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ORIGINAL ARTICLE

Impurity profiling and in-process testing of drugs for injection by fast liquid chromatography

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Abstract Liquid chromatography (LC) is considered by many as a mature technique. Nonetheless, LC technology continues to evolve driven by the need for high-throughput and high-resolution analyses. Over the past several years, small particle size packing materials have been introduced by several column manufacturers to enable fast and efficient LC separations. Several examples of pharmaceutical analyses, including impurity profiling of taxanes and atracurium besylate, *in-process* testing of peptides in injectable dosage form, using sub-2 μm column technology are presented in this paper, demonstrating some of the capabilities and limitations of the technology.

1. Introduction

Ease of use, adaptability to a wide range of detectors, availability of a wide selection of stationary phases, its high resolving power and speed of analysis have made the use of modern liquid chromatography (LC) very popular in the pharmaceutical industry. Liquid chromatography is typically employed throughout the entire pharmaceutical development process, from drug discovery to raw material analysis, impurity profiling, stability studies and final product testing [1–3]. The development of LC methods for analysis of drugs and their related substances is typically a time-consuming process in an analytical laboratory. Therefore, separation scientists are continually driven towards the use of more efficient LC technology to speed up the method development and validation process, as well as productivity in quality control laboratories. Efficiency and speed of analysis become particularly important while performing *in-process* pharmaceutical testing, a necessary but time-consuming step to achieve high quality standards in pharmaceutical product manufacturing [4,5].

Over the years, LC packing materials have evolved from irregular shaped 10 μm silica particles to small spherical particles, which are now less than 2 μm in diameter [6]. Spherical small

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particles can be packed more uniformly and homogeneously than their irregular shaped counterparts, thus providing better efficiency and resolution for chromatographic separations.

However column permeability tends to decrease significantly, which is accompanied with an increase in column back pressure [1,7]. High back pressure requires use of high-pressure LC systems allowing operation that exceeds 400 bars. The use of much shorter LC columns (< 50 cm in length) at low flow rates, or the use of high separation temperatures to reduce mobile phase viscosity may alternatively be used to reduce back pressure, often at the cost of adequate separation efficiency [6,8]. Although short columns with small particles provide rapid LC separations, the column plate number is not always sufficient to allow complex multi-components analysis. In this paper, several examples of pharmaceutical analyses, including impurity profiling of taxanes and atracurium besylate, *in-process* testing of peptides in injectable dosage form, using sub-2 μm column technology will be presented, demonstrating some of the capabilities and limitations of the technology.

2. Materials and method

The experimental results reported in this paper were obtained on Waters Acquity UPLC® (Milford, MA, USA) systems with either a Photodiode Array Detector or tunable UV (TUV) detector. The first example of application of a sub-2 μm column with a high pressure LC system to drug compound impurity profiling involved the use of an Acquity UPLC® BEH C18, 100 mm × 2.1 mm column with 1.7 μm hybrid silica particles (Waters, Milford, MA, USA). The second example involved a Zorbax Eclipse XDB-C18, 50 mm × 4.6 mm column with 1.8 μm particle size (Agilent Technologies, Santa Clara, CA, USA). Analyses of peptides were performed on an Acquity UPLC® BEH C18, 50 mm × 2.1 mm column with 1.7 μm hybrid silica particles, an Acquity UPLC® BEH 300 C18, 50 mm × 2.1 mm column with 1.7 μm hybrid silica particles with 300 Å pores, and a Kinetex® C18, 100 mm × 2.1 mm column with 1.7 μm core-shell particles from Phenomenex (Torrance, CA, USA). High-quality HPLC grade reagents and solvents were used throughout this study. Mobile phases and samples were filtered on 0.2 μm GHP membrane and syringe filters (Pall Corporation, Port Washington, NY, USA).

3. Results and discussion

3.1. Impurity profiling of drug substances and products with sub-2 μm LC columns

Impurity and degradation profiling is an integral part of the pharmaceutical quality control of drug substances and products. Impurity profiling often requires the use of longer LC columns, along with a mobile phase gradient [9,10]. A typical example involves the impurity profiling of an analog of Taxol® requiring the use of a 250-mm YMC™ ODS-AQ column with a water/acetonitrile gradient, to achieve the chromatographic separation of the taxane drug substance from its many impurities [11]. Such a challenging separation may take upto 90 min per sample injection, including mobile phase gradient and column re-equilibration.

Use of conventional LC is excessively time-consuming and could not practically be used for process testing during pharmaceutical manufacturing, which typically requires rapid testing methodologies. A Waters Acquity UPLC® BEH C18 column with 1.7 μm silica hybrid particles was used advantageously to achieve a 7-fold reduction in analysis time (Fig. 1). An equivalent gradient separation was achieved in 10 min with a sub-2 μm column [12], as compared to the 75 min run time with the conventional 3 μm ODS-AQ column. Such a short analysis run time with UPLC is much more compatible with impurity profiling while performing *in process* testing.

For a typical analysis sequence requiring replicate injections of blank, standard (for system suitability), and sample solutions, this represents a significant reduction in analysis time and total volume of mobile phases consumed: 2.8 h of analysis time for 12 injections and a total volume of mobile phases of 100 mL using the Acquity UPLC methodology, as compared to 18 h of analysis time and more than 1 L of mobile phase using the conventional LC method. This represents not only a significant gain in efficiency, but also a significant reduction in operating costs for solvents used to prepare mobile phases and a reduction in waste disposal.

Another example of a challenging LC separation using a sub-2 μm column involves the impurity and degradation profiling of atracurium besylate injection. Atracurium besylate is a muscle relaxant used in anesthesia [13]. This molecule has four chiral stereocenters and may occur in 10 different

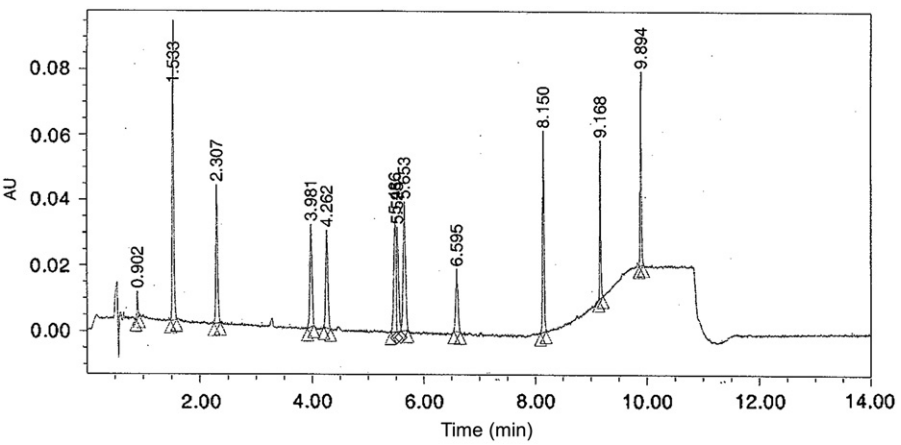

Figure 1 Example of chromatographic separation of a taxane from known impurities and degradants on a Waters Acquity UPLC® BEH C18 column (100 mm × 2.1 mm), using a water/acetonitrile gradient at a flow rate of 0.6 mL/min and UV detection at 228 nm.

stereoisomeric forms, which makes impurity and degradation profiling particularly challenging. Atracurium besylate injection is typically composed of 55.0–60.0% of the cis–cis isomer, 34.5–38.5% of the cis–trans isomer, and 5.0–6.5% of the trans–trans isomer [14]. The most biologically active isomer is the cis–cis isomer, also known as cisatracurium besylate [15].

The LC methodology in the United States Pharmacopeia for atracurium besylate describes use of a 250 mm × 4.6 mm octadecylsilane base-deactivated column with a 30 min mobile phase gradient at 1.0 mL/min [14]. However, we have demonstrated that separation of the main isomers of atracurium besylate can be readily achieved on a much shorter column, 50 mm × 4.6 mm with a 1.8 μm C18 stationary phase, in one third of the time with excellent resolution [16]; the resolution between the trans–trans, cis–trans, and cis–cis isomers in Fig. 2 is superior to 2.4. In addition to being more efficient, the dimensions of the Eclipse XDB column allow the fast LC methodology for atracurium besylate to be operated on a conventional system since the maximum column back pressure does not exceed 300 bars. However, care must be taken to minimize the internal volume of the system to reduce band spreading and optimize peak resolution. It is important to minimize connecting tubing as part of instrument optimization, selecting the narrowest diameter and shortest length tubing to prevent peak dispersion and loss in resolution.

Atracurium besylate injectable solution is refrigerated to extend its shelf-life, as it degrades when exposed to room temperature for just few hours. The solution is also particularly sensitive to solution alkalinity [17,18]. Major degradants of atracurium besylate include early-eluting laudanosine and a monoquaternary acrylate of laudanosine besylate [18], which elutes near the end of the mobile phase gradient. In Fig. 2, all degradants eluted within 8 min allowing rapid assay and degradation profiling. While laudanosine elutes at a relative retention time (RRT) of 0.27 with respect to cisatracurium besylate, monoquaternary laudanosine besylate elutes at RRT 1.26 after the cis–cis isomer of atracurium besylate. Therefore, this methodology may not only be successfully applied to *in process* assay of atracurium besylate, but also to the drug product degradation profiling.

3.2. *Use of sub-2 μm column technology for in process testing of drugs for injection*

In process testing after compounding and formulating dosage forms is becoming a common practice in manufacturing of drugs for injection. Testing prior filtration and vial filling allows *in process* determination indicating whether or not the final products will meet specifications, allowing process optimization and concentration adjustment to maintain high-quality drug manufacturing. Speed of analysis is particularly important while performing *in process* testing, since the manufacturing process must be interrupted until results from assay of the drug formulation are obtained. *In process* pharmaceutical testing may take full advantage of the small particle size column technology and high-pressure LC systems to gain separation speed, while maintaining adequate efficiency and resolution.

Unfortunately, application of sub-2 μm column technology is not always successful as exemplified in the analysis of octreotide acetate, a cyclic octapeptide mimicking natural somatostatin [19]. Separation of octreotide acetate from the phenol preservative in a multi-dose formulation was achieved using a Waters Acquity UPLC® BEH C18 column, in combination with a rapid mobile phase gradient allowing *in process* analysis to be performed in less than 3 min (Fig. 2). The unidose formulation contains mannitol and sodium acetate buffered with acetic acid at pH 4.2; the multi-dose formulation additionally contains 0.5% of phenol preservative.

Results from the dosage of the cyclic octapeptide in representative batches of several formulations and strengths are detailed in Table 1. Results from the Acquity UPLC methodology and conventional LC methodology involving a 250 mm × 4.6 mm YMC™ ODS-AQ column and a phosphoric acid–acetonitrile gradient are compared. Although sufficiently sensitive, inaccurate dosing of the octapeptide at low dosage strength was systematically observed with the Acquity UPLC methodology. While providing assay values consistent with conventional LC at 200 and 500 μg/mL, inaccuracy greater than 15% was observed in the peptide assay at 50 μg/L with a result lower than the specification limit [16]. Despite evident gain in efficiency, inconsistencies in assay values did not allow use of the UPLC methodology for *in process* testing of octreotide acetate formulation.

Non-linear response is not uncommon in the analysis of peptides and proteins. On-column system adsorption is particularly common in the analysis of proteins by capillary electrophoresis [20]. The use of low pH, high ionic strength buffers, and a higher sample concentration or injection volume may help reduce the effect of adsorption on the analysis of peptides and proteins by LC. While higher dosage strengths at 200 and 500 μg/mL are diluted by a factor of 4 and 10, respectively, with 0.1% aqueous phosphoric acid, the low dosage strength at 50 μg/mL must be injected without further dilution at pH 4.2 to maintain adequate sensitivity. Adsorption of octreotide acetate from the low-strength unidose formulation, injected at a higher pH, may explain the inaccurate dosage results obtained by UPLC. Use of a large pore Acquity UPLC BEH 300 C18 column did not help improving the accuracy of the measurement at low-dose of octreotide acetate, with measured inaccuracy of 12% as compared to the theoretical concentration [16]. The low internal volume of small particle size columns does not allow injection volumes above a few microliters without risking overloading the UPLC column. Use of conventional LC with a 50 μL injection volume proved to be more reliable for the determination of the low-dose peptide formulation.

Inaccurate measurement of the main impurity of the anti-platelet peptide drug eptifibatide was also observed using a Phenomenex Kinetex C18 sub-2 μm column [16]. In Fig. 3, the impurity eluting immediately after the main peak cannot be detected when injected alone at a concentration of 0.5 μg/mL, representing a concentration equivalent to 0.1% of the sample concentration of eptifibatide. Injected along with eptifibatide, the same impurity injected at the exact same concentration can be easily detected and measured with a signal-to-noise ratio of at least 10. Even at a concentration 10 times higher, representing an equivalent concentration of 1% or 5 μg/mL, significant loss in sensitivity is observed for eptifibatide main impurity.

When peptide analysis is involved, low concentration material appears to be difficult to recover from the high pressure LC system used along with sub-2 μm columns,

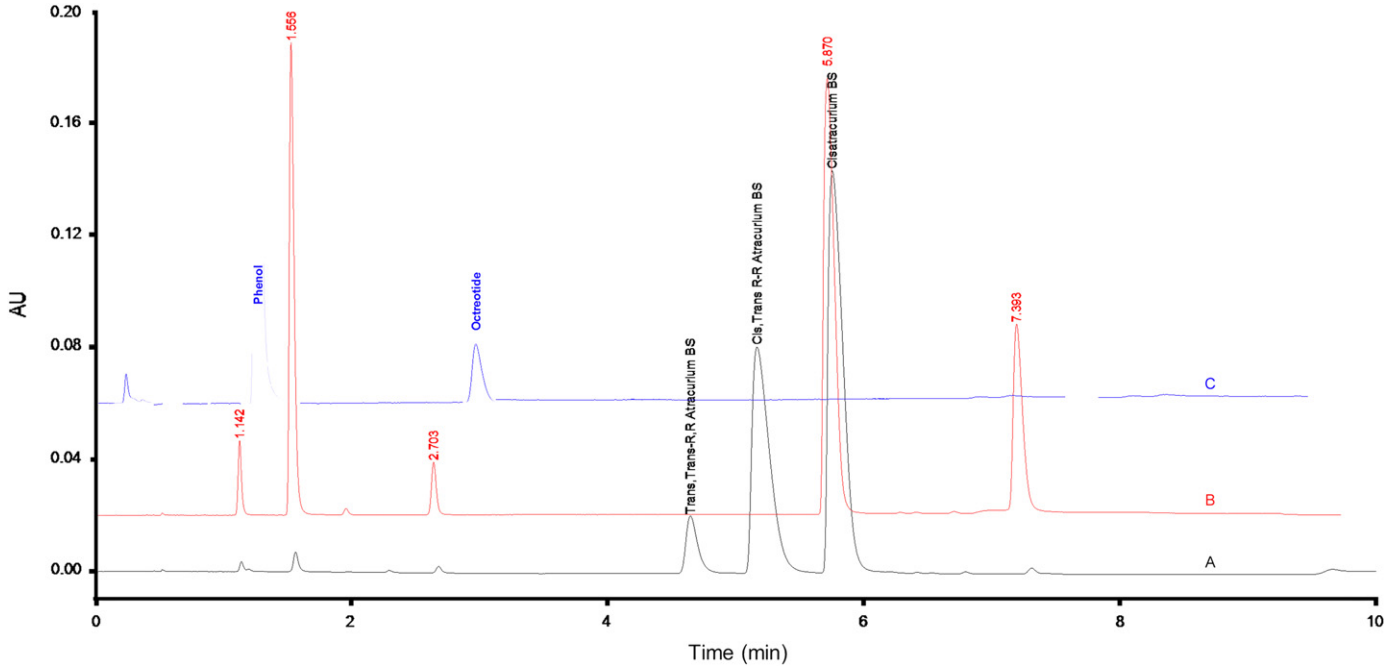

Figure 2

(A) Example of a chromatographic separation of atracurium besylate stereoisomers using an Agilent Technologies Zorbax Eclipse XDB-C18 50 mm × 4.6 mm column with 1.8 μm particle, using a potassium phosphate buffer–acetonitrile–methanol gradient at a flow rate of 1.0 mL/min and UV detection at 280 nm.

(B) Example of a chromatographic separation of a degraded solution of cis–cis atracurium besylate exposed to a temperature of 80 °C for 60 min. The same chromatographic conditions as those used in (a) were used. The peak eluting at 1.6 min corresponds to laudanosine (RRT = 0.27); the peak eluting at 7.4 min corresponds to monoacrylate laudanosine besylate (RRT 1.26).

(C) Separation of a peptide from phenol preservative in a multi-dose formulation. Experimental conditions: Waters Acquity UPLC® BEH C18 column (50 mm × 2.1 mm, with 1.7 μm hybrid silica particles), using a mobile phase gradient with 0.1% aqueous phosphoric acid and acetonitrile in 4 min, at a flow rate of 0.6 mL/min, with an injection volume of 1 μL and detection wavelength at 220 nm. Solution concentration: 50 μg/mL.

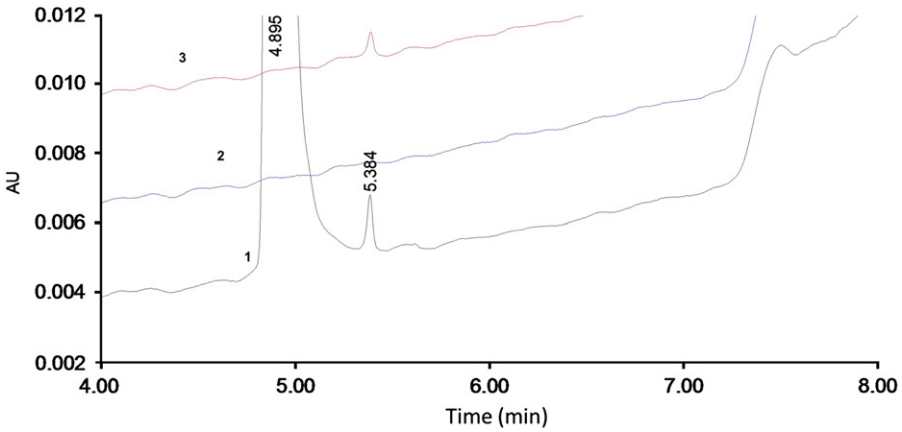

Figure 3 Impurity profiling of eptifibatide drug substance by UPLC.
Experimental conditions: Phenomenex Kinetex C18 column (100 mm × 2.1 mm, with 1.7 μm core-shell particles) using a mobile phase gradient with diluted trifluoroacetic acid and acetonitrile, at a flow rate of 0.5 mL/min, with an injection volume of 1 μL and detection wavelength at 220 nm. Solution concentration of eptifibatide: 500 μg/mL

(1) Eptifibatide spiked with 0.5 μg/mL of its main impurity, which elutes after 5.38 min.
(2) Under the same conditions, eptifibatide main impurity injected alone at 0.5 μg/mL cannot be detected.
(3) Eptifibatide main impurity injected at 5 μg/mL can be barely detected with a signal-to-noise ratio of 2:1.

Table 1 Dosage of an octapeptide in a multi-strength drug product formulation.

Octapeptide formulation	Specifications (μg/mL)	Dosage release testing (μg/mL)	Dosage testing by UPLC (μg/mL)	Difference (%)
Unidose low-strength batch #1	45–55	51	42	17.6
Multidose batch #2	180–220	209	206	1.4
Unidose high-strength batch #3	450–550	521	517	0.8

limiting detectability and method sensitivity. On-system adsorption of a small amount of peptide is suspected, leading to a loss in sensitivity at low concentration. Results from the analysis of octreotide acetate and Eptifibatide have indicated that response signal is lost when injecting only a few nanograms of peptide. Although potential adsorption sites could not be clearly identified, it is suspected that the low porosity column frits used with sub-2 μm stationary phases may be involved. Low porosity 0.2 μm column frits tend to clog more easily than larger pore 0.5–2 μm frits used with conventional LC columns, especially when analyzing biological molecules and samples with complex matrices.

4. Conclusion

Sub-2 μm column technology and high-pressure LC systems can advantageously be used in challenging analyses with significant gain in speed, without sacrificing separation efficiency and resolution. Useful mostly in the early phases of drug development until very recently, application to late stage pharmaceutical development and quality control is becoming increasingly important as exemplified here by several applications. When fast liquid chromatography is involved, sub-2 μm column technology and high-pressure LC have been particularly successful in purity analysis of hydrophobic drug substances, requiring high-resolving power and a rapid mobile phase gradient.

Although not always straightforward as any new technology has its own limitations, transfer of methodologies from conventional LC to small particle size column LC is likely to become increasingly important with the increased availability of high pressure systems in quality control laboratories and wider range of sub-2 μm stationary phases. Unfortunately when biological molecules and samples are involved, the limitations of the fast LC technology can seriously outweigh the gain in efficiency. Low porosity column frits are more prone to clog easily, requiring additional care in filtering mobile phases and sample preparation. Sensitivity issues may also be encountered, limiting the applicability of sub-2 μm column technology in the routine analysis of peptides; conventional LC being then a more reliable and robust option.

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