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# A novel HPLC method developed and validated for the detection and quantification of atorvastatin, fluvastatin, pitavastatin and pravastatin during transdermal delivery studies

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An HPLC method was developed and validated to quantify and identify several statins (atorvastatin, fluvastatin, pitavastatin and pravastatin) that were used during transdermal drug delivery. The method proved to be most effective with a Restek Ultra $C_{18}$, 250 x 4.6 mm, 5 µm column, a flow rate of 1.0 ml/min, UV detection at 240 nm and injection volume of 10 µl. The mobile phase used was acetonitrile/Milli-Q® water with 0.1% orthophosphoric acid starting with 30% acetonitrile, which increased linearly to 70% (after 4 min) for up to 10 min and then re-equilibrated to start conditions. This HPLC method indicated linearity (correlation coefficient ($R^2$) of 1) within the concentration range of 0.05-200.00 µg/ml and had an average recovery of 98-103%. Limit of detection (LOD) and limit of quantification (LOQ) showed that statins could still be identified at concentrations of 0.004-0.006 µg/ml with the exception of atorvastatin (quantifiable at 0.013-0.035 µg/ml). Specificity performed during method validation, confirmed that the method was suitable for accurate detection and quantification of the statins when included in the transdermal formulations with other excipients.

## 1. Introduction

Statins are used as first-line treatment for primary hyperlipidaemia (due to their ability to lower low density lipoproteins (LDL) and triglycerides), and have the ability to inhibit the enzymes responsible for the synthesis of endogenous cholesterol and isoprenoid formation (Schierwagen et al. 2017). When focusing on the oral route, statins may cause gastro-intestinal side-effects (nausea, vomiting, abdominal cramps, flatulence, difficulty swallowing, indigestion, constipation and diarrhoea) (Mancini et al. 2013), increase the risk of hepatotoxicity (with symptoms of hepatomegaly, jaundice, elevated liver aminotransferase, bilirubin and prothrombin levels) (McKenney et al. 2006) and reduce systemic bioavailability (either due to statin-stomach content interaction (Schachter 2004) or extensive clearance of the statins by means of liver enzymes (i.e. cytochrome P450) (Bellosta et al. 2004; Maji et al. 2013). In the attempt to overcome or bypass these shortcomings and side-effects of oral administered statins, the transdermal delivery route was investigated. A novel HPLC method was developed and validated for atorvastatin, fluvastatin, pitavastatin and pravastatin to adhere to requirements needed during *in vitro* skin permeation analysis; a sensitive, suitable and accurate method to detect exceptionally small concentrations.

## 2. Investigations, results and discussion

The method was validated according to linearity, precision (inter- and intra-day), accuracy, ruggedness (sample stability and system repeatability), specificity, LOD (limit of detection) and LOQ (limit of quantification); all results are summarised in the Table. Standard solutions (2.5-200.0 µg/ml) were prepared for each of the statins to determine the linearity (in duplicate). From the different concentration ranges obtained, analytical response plots (peak areas against analyte concentration (µg/ml)) were generated to obtain the linear regression equations, which were used to calculate the correlation coefficient ($R^2$). The $R^2$ values for each statin signified a strong relationship between the peak areas and analyte concentration (µg/ml) within the evaluated concentration range. Precision was determined in terms of intra-day (repeatability done in one day) and inter-day (intermediate precision done over three consecutive days) variation. When assessing precision, a relative standard deviation (%RSD) value was calculated on a minimum of nine samples (ICH 2005). Prior to testing the precision, standard solutions, placebo nano-emulsions and spiked solutions were prepared for each statin. Both intra- and inter-day precision were determined in triplicate. Samples tested were prepared and contained 0.16, 0.18 and 0.20% spiked solutions and 0.60, 0.80 and 1.00% placebo nano-emulsions, which were made up to volume (100 ml) with methanol and analysed by HPLC. Note that for inter-day precision, only the intermediate concentrations (0.18% spiked solutions and 0.80% placebo nano-emulsions) were analysed from days 2-3. The %RSD values calculated during intra- and inter-day precision for the statins were satisfactory, as they remained lower than 5%, respectively (APVMA 2004; Paithankar 2013). Accuracy is reported as recovery (%) and all statins proved to be accurate and within acceptable limits, since the mean recovery (%) ranged between 90 and 110% (APVMA 2004) with consistent %RSD of 0.7-0.9%. Sample stability assesses the ability of the validation method to analyse compounds when degradation products are present, which is measured in hourly intervals over 24 h. Sample stability results (%RSD) for the statins were stable, since the degradation value was less than 2% (Paithankar 2013). Repeatability in terms of accuracy and precision was prepared similar to the method used to determine sample stability. Each sample was injected six consecutive times into the HPLC, to determine retention times and peak areas of samples under the same conditions in one day. System repeatability of statins was successful, since the %RSD was less than 2% for the peak area and the retention times (Paithankar 2013). To determine the specificity, tests were performed to accurately establish the reaction of the tested statin's peak in the presence of impurities (other components) using the specific analytical method

**Table: Summary of the HPLC's validation parameters and results for the statins analysed**

| Validation parameters | | Accepted parameters | Atorvastatin | Fluvastatin | Pitavastatin | Pravastatin |
|---|---|---|---|---|---|---|
| **Linearity** | | $R^2$>0.98 | $R^2$=1 | $R^2$=1 | $R^2$=1 | $R^2$=1 |
| **Accuracy** | | Recovery (90-110%) | 99.2% | 103.0% | 98.0% | 100.2% |
| **Precision** | Intra-day | RSD≤5% | 0.68% | 1.56% | 0.83% | 2.48% |
| | Inter-day | | 0.72% | 0.90% | 0.78% | 3.35% |
| **Sample stability** | | RSD<2% | 0.94% | 0.77% | 0.27% | 0.65% |
| **System repeatability** | Peak area | RSD<2% | 0.78% | 0.25% | 0.20% | 0.31% |
| | Retention time (min) | RSD<2% | 0.05% | 0.03% | 0.06% | 0.06% |
| **Specificity** | | Fig. 1 specificity | | | | |
| **LOD (µg/ml)** | | RSD<15% | 0.116 | 0.006 | 0.005 | 0.004 |
| **LOQ (µg/ml)** | | RSD<20% | 0.035 | 0.019 | 0.015 | 0.013 |

(ICH 2005). Specificity of the analytical method is acceptable and the method is regarded as specific, when no peak hindrances (at the same retention time as the API) appear (Snyders et al. 1997). Specificity was determined through preparation of six samples: a) placebo (methanol only), b) standard (statin (20 mg) in 100 ml methanol) and four separate test tubes (standard containing the selected statin) that were spiked with 200 µl of c) hydrochloric acid (HCl), d) Milli-Q® water ($H_2O$), e) sodium hydroxide (NaOH) and f) hydrogen peroxide ($H_2O_2$). The Figure indicates that degradation peaks were detected in the samples; although, peak retention times of the statins remained unaltered and were 4.84, 5.84, 8.53 and 8.95 min for pitavastatin, pravastatin, atorvastatin and fluvastatin, respectively. The LOD of an analytical method represents the sensitivity and as the lowest detectable analyte in a sample; this is not necessarily a definite value (APVMA 2004; Snyder et al. 1997), while the LOQ is the lowest detectable amount of analyte present in a sample under specific conditions, this value can be accurately determined (APVMA 2004). To establish the LOD and LOQ values, a standard solution (0.5 µg/ml) was placed in HPLC vials (for each statin) and injected six consecutive times at four different injection volumes (2.5, 5.0, 7.5 and 10.0 µl). The LOD (= 3.3 x σ/S) and LOQ (= 10 x σ/S) of the statins were established through the calculation of the regression after data analysis, where σ indicates the standard variation of the *y*-intercept and *S* indicates the gradient of the slope (Swartz and Krull 2012). The LOD and LOQ results of the different statins fell within the accepted parameters, since the %RSD values were less than 15% and 20%, respectively (FDA 2018; Rathmann et al. 2015).

Subsequent to the validation of the analytical method, skin diffusion and tape stripping studies were performed. Analyses of the statins in the receptor phases, tape strips and skin samples were done utilising the newly validated method (see Fig.).

## 3. Experimental

An analytical instrument (Dionex UltiMate 3000 dual system with ternary gradient pumps, column ovens, an autosampler and diode array detectors) was used, which operated on Chromeleon 7.2 instrument control and data analysis software (Thermo Fisher Scientific Inc., Waltham, MA). A Restek Ultra $C_{18}$, 250 x 4.6 mm, 5 µm (Restek, Bellefonte, PA) column was inserted with a flow rate of 1.0 ml/min, injection volume of 10 µl and UV detection at 240 nm. The mobile phase was acetonitrile/Milli-Q® water (Milli-Q® Academic water purification system, Merck-Millipore, Midrand, RSA) with 0.1% orthophosphoric acid, which started with 30% acetonitrile; linearly increased to 70% after 4 min and held at 70% for up to 10 min and then re-equilibrated to start conditions. The solvent used during method development and vali-

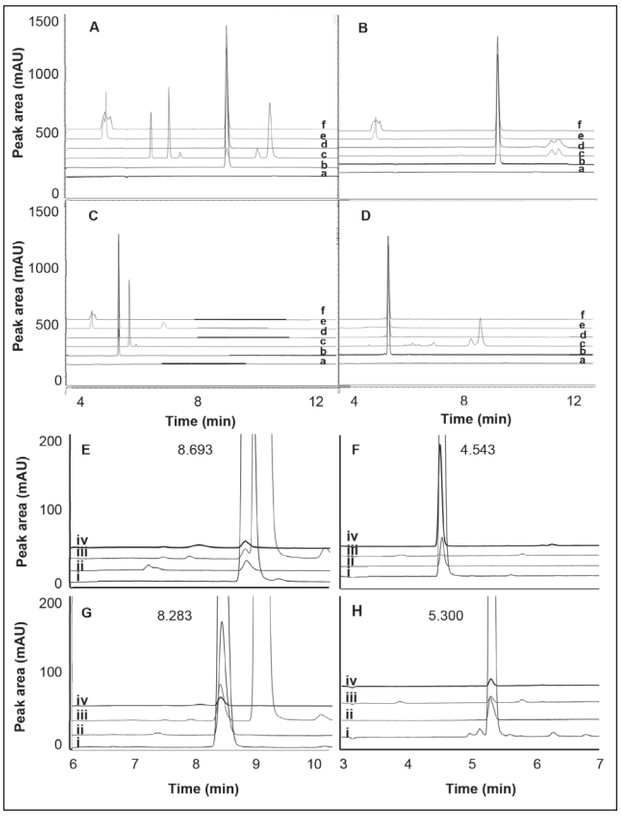


Fig.: HPLC chromatogram indicating the specificity data of: A) atorvastatin, B) fluvastatin, C) pitavastatin and D) pravastatin, while the samples are represented by: a) placebo and b) standard solution with selected statin, together with the statin sample solutions stressed with: c) HCl, d) Milli-Q® water, e) NaOH and f) $H_2O_2$, and the *in vitro* skin data of: E) fluvastatin, F) pitavastatin, G) atorvastatin and H) pravastatin, while the samples of the respective statins are: i) standard solution, ii) receptor phase (transdermal delivery), iii) tape strip (topical delivery) and iv) skin sample (topical delivery)

dation was methanol. The retention times were reported as 4.84, 5.84, 8.53 and 8.95 min for pitavastatin, pravastatin, atorvastatin and fluvastatin, respectively with a stop time of 15 min.

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