The introduction of ultra-high performance liquid chromatography (UPLC) has marked a radical change by opening new doors for analysts to fetch rapid analytical separation techniques without sacrificing high-quality results obtained earlier by high performance liquid chromatography (HPLC). Many laboratory experts have acknowledged that UPLC will ultimately supersede all the conventional HPLC techniques (1). The immaculate separation method of HPLC has many advantages like robustness, ease of use, changeable sensitivity and selectivity but the main limitation is lack of efficiency in comparison to gas chromatography or capillary electrophoresis (2, 3).

UPLC is a technique which comprises the above mentioned features and stands better than HPLC in many ways as it shows better chromatographic resolution, performs more sensitive analysis, consumes less time, reduces solvent consumption (19–23) and has high analysis speed (24–26).
Contribution of small particles in UPLC

The small particle size (i.e., less than 2 µm) of stationary phase is the basis of UPLC technique. The basal principle of this technique is governed by an empirical formula known as Van Deemter equation (27) that describes the relationship between linear velocity (flow rate) and plate height (HETP or column efficiency). This equation shows that enhancement of efficiency of UPLC technique cannot be accomplished without using smaller particle size than those used in conventional HPLC technique. In Figure 1, Van Deemter plot demonstrates that as the particle size decreases less than 2 µm there is a significant gain in the efficiency, and this condition is well maintained even if there is an increase in flow rate or linear velocities. This plot indicates that the usable flow range which shows good efficiency is much greater for smaller particles as compared to the larger particles (28, 29). The Van Deemter plot is governed by the equation: \( H = A + \frac{B}{v} + Cv \), where \( v \) is linear velocity and A, B and C are constants. A is independent of velocity and represents the “Eddy” mixing and when column particles are uniformly small, the value of A is the lowest. B is axial diffusion or the natural diffusion tendency of molecules and this effect is diminished at high flow rates, so this term is divided by \( v \). C is due to kinetic resistance to equilibrium in the separation process. The design and development of required 1.7 µm particles was a challenging task, contrary, researchers have shown keen interest in the development of these particles in order to capitalize their advantages. Despite, the availability of high efficiency non porous 1.5 µm particles in the market; they were not employed in UPLC columns due to their poor loading capacity and retention due to low surface area. UPLC required porous particles, which can withstand the high pressure in order to maintain their retention and capacity similar to that of HPLC (30, 31). Silica particles possess good mechanical strength but their application was limited by narrow pH application range and generally exhibit tailing during analysis of basic analytes (32). However, polymeric columns did not have any pH limitations but found to have low efficiency. In 2000, the first generation hybrid chemistry utilizes the classical sol-gel synthesis method to create durable columns that incorporated carbon in the form of methyl groups (33, 34). These columns exhibit several advantages such as mechanical strength, high efficiency and are operative over an extended pH range. However, they do not possess enough mechanical stability necessitated by UPLC. Consequently, the second generation bridged ethane hybrid (BEH) technology was developed (35ñ37). This technology increases the mechanical stability of 1.7 µm particles by bridging the methyl groups in the silica matrix as shown in Figure 2 that lead to the production of the columns which can withstand high pressure and pH. These BEH columns are highly efficient as efficiency of a column is directly proportional to its length and is inversely proportional to the particle size. The application of BEH columns resulted in the detection of additional drug metabolites, superior separation and improved spectral quality (38, 39).

Figure 1. Van Deemter plot illustrating the evolution of particle sizes over the last three decades
INSTRUMENTATION

The basic instrument in UPLC technique had to keep its tempo up in order to take advantage of the enhanced speed, superior resolution and greater sensitivity provided by small particles. A design with advanced technology in the pump, autosampler, detector, data system, and service diagnostics was required to fulfil the purpose. The basic instrumentation of UPLC is discussed below.

Pumping devices

An ideal pump for UPLC has a capacity of delivering solvent at higher pressure around 15000 psi for the optimum flow rate with maximum efficiency across 15 cm long column packed with 1.7 µm particles (40, 41). UPLC uses two serial pumps with pressure limit of 1000 bar (42, 43) and have inbuilt solvent selector valves, which have the capability to choose the accurate solvent ratio from up to four solvents (44).

Sample injector manager

The UPLC system with its flow through-needle design sample manager addresses three design challenges for reliable performance: robust sealing of the needle at higher pressure, minimizing the extra-column band spread for narrow peaks and performing pulse free injection process to protect column from extreme pressure fluctuations. When an injec-
Figure 3. The injection sequences of UPLC in both load and inject phase

Figure 4. UPLC column chemistries
tion is initiated, the inject valve diverts flow from the needle in order to collect sample from the vial as shown in Figure 3. The needle is inserted into the vial to withdraw the exact volume of the sample required and then it returns to the injection port. The needle is pushed against the internal sealing surface of the port and the injection valve turns and the sample is pushed down to the injection port. Dispersion of the sample can be minimized by keeping less distance between the injection port and the inject valve. After injecting the sample, the needle is washed for a specified amount of time to minimize sample carryover. Beside this, there are also direct injections for biological substances mentioned in the literature (45, 46).

<table>
<thead>
<tr>
<th>No.</th>
<th>Components analyzed</th>
<th>Column specifications</th>
<th>Mobile phase flow rate (mL/min)</th>
<th>Retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Metoprolol (MT), Atorvastatin (AT) and Ramipril (RM) from capsules dosage forms (49)</td>
<td>Zorbax® XDB-C18 (4.6 x 50 mm)</td>
<td>1.0</td>
<td>1.3, 2.1 and 2.6 for MT, AT and RM, respectively</td>
</tr>
<tr>
<td>2.</td>
<td>Aspirin (ASP), Clopidogrel bisulfate (CLP) and Atorvastatin calcium (ATV) from capsule dosage form (50)</td>
<td>Acquity BEH C18 column (2.1 x 100 mm, 1.7 μm)</td>
<td>0.35</td>
<td>0.59, 1.04 and 2.89 for ASP, CLP and ATV, respectively</td>
</tr>
<tr>
<td>3.</td>
<td>Fexofenadine in human plasma with carbamazepine as internal standard (51)</td>
<td>Acquity BEH C18 column (2.1 x 100 mm, 1.7 μm)</td>
<td>0.25</td>
<td>9.58 and 4.69 for fexofenadine and carbamazepine, respectively</td>
</tr>
<tr>
<td>4.</td>
<td>Secnidazole (SNZ), Fluconazole (FCZ) and Azithromycin (AZTM) in tablet dosage forms (52)</td>
<td>Acquity BEH Shield RP18 column (2.1 x 100 mm, 1.7 μm)</td>
<td>0.3</td>
<td>3.7, 4.1 and 6.2 for SNZ, FCZ and AZTM, respectively</td>
</tr>
<tr>
<td>5.</td>
<td>Narirutin, Hesperidin, Naringin, Neo hesperidin and Meranzin hydrate in Fructus aurantii-type preparations (53)</td>
<td>Acquity BEH C18 column (2.1 x 100 mm, 1.7 μm)</td>
<td>0.3</td>
<td>6.8, 8, 9, 10.1 and 11.6 for narirutin, hesperidin, naringin, neohesperidin, meranzin hydrate, respectively</td>
</tr>
<tr>
<td>6.</td>
<td>Diclofenac (54)</td>
<td>Acquity BEH C18 column (2.1 x 50 mm, 1.7 μm)</td>
<td>0.6</td>
<td>1.5</td>
</tr>
<tr>
<td>7.</td>
<td>Fourteen different samples of Rheum palmatum L (55)</td>
<td>Acquity BEH C18 column (2.1 x 50 mm, 1.7 μm)</td>
<td>0.4</td>
<td>In between 0–10 for all 14 samples</td>
</tr>
<tr>
<td>8.</td>
<td>Catechin, epicatechin, B2 and B5 (epicatechin-4a-8-epicatechin), C1 and tetramer D (epicatechin-4a-8-epicatechin-4a-8-epicatechin) in chocolates (56)</td>
<td>Acquity BEH C18 column (2.1 x 50 mm, 1.7 μm)</td>
<td>0.3</td>
<td>In between 0–3 for all six compound</td>
</tr>
<tr>
<td>9.</td>
<td>Hippuryl–histidyl–leucine (HLL) and hippuric acid (HA) for assaying angiotensin converting enzyme activity (57)</td>
<td>Acquity BEH C18</td>
<td>0.4</td>
<td>1.12 and 1.37 for HA and HHL, respectively</td>
</tr>
<tr>
<td>10.</td>
<td>Seven steroids; dienogest, finasteride, gestodene, levonorgestrel, estradiol, ethinylestradiol and norethisterone acetate (58)</td>
<td>Acquity BEH C18 column (2.1 x 50 mm, 1.7 μm)</td>
<td>0.55</td>
<td>Less than 2.5 for all seven steroids</td>
</tr>
<tr>
<td>11.</td>
<td>Aspirin (AP) and Dipyridamole (59)</td>
<td>Hypersil Gold C18, column (1.9 μm, 100 mm x 2.1 mm)</td>
<td>0.5</td>
<td>0.83 and 1.62 for AP and DL respectively.</td>
</tr>
<tr>
<td>12.</td>
<td>Raloxifene hydrochloride and its related impurities (60)</td>
<td>Extended C18, 50 mm x 3.0 mm ID with 1.8 microns particles</td>
<td>0.7</td>
<td>Retention time of different impurities was 0.70, 1.59, 2.46 and 6.05.</td>
</tr>
</tbody>
</table>
Columns

The UPLC columns are made up of small particles having size less than 2 µm. The role played by small particle size in UPLC technique has been mentioned above. The particles are bonded in matrix as the bonded stationary phase is required for providing both retention and selectivity. Four bonded stationary phase (Fig. 4) columns manufactured by ACQUITY are available in the market, which can be used by UPLC technique (47, 48).

- BEH C18 and C8 columns – These are straight alkyl chain, most preferred UPLC columns as they can be used over wide pH range. The trifunctional ligands produce low pH stability, which is combined with high pH stability of 1.7 µm BEH particles to produce the widest usable pH operating range.
- BEH Shield R18 columns – They provides selectivity to UPLC as it complements C18 and C8 columns.
- BEH Phenyl columns – They has tri-functional C6 alkyl ethyl between the phenyl rings and the silyl functionality.
- BEH Amide columns – The combination of trifunctionally bonded amide phase with BEH small particles provides exceptional column life time. They facilitate the use of a wide range of phase pH i.e., from pH 2 to 11.

Detectors

Two tuneable UV-visible photodiode array detectors namely ACQUITY PDA and ACQUITY PDA eλ detectors are generally used for the routine analysis and method development, which have the power of detecting and quantifying trace impurities. The data rates of these detectors are up to 80 Hz having low noise specification i.e., 10 Au with wide range of spectra analysis up to 500 nm (PDA detector) and 800 nm (eλ detector). To avoid band spreading and concentration variance, low volume light guiding flow cells are used comprising of Teflon AF, which eliminates the internal absorption by using total internal reflection principle to enhance the light transmission efficiency.

Applications

This technique has been successfully applied to pharmaceutical analysis of numerous drugs, a few of them are enlisted in Table 1 (49–60), with the improvement in the factors like retention time and mobile phase consumption. Narasimham and Barhate developed and validated UPLC method for the simultaneous determination of β-blockers and diuretic drugs in pharmaceutical formulations with the objective of reducing analysis time and maintaining good efficiency (61). The chromatographic separations of all the drugs were achieved on a Waters Acquity BEH C18, 50×2.1 mm, 1.7 µm UPLC column within a short runtime of 2.6 min. The results obtained were compared with the previously developed HPLC methods (Table 2). The elution time of all the drug compounds in UPLC were observed to be reduced by 10-fold. The higher peak capacity, i.e., 1494 in UPLC as against in HPLC i.e., 942, conforms the better gradient separation efficiency and resolving power of UPLC system. Furthermore, the applications of this technique are discussed below:

Rapid analysis of products

Before the introduction of this technique, chromatographers were compromising with the factors like low speed and resolution. Even in the case of natural products more resolution was required to get apt knowledge of the additional peaks. UPLC can be extensively used in the analysis of natural products and herbal medicines. In 2004, the first commercially available UPLC system was the ACQUITY UPLC that fulfilled all the requirements needed for the separation of various pharmaceutical products

<table>
<thead>
<tr>
<th>Drug component</th>
<th>Retention time</th>
<th>Capacity factor</th>
<th>USP tailing</th>
<th>Resolution</th>
<th>Peak capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
<td>UPLC</td>
<td>HPLC</td>
<td>UPLC</td>
<td>HPLC</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>13.4</td>
<td>1.9</td>
<td>4.3</td>
<td>8.9</td>
<td>1.4</td>
</tr>
<tr>
<td>Amiloride</td>
<td>10.2</td>
<td>1.1</td>
<td>3.1</td>
<td>4.7</td>
<td>1.1</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>12.8</td>
<td>1.3</td>
<td>4.1</td>
<td>5.4</td>
<td>1</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>17.6</td>
<td>2.2</td>
<td>6</td>
<td>10.4</td>
<td>1.2</td>
</tr>
<tr>
<td>Propranolol</td>
<td>16.4</td>
<td>2.1</td>
<td>5.6</td>
<td>9.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Felodipine</td>
<td>24.3</td>
<td>2.6</td>
<td>8.7</td>
<td>11.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>
related small organic molecules, proteins and peptides (62).

Identification of metabolites

When a new chemical entity (NCE) reaches the development stage, identification of its metabolites becomes a perpetual process. The detection of all circulating metabolites of a candidate drug is necessary. The identification of major metabolites is done by performing in vitro discovery studies. The weak spots of metabolites of drug candidate molecules are recognized and hence protected by altering the compound structure. Plumb et al. have explored the application of UPLC-MS for analysis of metabolites of candidate drug (63, 64). They also examined the application of UPLC-MS in profiling metabolic pathways for functional genomics studies (65). Their studies revealed that higher resolution of UPLC in terms of specificity and spectral quality diminishes the risk of missing any detection of potentially important metabolites.

Bioequivalence studies

The detection of drug in biological sample is very important to study the pharmacokinetics, toxicity and bioequivalence of drug. Blood, plasma and urine can be used as biological matrices which consist of sample drug in them (66). Previously LC-MS was used for detecting drug in these biological matrices. But nowadays UPLC-MS is used as it has some advantages over LC-MS such as:

- UPLC-MS provides unprecedented performance and compliance support.
- It has excellent chromatographic resolution and sensitivity.
- UPLC can accommodate more number of samples than HPLC so sample throughput is enhanced.
- It gives not only high quality results but also ensures the safety of data by using security based data collection software.
- Tolley et al. (67) in their studies have concluded that higher efficiency, better resolution and higher peak capacity provided by UPLC technique is particularly important for the analysis of proteins and peptides.

Detection of impurities

The detection of impurities in raw material as well as in final product is the most vital phase of the drug development process. Earlier studies have accounted for excellent detection of impurities by HPLC as it has sufficient resolution for the determination of the lowest level of impurities with good reproducibility results, but due to the presence of excipients, there is prolonged HPLC analysis so it becomes necessary to perform several analytical runs to get the required data. This curb can be overcome by using UPLC technique as it offers exact required data and is operational at alternate low and high collision energies. The fast change of collision energy produces both precursor and product ions of all analytes present in the sample, which allows rapid identification and profiling of impurities. Lippert et al. (68) pointed that in some cases, the impurity and compound are of the same molecular weight and have similar structures so they could not be differentiated by MS or LC/MS necessitating higher chromatographic resolution which can be provided by UPLC.

Table 3. Comparative study of parameters used in HPLC and UPLC technique.

<table>
<thead>
<tr>
<th>No.</th>
<th>PARAMETER</th>
<th>HPLC</th>
<th>UPLC</th>
<th>INFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Particle size</td>
<td>3–5 µm</td>
<td>Less than 2 µm</td>
<td>Reduction in analysis time</td>
</tr>
<tr>
<td>2</td>
<td>Mobile phase flow rate</td>
<td>More</td>
<td>Less</td>
<td>Lesser mobile phase consumption</td>
</tr>
<tr>
<td>3</td>
<td>Injection volume</td>
<td>5 µL (Std. in 100% methanol)</td>
<td>2 µL (Std. in 100% methanol)</td>
<td>Can deal with even small traces of sample</td>
</tr>
<tr>
<td>4</td>
<td>Column</td>
<td>ALLTIMA C18, ZORBAX C8</td>
<td>ACQUITY UPLC BEH C18 and C8</td>
<td>Can withstand high pressures and have high mechanical stability and efficiency</td>
</tr>
<tr>
<td>5</td>
<td>Column dimension</td>
<td>150 × 3.2 mm</td>
<td>150 × 2.1 mm</td>
<td>Higher resolution</td>
</tr>
<tr>
<td>6</td>
<td>Column temperature</td>
<td>30°C</td>
<td>65°C</td>
<td>Increased selectivity, lower solvent viscosity and increase mass transfer rate</td>
</tr>
<tr>
<td>7</td>
<td>Maximum back pressure</td>
<td>35–40 MPa</td>
<td>103.5 MPa</td>
<td>Faster separation</td>
</tr>
</tbody>
</table>
Dissolution testing

Dissolution testing is very important to control the quality and release of drug. In sustained release dosage formulations dissolution is the rate limiting step so this becomes very important in such cases. The dissolution profile of a drug signifies reliability and batch to batch uniformity of API (Active Pharmaceutical Ingredients) in the formulations. Nowadays, newer and relatively high potent formulations are coming in the market which requires enhanced analytical sensitivity. This can be fulfilled by UPLC which provides online sample acquisition. By using UPLC, dissolution testing can be fully automated as it can perform functions like data acquisition, analysis of samples aliquots, management of test results and distribution.

Besides the above mentioned applications, there are some conditions in which UPLC can be applied successfully, few of them are enlisted below:

- Identification and authentication of purity of newly synthesized pharmaceutical compound for which chemists need to be sure that they have synthesized the expected compound. The throughput of UPLC system’s remote status monitoring software along with the fast scanning MS allows the chemist to obtain high quality comprehensive data regarding the candidate compound in the shortest possible time.
- In performing the forced degradation studies or stress testing which are carried out under extreme conditions like peroxide oxidation, acid and base hydrolysis, photo-stability etc. The speed, resolution, and sensitivity of UPLC separations, when combined with the high-speed scan rates of UPLC-specific photodiode array and MS detection, makes the identification of degradation products more effective and time required to develop stability-indicating method is shortened.
- In performing highly accurate and well regulated, quantitative analyses performed in QA and QC laboratories in pharmaceutical industries.

UPLC – a real hope or just hype

UPLC brought new opportunities in pharmaceutical industries to get rapid analytical separation results while retaining method accuracy and resolution quality. Some laboratory experts believe that this technique will eventually replace all the conventional HPLC methods and consider it as a HOPE, while some critics says that UPLC cannot deliver long term and cost effective benefits and termed UPLC as a HYPE. This point of contradiction can be well elucidated by comparing the merits and demerits of the UPLC.

Merits

- There is a fivefold decrease in the analysis time for many separations in comparison to analysis time taken by other chromatographic techniques (69).
- Consumption of solvent is very low as run time is reduced in UPLC.
- Resolution and sensitivity of UPLC is greater than those of other conventional techniques.
- Sample output is enhanced so manufactures can produce more products according to required specification and hence there is reduction in failure of batches.
- Overall operation cost is reduced.
- Scope of multi residue methods is enhanced.
- Some parameters of UPLC which differ from HPLC and will turn in favor of chromatographic techniques are enlisted in Table 3.

Demerits

- UPLC operates at ultra high pressure up to 1000 bars so column requires more maintenance and life of column is reduced (70).
- Phases 1.7 µm sized are non regenerable and thus have limited use.

UPLC has better column technology, which can work under extremely high pressure conditions, have faster rate of detection and moreover, is based on already established HPLC technique so there is a hope that UPLC will increase the quality of pharmaceutical analyses and productivity of research scientists. While it may take some years for UPLC to become full time analytical technique, we remain optimistic that this technique will live up to expectations.

CONCLUSION

UPLC is based on much proven technology of HPLC technique and it not only enhances but flourishes the utility of conventional HPLC technique when many scientists started experiencing separation obstacles with it. The information provided per unit work by UPLC is much greater than HPLC as it has higher speed, resolution and sensitivity. Also the analysis time and solvent consumed in UPLC is less than in all previously used chromatographic techniques. On the contrary, in UPLC the factors like resolution, tailing factors and peak area/peak retention time repeatability are similar to those of HPLC. So, the transfer of an existing HPLC method to new UHPLC method is preferable as well as profitable in
the pharmaceutical industry and this has been successfully demonstrated in the literature (71, 72). Extraction of above data has revealed that UPLC will prove to be an essential and crucial tool for enhancing the quality of pharmaceutical analysis and also the productivity of the research scientists.

REFERENCES

Received: 18. 03. 2011