UPLC® method development and validation for the assay of the photolabile drug nifedipine and its trace level degradation products

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Introduction

Quality assurance regulations require that pharmaceutical drugs are highly pure. This is in order to ensure the safety and efficacy of medicines for patients, two fundamental issues of importance in treatment. The safety of a drug is determined by both its pharmacological and toxicological profile as well as impurities present in its dosage form. Impurities may have pharmacological and toxicological effects that outweigh any benefit from the dosage form administration. While the use of a drug to treat a patient is a balance of risk and benefit, impurities in pharmaceuticals provide only risk.

Therefore, pharmaceutical companies must characterise what molecules may be present and be able to identify them at low levels or as low as reasonably practicable (ALARP). This involves reducing risk to an acceptable level without disproportionate resource expenditure. Molecule characterisation involves the identification, structural elucidation and quantitative determination of impurities and degradation products. Impurity analysis is therefore a very important field in pharmaceutical characterisation as it allows assurance that a drug is safe and of sufficient quality (Görög, 2000).

Due to newer analysis methods becoming available and the need to detect impurities at ever lower levels, ongoing method development and transfer of newer techniques to the quality control laboratory is a reasonable regulatory expectation. However pharmaceutical companies can be innately conservative, with a to change and adaptation to new techniques that can require significant resource investment. Although current techniques may appear adequate, when dealing with impurities that can impact patient safety, in particular genotoxic impurities, this resource allocation is prudent.

Impurities present in drug products are classified as organic, inorganic and residual solvents by the United States Food and Drug Administration (FDA) (FDA, 2008A). The source of impurities can be from the manufacturing process and/or during storage including by contact with packaging. Possible impurities include: starting materials, by-products, intermediates, degradation products, reagents, ligands and catalysts (The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2006). Potential impurities may be known or unknown, volatile or non-volatile. Additionally, there may be impurities from products of incomplete or over reaction, enantiomeric impurities, impurities in materials used for synthesis and impurities in excipients (Nageswara Rao and Nagaraju, 2003).

Genotoxic impurities are a special case of impurities defined by the ICH as "a broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced." (ICH, 2011). Genotoxic impurities, even at low concentrations pose significant health risks as they can

potentially be mutagenic and thus damage DNA. Therefore, they pose a cancer development risk for patients (Jacobsonkram and McGovern, 2007). Due to the potential for adverse safety posed by these types of impurities the European Medicines Agency and FDA have given emphasis on tackling this issue by introducing a threshold of toxicological concern of 1.5 µg/day for genotoxic impurities in new commercial drugs (FDA, 2008B) (EMA, 2006). The International Council for Harmonisation provides a more detailed complementary document for guidance on genotoxic impurities titled ICH M7 (ICH,2015). Increasingly, the analyst must relate to this new paradigm and augment historical units for determination of impurities.

The object of this work was to provide a valid method capable of separating nifedipine and its by-products (Figure 1) in an efficient manner using UPLC coupled with diode array detection.
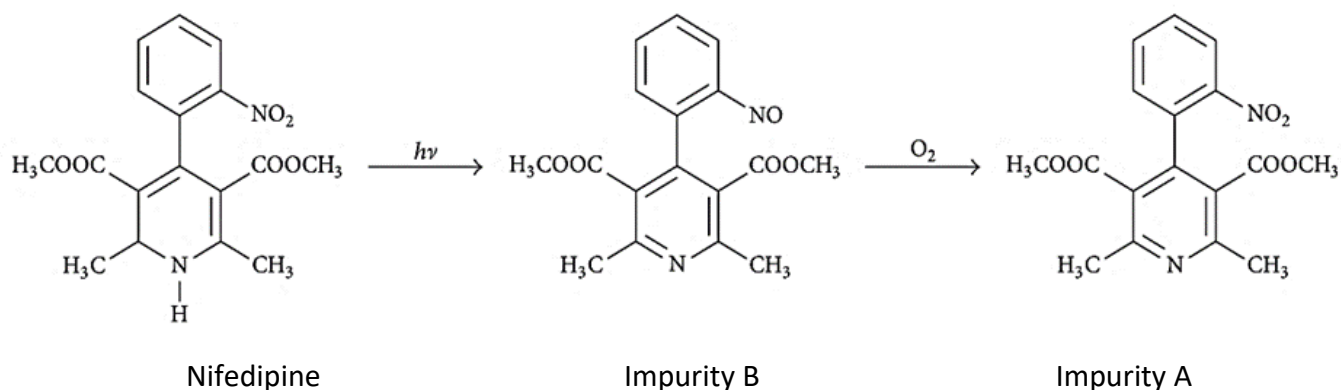

Nifedipine   Impurity B   Impurity A

Figure 1: Nifedipine photolysis followed by oxidation producing major breakdown products (Ahmad et al., 2016).

Nifedipine is photolabile and undergoes photodehydrogenation by intra-molecular mechanisms to nitro- and nitroso-pyridine analogues (Handa, Singh and Singh, 2014). These analogues are not known genotoxins but it is prudent to monitor the reduction of a nitro group to its nitroso analogue. Due to the unpredictability of photolytic degradation these products have historically had different thresholds for their presence in lots passing through the supply chain as an active pharmaceutical ingredient or finished product.

No UPLC method existed in the literature for the analysis of photo-degradants in nifedipine. The aim was to make available a *pharmacopeial* assay method validated using the ICH Q2(R1) *Validation of Analytical Procedures* document, using the isocratic mode to maximise baseline stability and enabling identification of photo-induced degradation products of nifedipine. Q2(R1) is a tripartite harmonised ICH Guideline that informs on the required validation parameters for a validated method.

ICH limits for impurities in new drug products are shown in Table 1 (ICH, 2006). Identification below limits may be necessary for unusually potent or toxic impurities. Impurities should be reduced to the lowest level reasonably possible but it is acknowledged that impurities cannot be reduced to zero thus specifications are used.

The maximum daily dose of nifedipine is 90 mg. The approach outlined by the ICH in the document Q3B(R2) is an identification threshold of 0.2% or 2 mg total daily intake of impurity (TDI) (whichever is lower) for 10-2000 mg (ICH, 2006). Therefore, for a 100 µg/mL (ppm) solution of drug in the laboratory, impurity levels above 0.2 ppm must be detectable.

Table 1: Q3B(R2) Impurities in new drug product's limits.

Maximum Daily Dose		Threshold
	Reporting thresholds	
≤ 1 g		0.1%
> 1 g		0.05%
	Identification thresholds	
< 1 mg		1.0% or 5 µg TDI, whichever is lower
1 mg - 10 mg		0.5% or 20 µg TDI, whichever is lower
>10 mg - 2 g		0.2% or 2 mg TDI, whichever is lower
> 2 g		0.10%
	Qualification thresholds	
< 10 mg		1.0% or 50 µg TDI, whichever is lower
10 mg - 100 mg		0.5% or 200 µg TDI, whichever is lower
>100 mg - 2 g		0.2% or 3 mg TDI, whichever is lower
> 2 g		0.15%

The increases in analytical technology in the last 30 plus years have allowed development of newer, superior methods to determine purity of medicines. High performance liquid chromatography (HPLC) is now the method of choice for impurity analysis as it can replace all non-specific assay methods with a highly specific and precise one. For the last 20 years HPLC has been used for nearly all organic impurity determinations (Nageswara Rao and Nagaraju, 2003).

Validated HPLC methods are especially required due to their powerful precision, specificity and accuracy. However, adequate system suitability testing must be employed to ensure optimal set up.

Recent developments have seen a shift towards newer liquid chromatography systems called ultra-performance or UPLC. Waters released the first such instrument machine equipped with the capability to function at higher operating pressures in 2004. It was designed to exploit the performance advantages of a sub 2 µm stationary phase. Smaller particle sizes of UPLC cause the Van Deemter curve to flatten allowing a more usable range of flow rates with subsequent faster analysis times. Van Deemter plots of efficiency *vs* flow rate for UPLC columns show none or little deterioration with these increased flow rates due to the smaller particles having shorter diffusion path lengths with the analyte not spending long inside the particle where diffusion could happen (Figure 2).
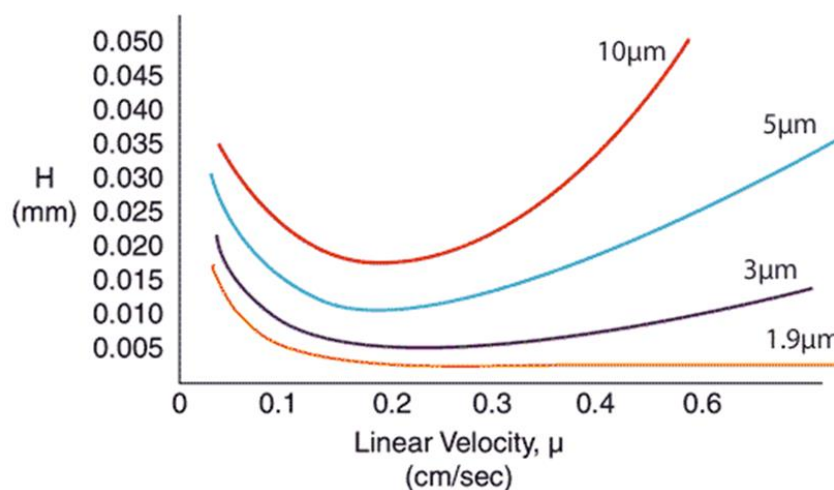

Figure 2: Van Deemter plot of various particle size columns (Restek.com, 2017).

Reducing particle size in columns to increase separation efficiency had reached a limit due to instrument band spreading and a limited pressure range. Higher pressures were required to be tolerable as smaller particles result in a higher density, increasing resistance to flow. By providing access to higher pressures of 15,000 psi these limitations were overcome. Waters approach has since been replicated by competitors, resulting in the more widespread use of such systems. UPLC has advantages of improved analysis speed, thereby increasing sample throughput, resolution and sensitivity (Waters.com, 2016). Therefore, it is fast becoming the analytical technique of choice.

It is critical new analytical methods are validated in order to ensure accurate data sets and regulatory compliance. Failure to validate can lead to issuance of FDA form 483 or similar, which indicates excellence is not being achieved and beyond the laboratory can have negative reputational consequences.

ICH Q2(R1) document outlines the parameters that must be investigated and recorded as well as recommended methods for examining each. These include robustness, linearity and range, limit of detection (LOD), and limit of quantitation (LOQ). Chromatographic specific parameters that are of daily importance for the resultant validated method include those system suitability aspects which examine separation efficiency (N), capacity factor (K), selectivity (α) and resolution (R).

Separation efficiency (N) is a measure of the sharpness of peaks. It reflects column performance and expresses the number of theoretical plates in a column. A perfect peak would be like a pencil line however due to dispersion effects this is not the case in practice. A plate is a distance where the sample components achieve one equilibration between the mobile phase and stationary phase in the column. More theoretical plates equate to a more efficient separation, therefore knowledge of N is a key component in evaluating a method.

Capacity factor (k) is the ratio of analyte retention time to an unretained molecule's retention time. The unretained molecule has no affinity for the stationary phase and elutes with the solvent front. It is generally independent of the equipment, being instead a function of mobile phase and column choice. This makes it a useful point of reference when comparing the retention of peaks that were obtained using a different chromatographic system.

Selectivity (α) is a measure of the chromatographic system's ability to separate two components. It is a measure of the ratio of capacity factors of each, therefore a function of their distance. Selectivity is dependent on the analyte, column and mobile phase with alterations to each affecting its value.

Resolution (R) is perhaps the most crucial factor to consider when evaluating a chromatogram with the main goal being to have the best resolution possible in the shortest time. It is determined by the difference between retention times of each peak divided by the average width of both peaks at the baseline. Resolution is influenced by N, K and α.

Chemicals and Reagents

Nifedipine (1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester), impurity A (2,6-Dimethyl-4-(2´-nitrophenyl)-3,5-pyridinecarboxylic acid dimethyl ester) and impurity B (Dimethyl 2,6-dimethyl-4-(2-nitrosophenyl)-3,5-pyridinedicarboxylate) were sourced from Sigma-Aldrich. Formic acid was supplied by Waters. HPLC grade 99.8% acetonitrile sourced from Macron Fine Chemicals was used as was Elga deionised 18 megohm water.

Experimental

Table 2: Final Chromatographic system details.

| UPLC	Waters ACQUITY
Column	ACQUITY UPLC BEH C18 1.7 µm.
Column dimensions	2.1 x 50 mm
PDA Detector wavelength	237 nm (Recording in the range 210-400
UV Block filter	nm)
	Below 210 nm
Injection size	5 µL
Mobile phase	Water/acetonitrile (73:27)
Flow rate	0.5 mL/min
Run time	5 mins
Sample loop	Partial loop
Injection volume of loop	20 µL
Weak wash	Water/acetonitrile (95:05)
Strong wash	Water/acetonitrile (50:50)
Sampling rate	20 pts/s
Auto sampler temperature	7°C
Column temperature	45°C

Results
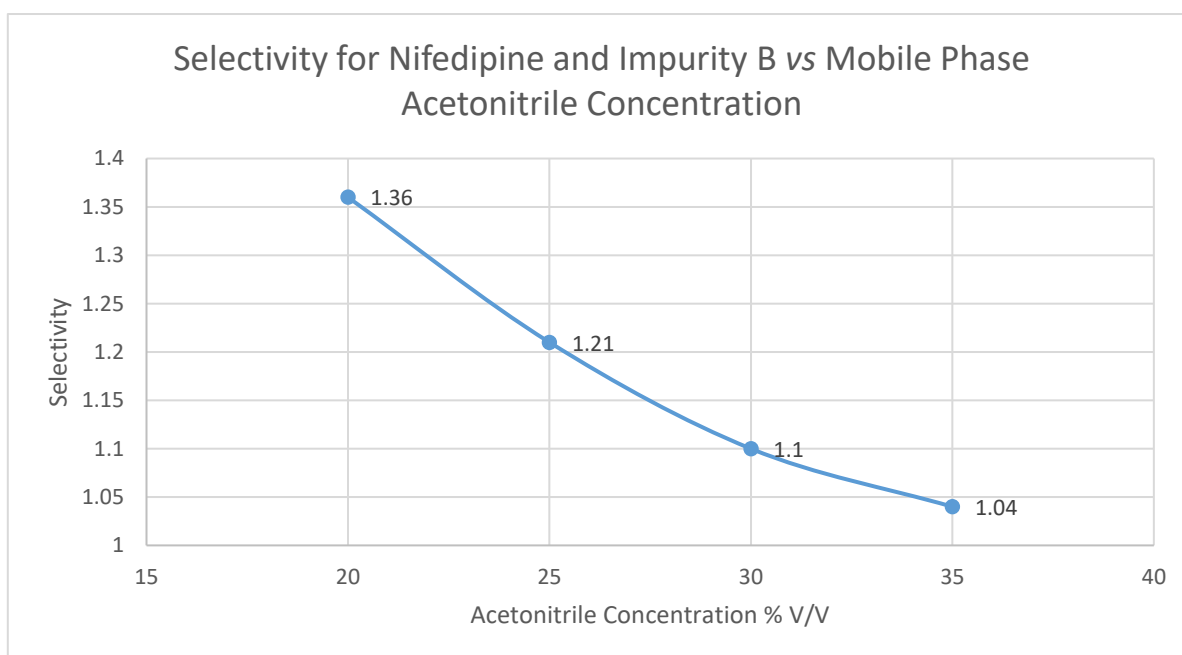
Figure 3: Graph of selectivity vs acetonitrile concentration with remainder made up of Elga deionised 18 megaohm water.
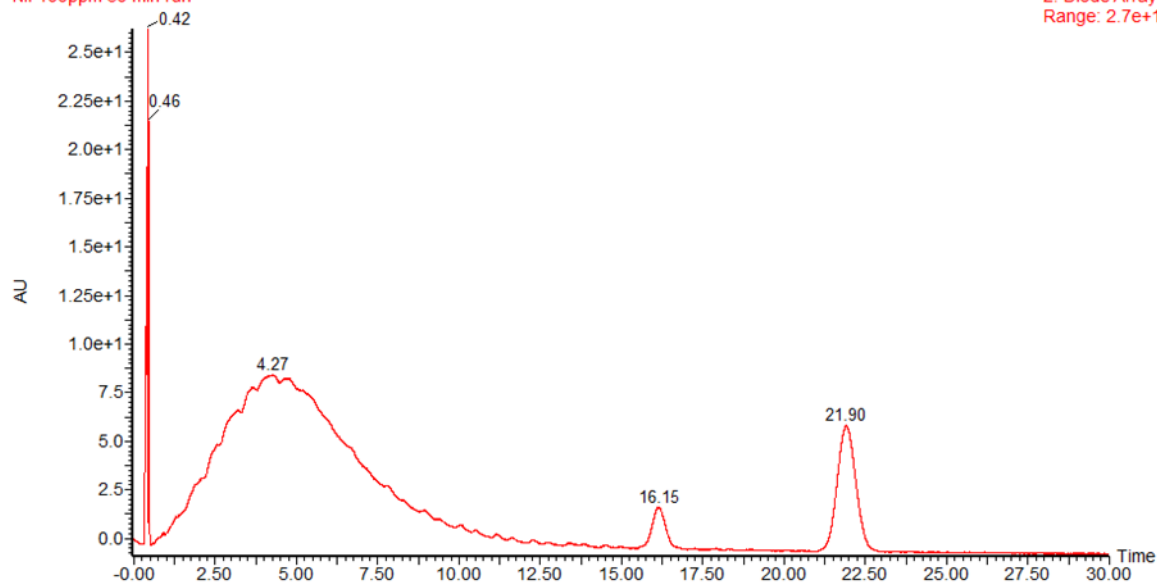
Figure 4: 100 ppm Each of nifedipine, impurity A and impurity B. Mobile phase: water/acetonitrile (80:20) with 0.1% formic acid. Column temperature 35°C. Flow rate: 0.4 mL/min. Injection: 8 µL.
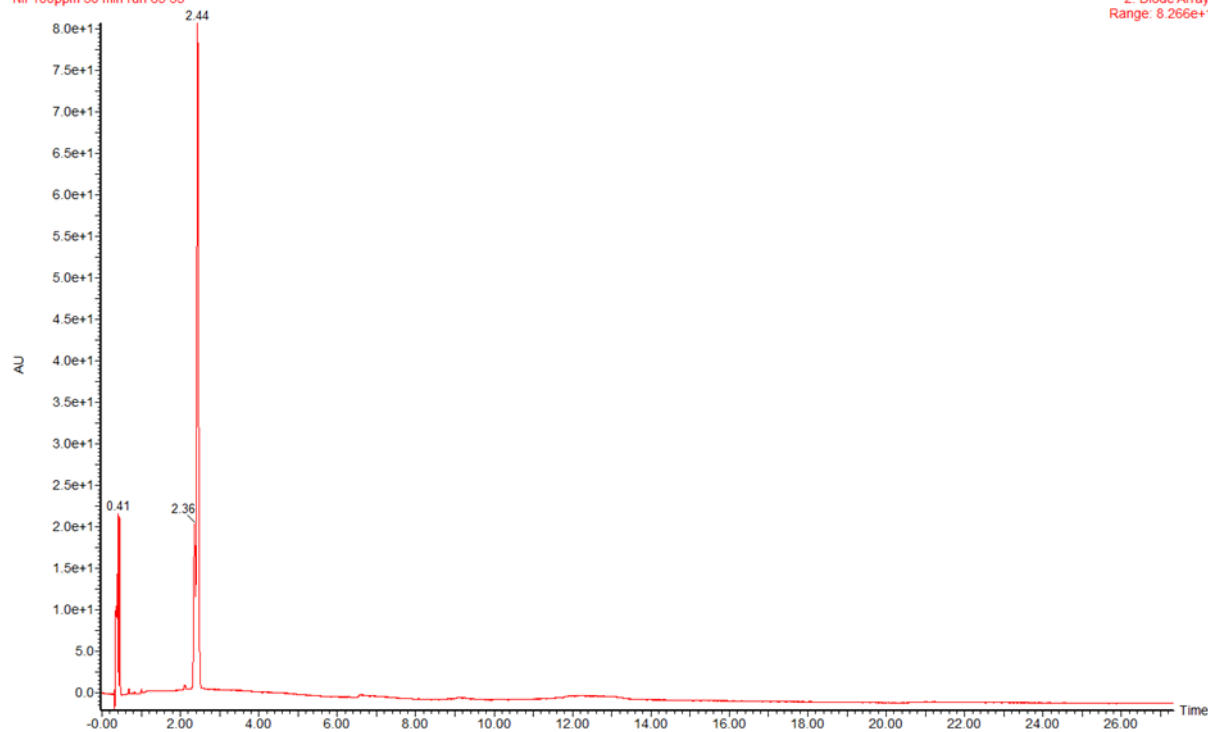

Figure 5: 100 ppm Each of nifedipine, impurity A and impurity B. Mobile phase: water/acetonitrile (65:35) with 0.1% formic acid. Column temperature 35°C. Flow rate: 0.4 mL/min. Injection: 8 µL.

Figure 6: 100 ppm Each of nifedipine, impurity A and impurity B. Mobile phase: water/acetonitrile (75:25) with 0.1% formic acid. Column temperature 35°C. Flow rate: 0.4 mL/min. Injection: 5 µL.
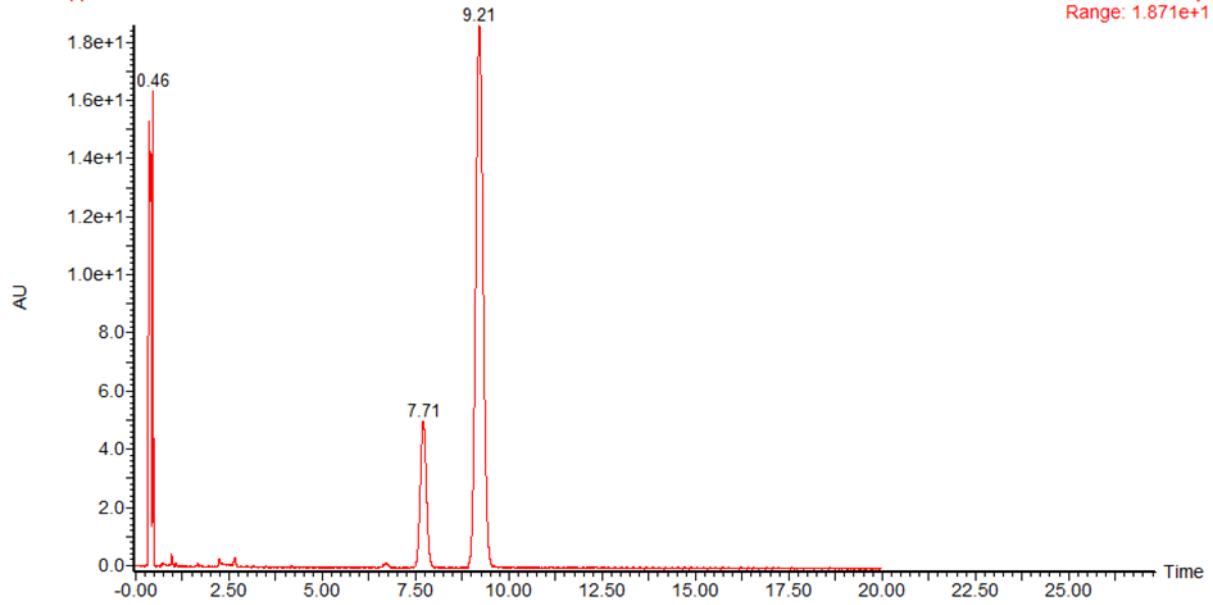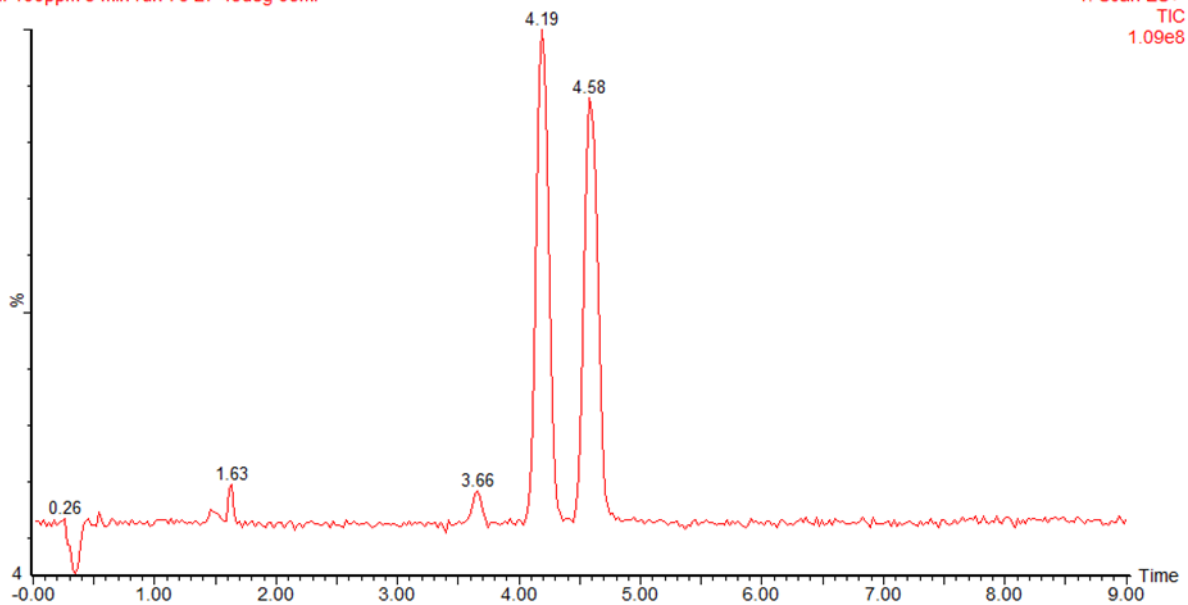

Figure 7: 100 ppm Each of nifedipine, impurity A and impurity B. Mobile phase: water/acetonitrile (73:27) with 0.1% formic acid. Column temperature 45°C. Flow rate: 0.5 mL/min. Injection: 5 µL.
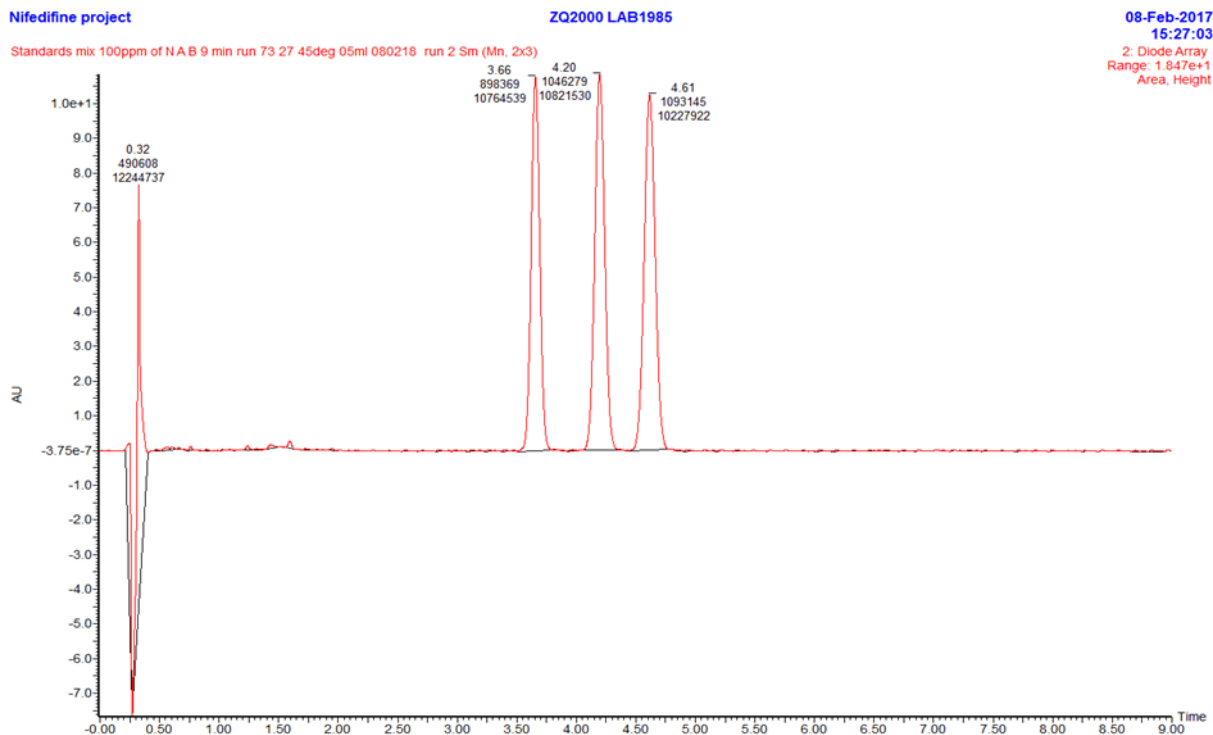
Figure 8: <mark>*Developed method*</mark> *with 33.3 ppm each of nifedipine, impurity A and impurity B. Mobile phase: water/acetonitrile (73:27) with 0.1% formic acid. Column temperature 45°C. Flow rate: 0.5 mL/min. Injection: 5 µL.*

Table 3: Table of selectivity and resolution for Fig 7.

Peak	α	R
Impurity A to B	1.16	2.07
Impurity B to Nifedipine	1.10	1.5

Table 4: Table of capacity factors and theoretical number of plates for Fig 7.

Peak	K	N/m
Impurity A	10.43	195,195
Impurity B	12.13	257,042
Nifedipine	13.44	193,336

Discussion

A mixture consisting of nifedipine, impurity A and impurity B was run for the purpose of optimising the system on a qualitative basis so as to establish a functional method capable of being assessed through relevant, advantageous, system suitability criteria. This was analysed with alteration of various conditions which included mobile phase ratio, column temperature, and flow rate.

In this investigation, a balance was required of speed, resolution and elution time of the photo degradant impurities. Acetonitrile at 27% was ultimately determined after development steps to be optimal, giving the desired balance. The higher the acetonitrile concentration, the higher the affinity of the non-polar mobile phase for non-polar nifedipine reducing its retention time significantly due to less time spent bound to the non-polar stationary phase. The acetonitrile competes with the stationary phase for nifedipine; Figure 3 shows how the selectivity changes with mobile phase strength changes as acetonitrile concentration is

altered. This therefore has a significant effect on retention time requiring the aforementioned balance of speed, resolution and elution time.

Figures 4-7 inclusive show the transformative effects of quite small alterations of chromatographic conditions and informed the selection of conditions that achieved the requisite balance of chromatographic efficiency, speed and limit of detection (LOD).

In Figure 5 the mobile phase was changed from a starting ratio of water/acetonitrile (80:20) to water/acetonitrile (65:35). A dramatic effect was observed with nifedipine's retention time reducing to 2.44 minutes, an almost 20-minute reduction. This was due to the selectivity changing.

Further adjustments to the mobile phase of water/acetonitrile (75:25), gave a more acceptable chromatogram with sharp, symmetrical peaks and a good resolution of 2.93 and a drug retention time of 9.2 mins, seen in Figure 6. However, the peak for impurity A was poor and difficult to identify.

As temperature affects retention and selectivity (with improved mass transfer kinetics), it was increased. Changing the column temperature to 45°C improved impurity A's peak height, seen in Figure 7. The flow rate was also changed from 0.4 mL/min to 0.5 mL/min. This was afforded due to the reduced viscosity and subsequently reduced backpressure gained from increasing temperature.

The outcome was a further significant decrease in the retention time to 4.6 mins for nifedipine. The peaks also sharpened up with the bonus of impurity A's height increasing. However, the resolution was not optimal so the mobile phase was slightly altered again to water/acetonitrile (73:27) and shown in Figure 8. This 'spread' the peaks and still had an acceptable retention time of 4.6 mins for nifedipine which eluted last, an ideal scenario for most drug impurity analysis. When acetonitrile is increased in concentration it has a stronger binding effect on nifedipine and its degradation products which results in earlier elution (Plumley et al., 2009). This series of alterations culminated in the final chromatographic system setup (Table 2).

Table 3 shows the selectivity values. Values between 1.10 and 1.16 were obtained. A value of 1 would indicate coeluting peaks. The value on paper was a little low but the resolution obtained in Fig 7 demonstrates no issues as does the high plate count. The initial wait of 3.5 mins before elution began is responsible for this.

Table 4 shows the chosen system had a high number of theoretical plates. This indicates the high separation efficiency of the system. The small particle size of the column is a significant contributing factor to the high N value.

Resolution (R) is certainly the most crucial factor to consider when evaluating a chromatogram with the main goal being to have the best resolution possible from potential impurities in the shortest time. An R value of 1.5 is ideal as it ensures the analytes are well separated allowing accurate peak areas to be determined while doing so in the minimum time required, therefore increasing throughput. The resolution between impurity A & impurity B was 2.07 and between impurity B & nifedipine, 1.5, as shown by Figure 8. This indicates the peaks were well resolved and that when analysing a manufactured tablet or capsule for impurities, that they can be separated and accurately quantified. This is especially critical when dealing with substances that are potentially toxic and failure to resolve them can have grave consequences for product quality and patient safety. That is why the method development template and process of the type described here is critical to undertake, to ensure that the system can conduct the analysis required, especially when the analyst possesses a reference portion of the impurity.

Precision was determined over 3 runs with the coefficient of variance calculated as 0.32% for nifedipine 0.45% for impurity B and 0.93% for impurity A. The lower level of variation for an active pharmaceutical ingredient compared with an impurity is not surprising with more variability to be expected of impurities. These figures were low indicating the system benefited from the isocratic approach and produced precise data. Linearity tests were conducted over the range of 0.1-20 ppm using impurity A, impurity B and

nifedipine in a mix. Excellent linearity was observed over this range with R^2 values being between 0.9999 and 1.0000. The limits of detection and quantitation was determined through the use of signal to noise ratios using matching blank solutions (figures 9-10). The LOD was 0.2 ppm and LOQ 0.5 ppm. These values mean that the method has a high sensitivity and the ability to quantitate at low levels which can be somewhat unusual for a photo diode array (PDA) detection. In this instance degradation of photolabile nifedipine could be monitored for a drug solution concentration of 100 µg/mL. These attributes are more and more vital for impurities' analysis as pharmaceutical medicines become more potent and are dosed at lower levels.

As part of a method robustness examination, the mobile phase composition in particular, column temperature and flow rate require control due to their potential effect on retention times.
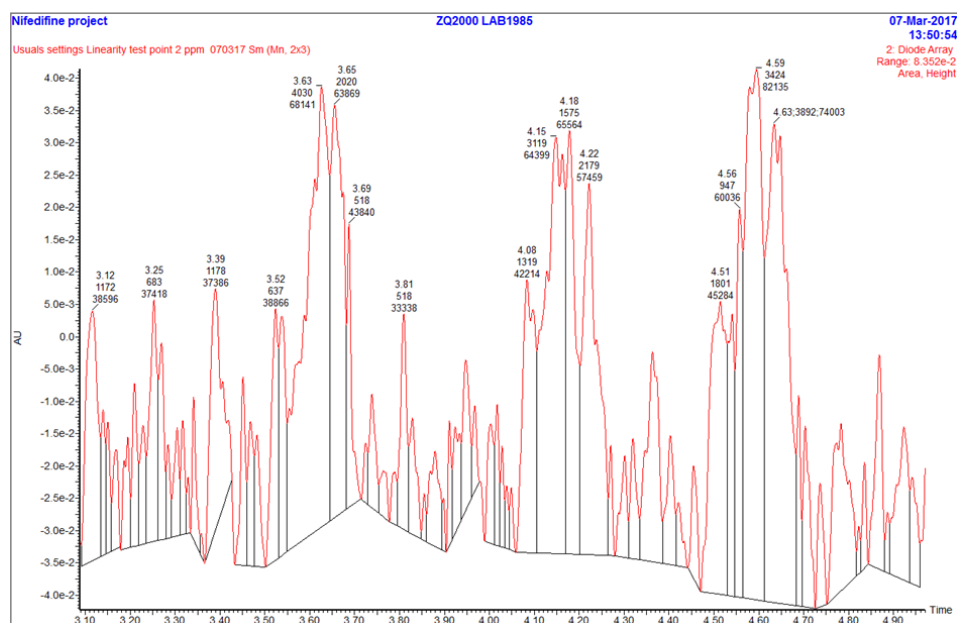

Figure 9: 0.2 ppm Each of nifedipine, impurity A and impurity B. Mobile phase: water/acetonitrile (73:27) with 0.1% formic acid. Column temperature 45°C. Flow rate: 0.5 mL/min. Injection: 5 µL.
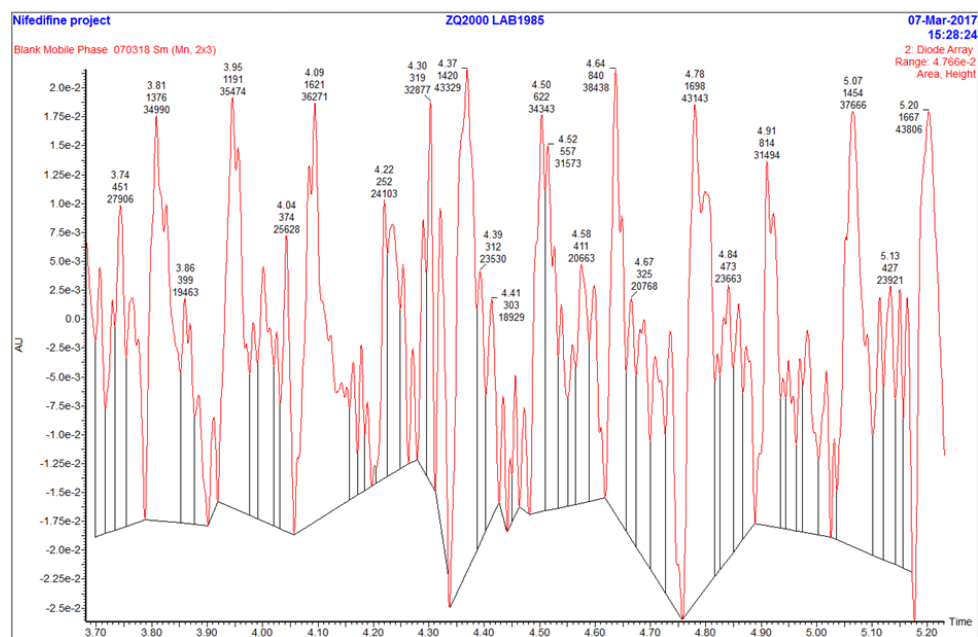

Figure 10: Blank mobile phase. Mobile phase: water/acetonitrile (73:27) with 0.1% formic acid. Column temperature 45°C. Flow rate: 0.5 mL/min. Injection: 5 µL.

As 100 ppm concentrations were analysed for method development purposes and at a 0.2 ppm detection limit this works out as 0.2% detection at this concentration. However, this is right on the limit of PDA capability and some cushion would be desirable by either increasing detection performance, increasing sample concentration administered or both. As it stands the validated system is within the permissible specifications and no impurity was noted in some nifedipine drug products sampled for challenge.

These results show the benefits of investigating the development of new methods on newer chromatographic analysis techniques as superior data sets can hope to be achieved by the analyst.

Conclusion

The developed method is able to distinguish between nifedipine and its degradation products with a low LOD of 0.2 ppm for identification, and a LOQ of 0.5 ppm allowing quantitation to be performed with low sample amounts. This method gave excellent peaks with little tailing, flat baselines and very good resolution all within a short retention time of 5 minutes. ICH method validation was performed satisfactorily demonstrating the suitability of this method for use in nifedipine impurity analysis.

About the authors

James Phelan has recently completed a B.Sc. (Honours) Pharmaceutical Science degree at Athlone Institute of Technology achieving first-class honours. He recently commenced working at BioClin Research Laboratories in the Bioanalysis department using LC-MS/MS.

James J. Roche worked for ten years in Elan Corp before transferring to Athlone Institute of Technology where he co-ordinates the B.Sc. (Honours) in Pharmaceutical Sciences and lectures in analytical chemistry in the Faculty of Science and Health. Eligible for nomination as a qualified person for the pharmaceutical industry, Jim is a Chartered Chemist, a Member of the Royal Society of Chemistry and a Member of the Institute of Chemistry of Ireland.

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