusion cell ($n = 5$). Samples (0.4 ml) were withdrawn from the receptor compartments at regular intervals throughout the study period and replenished with a fresh temperature-equilibrated receptor fluid. These samples were spiked with 100 μl of the internal standard Gal-HCl solution (40 μg ml$^{-1}$), then derivatised at the optimized conditions (80 °C, 30 min, 1% v/v PITC methanolic solution) and analyzed for GL-HCl content by HPLC as above.

*2.2.9. Calculations and data analysis*

Data are presented as mean ± standard deviation (S.D.) with the Student's *t*-test used for statistical comparisons; values of $P < 0.05$ were considered statistically significant. Additionally, linearity of response for our calibration curves used least squares as recommended in the ICH guidelines [14] but also, as a more robust estimator of response function linearity a lack-of-fit test was performed.

## 3. Results and discussion

### 3.1. Investigating factors that may influence PITC–glucosamine reaction

PITC has been used extensively in analytical determinations of amino acids and related structures [17]. The reaction between PITC and various primary amines under alkaline conditions produces a stable phenylthiocarbamyl adduct, which can be separated by reverse-phase high performance liquid chromatography and monitored by ultraviolet detection at 254 nm [18]. PITC has been used by Liang et al. [12] to determine glucosamine in dog plasma, raw materials and dosage forms. However, turbidity and irreproducibility using this method were reported [11]. To investigate the source of this turbidity, triplicate samples of reagent solution, PITC with water, PITC with phosphate buffer and PITC with TEA solution were evaporated at 50 °C under nitrogen, then reconstituted with the same volume of HPLC mobile phase, vortexed and then tested for turbidity. Turbidity was observed only with the PITC/phosphate buffer solution and the PITC/TEA solution, suggesting that the turbidity is related to insoluble PITC by-products produced upon mixing with the alkaline buffer and which increased with PITC concentration. Furthermore, the reaction of PITC with glucosamine at room temperature was irreproducible with extensive inter-experimental variations. Indeed, evaporation of the reaction mixture at room temperature provided a lower UV response in comparison to evaporation at 50 °C under nitrogen, suggesting that undertaking the reaction at room temperature was slow and incomplete, and that temperature influences the reaction rate. Consequently we investigated the influence of reaction temperature in addition to reaction time and reagent concentration on the derivatisation process.

*3.1.1. Influence of reaction temperature*

The yield of the PTC–GL adduct was determined following reaction at room temperature, 30, 60, 80 and 100 °C for 30 min using 5% v/v PITC solution. Subsequently the product was evaporated at 50 °C under nitrogen (Fig. 1). The solid residue was then dissolved in 400 μl of HPLC mobile phase, filtered through a PTFE syringe filter (0.45 μm) before analysis. Of the five reaction temperatures, the maximum yield was obtained at 80 °C. At 100 °C the reaction media turned brown and the PTC–GL peak

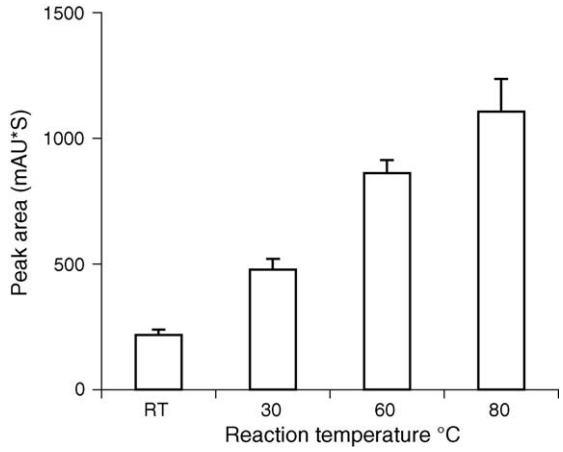


Fig. 1. Influence of temperature on PITC–glucosamine reaction yield (mean ± S.D., $n = 3$).

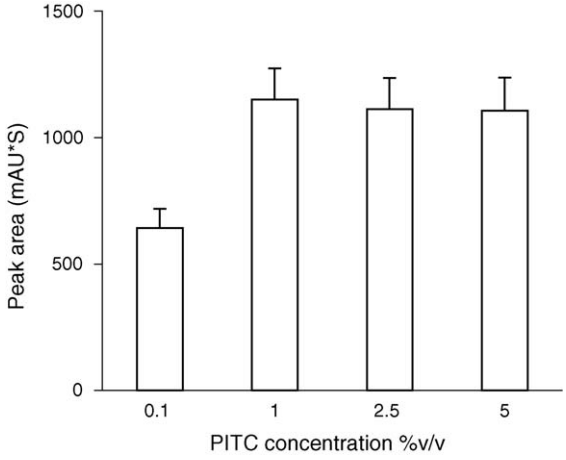


Fig. 3. Influence of PITC concentration on PITC–glucosamine reaction yield (mean ± S.D., $n = 3$).

was lost from the chromatograms suggesting that GL degraded at this elevated temperature. Accordingly, 80 °C was selected as the optimum reaction temperature, providing a statistically significant ($P < 0.05$) increase in yield as compared with the lower temperatures.

### 3.1.2. Influence of reaction time

Derivatisation was conducted at 80 °C using 5% v/v PITC solution for 10, 20, 30, or 60 min. The highest yield was obtained after reacting for 60 min (Fig. 2). However, no statistically significant difference ($P > 0.05$) was observed between 30 and 60 min reactions. Consequently, 30 min was considered to be the most appropriate reaction time to select whilst wishing to minimize potential degradation of glucosamine.

### 3.1.3. Influence of PITC concentration

It was previously shown that turbidity increased by increasing PITC concentration, but reducing the concentration of PITC could affect reaction efficiency. Therefore, the minimal reagent concentration that could be used without adversely affecting reaction efficiency was determined. PITC methanolic solutions ranging from 0.1 to 5% v/v were used for derivatisation at 80 °C for 30 min. The results (Fig. 3) show no significant difference ($P > 0.05$) in the PTC–GL adduct yield between 1 and 5% v/v PITC solutions. Consequently 1% v/v PITC solution was selected as an efficient yet minimal concentration.

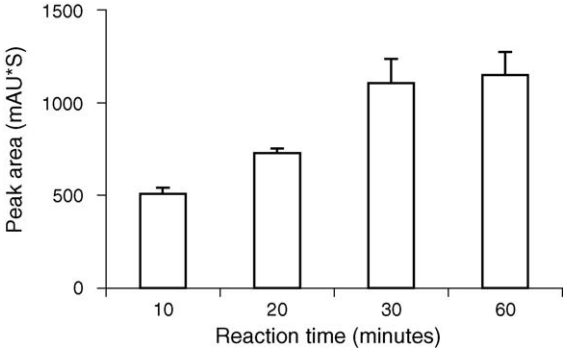


Fig. 2. Influence of reaction time on PITC–glucosamine reaction yield (mean ± S.D., $n = 3$).

## 3.2. Instrument calibration

Using the optimal derivatisation conditions 80 °C, 30 min using 1% v/v PITC methanolic solution, three replicates of the standard solutions (0, 0.5, 1, 5, 10, 20, 50, 100, 500 and 1000 μg ml$^{-1}$) of GL-HCl along with an internal standard of Gal-HCl were derivatised and analyzed. Under the chromatographic conditions used, PTC–GL and PTC–Gal adducts provided retention times of 9.7 ± 0.1 and 8.9 ± 0.1 min, respectively. The retention factors ($k'$), calculated from:

$$k' = \frac{t_R - t_M}{t_R} \quad (3)$$

where $t_R$ is the retention time of the analyte and $t_M$ is the time taken for the mobile phase to elute from the column, in our case 1.73 min. Thus, $k'$ was 4.61 and 4.14 for PTC–GL and PTC–Gal, respectively.

The linearity of the analytical procedure was evaluated by plotting the glucosamine/galactosamine adducts peaks areas ratio versus the nominal concentration of GL-HCl. A linear correlation between 0 and 1000 μg ml$^{-1}$ resulted ($R^2 = 0.99$). Further, a lack-of-fit test showed that ($F_{exp}$) > ($F_{critical} = 4.2$) again demonstrating that a linear relationship exists between the UV response and GL-HCl over the above concentration range.

## 3.3. Method validation using GL-HCl standard solutions prepared in a biological matrix

The analytical method was developed to quantify GL-HCl after in vitro transdermal permeation. Therefore, GL-HCl standard solutions (0.5, 1, 5, 10, 20, 50, 100, 500 and 1000 μg ml$^{-1}$) were prepared in skin extract containing 0.02% sodium azide, which was used as a bacterial and enzyme inhibitor. Samples of skin extract containing the internal standard were used as a control.

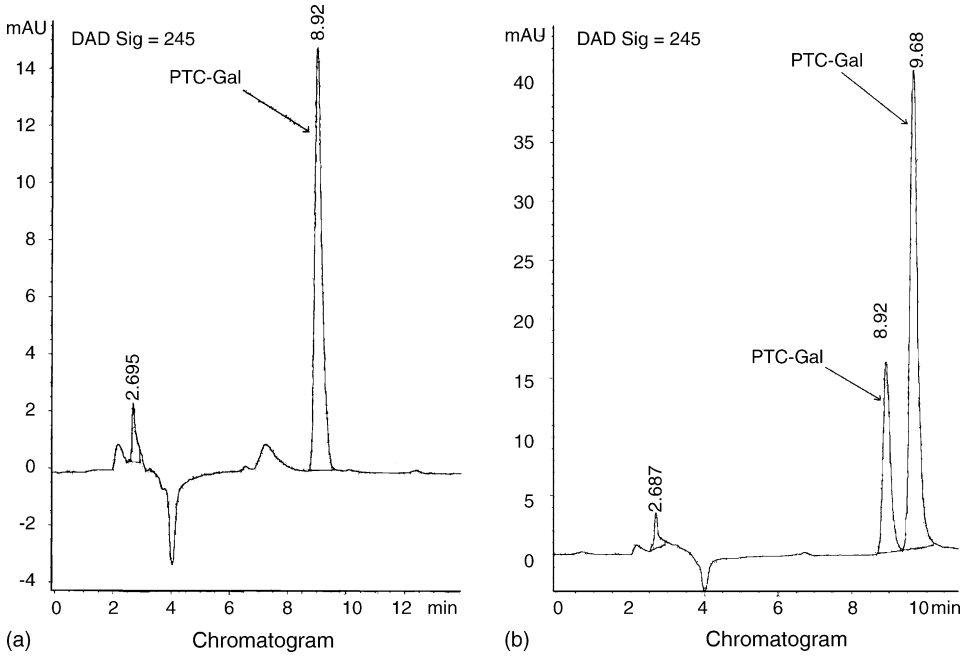


Fig. 4. Chromatograms obtained from analysis of samples prepared in skin extract fluid: (a) blank sample containing 10 μg ml$^{-1}$ of Gal-HCl as internal standard and (b) sample contains GL-HCl (20 μg ml$^{-1}$) and Gal-HCl (10 μg ml$^{-1}$).

The samples ($n = 3$) were spiked with Gal-HCl (internal standard), then derivatised and analyzed. Fig. 4a, from the sample containing the biological matrix and internal standard but with no glucosamine, shows that the detector responds to the internal standard and that any leached materials from the skin do not interfere with the analysis since the baseline is clean and flat in the region of the PTC–GL response (Fig. 4b). Additionally, Fig. 4b shows that the internal standard and glucosamine responses are sufficiently resolved using our mobile phase and column. The lack of interference from endogenous components of skin or PITC by-products demonstrates that the method is appropriately selective for monitoring glucosamine permeation through human skin.

The calibration curve prepared using the biological fluid also showed linearity over the whole concentration range (1–1000 μg ml$^{-1}$) with a least squares regression coefficient ($R^2$) = 0.9973. The limit of detection (LOD, S/N = 3:1) in the skin extract was 0.3 μg ml$^{-1}$ and the lower limit of quantification (LLOQ, S/N = 10:1) was 1 μg ml$^{-1}$. This sensitivity was statistically similar to the LOD and LLOQ determined for glucosamine in the aqueous solutions used to calibrate the instrument.

The intra- and inter-day precision and accuracy for determining glucosamine in the biological extract were also determined (Table 1).

Addition of the internal standard to the assay allowed reduction in the variability previously reported. The assay provided good intra- and inter-day precision with %R.S.D. < 12. The intra- and inter-day accuracy was also appropriate with the maximum %RE −5.60 and −8.00, respectively, which is within the limits described in the ICH guideline [14].

In summary, the method has been shown to be selective, precise and accurate and is sufficiently sensitive for assaying

Table 1
Intra- and inter-day precision and accuracy of HPLC analysis of glucosamine hydrochloride ($n = 5$)

| Prepared concentration of GL-HCl (μg ml$^{-1}$) | Measured concentration of GL-HCl (μg ml$^{-1}$) (mean ± S.D.) | %R.S.D.[a] | %RE[b] |
|---|---|---|---|
| Intra-day | | | |
| 1 | 0.97 ± 0.11 | 11.3 | −3.00 |
| 10 | 9.54 ± 0.17 | 1.78 | −4.00 |
| 100 | 94.4 ± 0.78 | 0.83 | −5.60 |
| 1000 | 957 ± 13.0 | 1.36 | −4.30 |
| Inter-day | | | |
| 1 | 0.96 ± 0.11 | 11.5 | −4.00 |
| 10 | 9.20 ± 0.21 | 2.28 | −8.00 |
| 100 | 93.7 ± 1.27 | 1.36 | −6.30 |
| 1000 | 989 ± 15.6 | 1.58 | −1.10 |

[a] Relative standard deviation.
[b] Relative error.

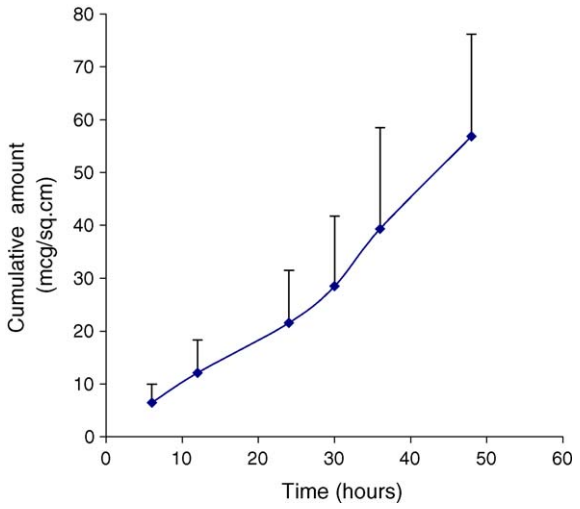


Fig. 5. Cumulative amount of glucosamine permeating per unit area of human epidermal membrane ($\mu g\,cm^{-2}$), as a function of time (h) after topical application of saturated aqueous solution of glucosamine hydrochloride ($n = 5$).

GL-HCl following in vitro transdermal permeation. This method was also used to evaluate the stability of GL-HCl in skin extract [19].

### 3.4. Permeation of glucosamine through HEMs

GL-HCl is a relatively hydrophilic compound with logarithm of its apparent partition coefficient ($\log K_{o/w}$) of −1.91 [8]. With a $pK_a$ of 7.75 at 37 °C [20], it is fully ionized in aqueous solution and the molecular weight (MW) of the GL ion is 180.17. These parameters can be used to predict the transdermal permeation of the GL ion through human skin using quantitative structure–activity relationships. One such model is the revised Potts and Guy equation, which has been extensively used for initial estimation of the skin permeability coefficient for chemicals possessing a $\log K_{o/w}$ in the range −1 to 5 [21]:

$$\log K_p = -1.326 + 0.6097 \log K_{o/w} - 0.1786 \times MW^{0.5} \quad (4)$$

where $K_p$ is the permeability coefficient ($cm\,h^{-1}$) of the permeant.

Although the highly hydrophilic fully ionized compound glucosamine falls outside the ideal $\log K_{o/w}$ range, the above model is simple to use for initial predictions and generally provides reasonable estimates for many permeants through human skin [22]. Using Eq. (4), the predicted permeability coefficient for glucosamine from a saturated aqueous solution is $4.03 \times 10^{-5}\,cm\,h^{-1}$. This value was then compared with experimental values.

The mean permeation profile of GL-HCl from a saturated aqueous solution through human epidermal membranes is shown in Fig. 5. The pseudo-steady state flux ($J_{ss}$) was calculated from the linear portion of the curve, whilst extrapolation of the linear portion to the time axis yields the lag time. The apparent permeability coefficient ($K_p$) was calculated from:

$$K_p = \frac{J_{ss}}{C_0} \quad (5)$$

where $C_0$ is the concentration of GL-HCl in the donor solution.

Experimentally, glucosamine flux through human skin was $1.497 \pm 0.42\,\mu g\,cm^{-2}\,h^{-1}$ with an apparent permeability coefficient of $5.66 \pm 1.6 \times 10^{-6}\,cm\,h^{-1}$ and a lag time of $10.9 \pm 4.6$ h (mean ± S.D., $n = 5$). This experimental permeability coefficient was about eight-fold lower than the predicted value, though predicted and experimental values seldom show perfect agreement since the model employs simple physical parameters (such as the octanol water partition coefficient) to predict permeation through a highly complex heterogeneous biological tissue. Thus, correlation within an order of magnitude is widely viewed as satisfactory.

Commonly, many researchers use radioisotopes to monitor permeation of compounds through human skin; fluxes tend to be low, many samples require analysis and most model compounds are readily available with radiolabels. However, this raises concerns, especially employing tritium labels which can exchange with, for example water; the flux of water through human skin is relatively rapid and thus even low levels of tritium exchange can have a significant impact on apparent flux values. Further, the skin is metabolically active and, with isotopic methodologies, it is merely the label that is recorded and hence metabolism of the permeating compound cannot be assessed. Our HPLC methodology, modified from the literature [12] to reduce irreproducible results [11] overcomes the above difficulties. In our previous determinations of glucosamine permeation using a tritium labelled permeant we did indeed find exchange of the isotope onto water [8]. Interestingly, when we removed the exchanged water from our samples we found an apparent glucosamine permeability coefficient of $6.7 \pm 1.6 \times 10^{-6}\,cm\,h^{-1}$ in excellent agreement with the HPLC data herein, indicating minimal metabolism of glucosamine during permeation through skin.

## 4. Conclusion

The temperature and time of the derivatisation reaction were found to be crucial factors in optimizing adduct yield, whilst a minimal reagent concentration was determined to minimize generation of turbid solutions. The optimized protocol allowed the formation of a reproducible adduct which was then used in a relatively simple, precise, accurate and appropriately sensitive HPLC assay. Glucosamine determinations were linear ($R^2 > 0.99$) over a wide range of concentrations (0–1000 μg/ml). The method was employed successfully to quantify glucosamine after in vitro permeation through human skin with no interference from skin components and thus will be valuable for routine quantitation of glucosamine either following in vitro transdermal permeation or in raw materials and dosage forms.

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