Development of a stability indicating HPLC method for the Pheroid™ delivery system

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“For from Him and through Him and to Him are all things. 
To Him be the glory forever!” Romans 11:36
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ABSTRACT

Stability plays an important role in the development of a new drug product. High Performance Liquid Chromatography (HPLC) is considered a stability indicating method of analysis. It is widely used in the pharmaceutical industry for the quantification of small organic molecules during stability testing.

Previous stability studies conducted on Pheroid™- based drug products, experienced problems with the generation of reliable data by means of HPLC analysis. With these studies it was concluded that the inconclusive results could either be attributed to the stability of the delivery system itself and the compatibility of the active pharmaceutical ingredients (API’s) with the delivery system, or to the usage of unsuitable HPLC methods. The aims of this study were to:

i. determine if the Pheroid™ delivery system changes significantly over time at accelerated storage conditions and how these changes influence the HPLC analysis,

ii. determine the effect of the anti-oxidant tert-butylhydroquinone (TBHQ) on the stability and HPLC analysis of the Pheroid™ delivery system, and

iii. to suggest a suitable approach for the analysis of Pheroid™- based drug products.

Pheroid™ microsponges, containing no API’s, were prepared and stored for a period of three months at 5°C, 25°C+60%RH, 30°C+65%RH and 40°C+75%RH. Two of the four Pheroid™ formulations contained an extra anti-oxidant, namely TBHQ. Monthly HPLC analyses were done using existing methods for mefloquine and artesunate. In addition to HPLC analysis, particle size analysis and Confocal Laser Scanning Microscopy (CLSM) were undertaken to support the HPLC results and provide information concerning the overall stability of the Pheroid™ delivery system.

After the completion of the above analyses, experiments were carried out to determine whether adjustments to some of the key chromatographic parameters could improve the separation of Pheroid™- based samples. The parameters that were subjected to change included the organic solvent, isocratic versus gradient separation, pH and detection wavelength. Two pro-Pheroid vesicles formulations were prepared and stored for a three month period at 40°C+75%RH only. No API
was added to the one formulation while the other contained 2 mg/ml of mefloquine hydrochloride.

Results obtained indicated that the Pheroid™ formulations changed after exposure to elevated temperature and humidity. The number of detectable peaks increased, longer run times became necessary and solubility in the sample solvent (methanol) decreased. Solubility of the Pheroid™ formulations in methanol was preserved to some extent by the presence of TBHQ. Physical signs of instability like discoloration and creaming were noted for TBHQ-containing formulations. TBHQ also seemed to have influenced the particle sizes, particle size distributions and structure of the Pheroid™ microsponges.

With adjustments made to the HPLC method it was found that:

i. the sample solvent is incompatible with the HPLC system,

ii. very hydrophobic compounds are present in the Pheroid™-based samples,

iii. acetonitrile and methanol are unsuitable for both gradient and isocratic separation of Pheroid™-based samples,

iv. more Pheroid™ components absorb at shorter wavelengths, and

v. small changes in the pH values usually implemented do not influence the retention and selectivity of the Pheroid™ components.

The Pheroid™ delivery system proved to be too complex and hydrophobic for reversed phase HPLC analysis. Preparation of the sample by only diluting the Pheroid™ formulations with pure methanol was not optimal. These samples introduced compounds to the column of which some caused interferences with the analyte peak while others were difficult to elute from the column. To continue using HPLC for the analysis of Pheroid™-based drug products, it is therefore recommended that attention should be given to the development of a more appropriate sample preparation procedure, like solid phase extraction or liquid-liquid extraction, one that will eliminate the effects of the Pheroid™ components.

Physical instabilities noticed with the addition of TBHQ, suggest that there should also be attended to the compatibility and stability of each of the components in the Pheroid™ delivery system during formulation development.

**Key words:** HPLC, method development, sample preparation, stability, compatibility, Pheroid™ technology, TBHQ, mefloquine.
Stabiliteit speel 'n belangrike rol in die ontwikkeling van 'n nuwe geneesmiddel produk. Hoë-doeltreffendheid vloeistofchromatografie (HOVC) word beskou as 'n stabiliteits aanduidende analise metode. Dit word algemeen tydens stabiliteits studies in die farmaseutiese bedryf vir die kwantitatiewe bepaling van klein organiese molekules, aangewend.

Met die uitvoering van vorige stabiliteits studies op Pheroid™ gebaseerde geneesmiddel produkte, was die generering van betroubare data met behulp van HDVC, 'n uitdaging. Daar is tot die gevolgtrekking gekom dat die onbesliste resultate óf toegeskryf kon word aan gebrekkige stabiliteit van die Pheroid™ aflewering sisteem en onverenigbaarhede tussen aktiewe bestanddele en die aflewering sisteem, óf aan die gebrek aan geskikte HDVC metodes. Die doelwitte van hierdie studie was dus om te bepaal:

i. of die Pheroid™ aflewering sisteem noemenswaardig verander oor tyd met blootstelling aan versnelde bergingskondisies, en die effek daarvan op die HDVC analyse;

ii. wat die effek van die anti-oksidant tert-butielhidrokinoon (TBHK) op die stabiliteit en HDVC analyse van die Pheroid™ aflewering sisteem is; en

iii. om 'n geskikte benadering vir die analyse van Pheroid™ gebaseerde geneesmiddel produkte voor te stel.

Pheroid™ mikrosponsies, sonder enige aktiewe bestanddele, is vervaardig en geberg by 5°C, 25°C+60%RH, 30°C+65%RH en 40°C+75%RH vir 'n periode van drie maande. 'n Ekstra anti-oksidant, naamlik TBHK, is by twee van die vier Pheroid™ formulerings gevoeg. Maandelikse HOVC analises is uitgevoer volgens bestaande metodes vir meflokien en artesunaat. Om HOVC analises te ondersteun en inligting te verskaf rakende die algehele stabiliteit van die Pheroid™ aflewering sisteem, is deeltjiegrootte analyse en Konfokaal Laser Skanding Mikroskopie (KLSM) ook uitgevoer.

Na die voltooiing van die bogenoemde analises is daar geëksperimenteer met die aanpassing van sleutel chromatografiese parameters, om 'n moonlike verbetering in die skeiding van Pheroid™ gebaseerde monsters te ondersoek. Die parameters wat
onderwerp is aan verandering sluit die organiese oplosmiddel, isokratiese- teenoor gradiënt eluering, pH en die golflengte in. Twee pro-Pheroid vesikels formulerings is vervaardig en vir 'n tydperk van 3 maande geberg by slegs 40°C+75%RH. Geen aktiewe bestanddeel is by die een formulering gevoeg nie, terwyl die ander formulering 2 mg/ml meflokien hidrochloried bevat het.

Die resultate wat verkry is, dui daarop dat die Pheroid™ formulerings wel verander het na blootstelling aan verhoogde temperature en humiditeit. Die aantal pieke het toegeneem, die analise tye moes verleng word en die oplosbaarheid in die monster-oplosmiddel (metanol) het verswak. Die oplosbaarheid van die Pheroid™ formulerings in metanol het egter langer behoue gebly in die teenwoordigheid van TBHK. Waarnemings van fisiese onstabiliteit, soos verkleuring en oproming, is gemaak vir TBHK bevattende formulerings. Dit wil ook voorkom asof TBHK die deeltjiegroottes, deeltjiegrootteverspreiding en struktuur van die Pheroid™ mikroponsies beïnvloed het.

Daar is bevind met die aanpassings wat gemaak is aan die HDVC metode dat:

i. die monsters-oplosmiddel onverenigbaar is met die HDVC sisteem,

ii. verbindings met uitermatige hidrofobisiteit teenwoordig is in Pheroid™ gebaseerde monsters,

iii. asetonitriel en metanol ongeskik vir beide gradiënt en isokratiese skeiding van Pheroid™ gebaseerde monsters is,

iv. meer van die Pheroid™ komponente absorbeer UV by korter golflengtes, en

v. dat klein veranderinge in die pH waardes wat gewoonlik gebruik word, nie die retensie en selektiwiteit vir die Pheroid™ komponente beïnvloed nie.

Dit blyk dat die Pheroid™ aflewering sisteem te kompleks en hidrofobies is vir analise met behulp van omgekeerde fase HDVC. Die voorbereiding van monsters deur die Pheroid™ formulerings slegs te verdun met metanol, was nie optimaal nie. Verbindings wat met die analietpiek inmeng en verbindings wat moeilik uit die kolom gespoel word, was gevolglik ook in die monster teenwoordig. Om die gebruik van HDVC vir die analyse van Pheroid™ gebaseerde geneesmiddel produkte vol te hou, word die ontwikkeling van 'n meer gesikte monstervoorbereidings prosedure soos soliede-fase of vloeistof-vloeistof ekstraksie wat die effek van die Pheroid™ komponente sal elimineer, aanbeveel.
Aangesien TBHK die fisiese stabiliteit van die Pheroid™ formulerings beïnvloed het, behoort die verenigbaarheid en stabiliteit van elk van die afsonderlike komponente in die Pheroid™ aflowering sisteem, aandag te geniet tydens produk ontwikkeling.

*Sleutelwoorde:* HDVC, metode ontwikkeling, monster voorbereiding, stabiliteit, verenigbaarheid, Pheroid™ tegnologie, TBHK, meflokiën.
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ABBREVIATIONS

\(\alpha\) selectivity

\(\lambda_{\text{max}}\) wavelength of maximum UV absorbance

API active pharmaceutical ingredient

CLSM Confocal Laser Scanning Microscopy

COA Certificate of Analysis

DMSO dimethyl sulfoxide

FDA The Food and Drug Administration, USA

FL fluorescence

HDVC hoë-doeltreffendheid vloeistofchromatografie

HPLC high performance liquid chromatography

HSA hexane sulphonic acid

ICH International Conference on Harmonisation

IEC ion-exchange chromatography

IP The International Pharmacopoeia

IPG Impurity Profiling Group

\(k'\) capacity/retention factor

KLSM Konfokaal Laser Skandering Mikroskopie

LC liquid chromatography

MS mass spectrometry

\(N\) efficiency/plate number

NARP non-aqueous reversed phase

NPC normal phase chromatography

PDA photodiode array

RH relative humidity/relatiewe humiditeit

RI refractive index
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>RPC</td>
<td>reversed phase chromatography</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reversed phase high performance liquid chromatography</td>
</tr>
<tr>
<td>$R_s$</td>
<td>resolution</td>
</tr>
<tr>
<td>% RSD</td>
<td>percentage relative standard deviation</td>
</tr>
<tr>
<td>SEC</td>
<td>size-exclusion chromatography</td>
</tr>
<tr>
<td>T</td>
<td>tailing factor</td>
</tr>
<tr>
<td>TBHK</td>
<td>tert-butylhidrokinoon</td>
</tr>
<tr>
<td>TBHQ</td>
<td>tert-butylhydroquinone</td>
</tr>
<tr>
<td>TEA</td>
<td>triethylamine</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>USP</td>
<td>United States Pharmacopoeia</td>
</tr>
<tr>
<td>UV/Vis</td>
<td>ultraviolet/visible</td>
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</table>
The HPLC analysis of previous stability studies conducted on Pheroid™- based drug products, rendered inconclusive results, with only a few being successful. These studies concluded that the stability of the delivery system itself and compatibility of the API's with the delivery system may be in question, or that the HPLC method used is not suitable for the analysis of Pheroid™- based drug products.

To determine what the contributing factors for these poor results were, both HPLC method performance and product stability were evaluated. The conditions, under which previous HPLC analyses were performed, were recreated by subjecting the Pheroid™ delivery system to the same storage conditions used for these stability studies. Two existing HPLC methods were implemented and evaluated in terms of their performance (Chapter 3). Further tests were conducted where adjustments were made to some of the key chromatographic conditions (Chapter 4).

In addition to HPLC analysis, particle size analysis and Confocal Laser Scanning Microscopy (CLSM) were undertaken to support the HPLC results and provide information on the overall stability of the Pheroid™ delivery system.

The aims for this study can be summarised as follows:

1. To determine if the Pheroid™ delivery system changes significantly over time at accelerated storage conditions and how these changes influence the HPLC analysis.

2. To determine the effect of the anti-oxidant tert-butylhydroquinone (TBHQ) on the stability and HPLC analysis of the Pheroid™ delivery system.

3. To suggest a suitable approach for the analysis of Pheroid™- based drug products.
1.1 INTRODUCTION

The eventual degradation of a drug substance or drug product is a given. Rhodes (2007:12) lists the modes of degradation as being chemical, physical and biological in nature. Potential degradation or instability of a drug substance or product may influence the active pharmaceutical ingredient’s (API) content, its bioavailability, dosage uniformity, the product’s shelf life and may even lead to the formation of potentially toxic compounds. Stability studies are consequently performed to establish a specific drug’s stability on these various levels.

The most important reason for stability studies is the patient’s welfare. Regulatory authorities such as the Food and Drug Administration (FDA) and the International Conference on Harmonisation (ICH), have set forth a set of requirements and guidelines to be followed by pharmaceutical companies in the development of new drug products and substances to ensure that every pharmaceutical product that reaches a patient is safe, effective and of good quality (Rhodes, 2007:11).

The implementation of a validated stability indicating assay during stability studies is stipulated by the ICH (2003:7). Most of the pharmaceutical companies turn to high performance liquid chromatography (HPLC) to comply with the above requirements. This technique is both quantitative and highly discriminative (sec. 1.8). Reversed phase HPLC constitutes more or less 85% of pharmaceutical analyses (Hong & Shah, 2007:332). Due to its popularity and wide application, HPLC has also been the method of choice during stability studies performed on Pheroid™- based drug products.

The innovative Pheroid™ technology holds great potential in the pharmaceutical industry (refer to Chapter 2). A hurdle that still has to be overcome in the development of these products is proof of stability. HPLC methods previously implemented have failed to establish the stability of an API in the Pheroid™ delivery system (Cassim, 2007:156; Kuhn, 2008:75).
Therefore the aim of this study was to determine whether the HPLC method itself needs some adjustment in order to provide this proof of stability. The recommended steps for developing a stability indicating HPLC method are discussed in the sections that follow.

1.2 WHAT IS A STABILITY INDICATING METHOD?

According to the FDA (2000:4) a stability indicating method is “a validated, quantitative, analytical procedure, which can detect changes that may occur with time in the pertinent properties of the drug substance and drug product; and is able to accurately measure the active ingredients without interference from degradation products, impurities and excipients that may be present”.

In addition to the quantitative determination of the API content, the ICH (2003:7) also requires that any compound of the drug product which could change over time and may jeopardise safety, quality or efficacy, should also be quantified.

Bakshi and Singh (2002:1027) took these requirements into account with their definition of a stability indicating assay. They state that the term “stability indicating” has been used very liberally in literature. They distinguish further between specific and selective stability indicating methods, with the difference being in the quantitative measurement of the degradation products. According to them a specific stability indicating assay method separates the API from its degradation products and the excipients, so that a quantitative measurement of the API content is possible. A selective stability indicating assay method on the other hand, is able to separate the API and degradation products from each other and not only quantitatively measure the API content, but also measure the content of the different degradation products. According to this, a selective stability indicating assay method would thus meet the requirements of the ICH (Bakshi & Singh, 2002:1028).

1.3 STRATEGY FOR METHOD DEVELOPMENT

Simplicity and a minimum of experimental runs are both key elements of a sound method development strategy. The approach to method development can be either theoretical or empirical depending on what is known about the sample (Snyder et al., 1997:2,5). Snyder et al. (1997:5) postulates that the best approach is empirical, but with consideration of theoretical information.
Method development is known to be costly and time consuming. The introduction of software like DryLab™ made it possible for analysts to develop and optimise methods in shorter time periods by performing a minimum of experimental runs (Hong & Shah, 2007:351). DryLab™ technology and similar aids are not always available and therefore a method then needs to be developed by means of trial-and-error. This study required a trial-and-error approach.

The recommended strategy for HPLC method development remained more or less the same over the past twenty years as demonstrated in Table 1.1. Although two of the three publications elaborated a bit more, there seems to be five main steps in the method development process as illustrated by Dong (2006:195).

### Table 1.1: Strategies for HPLC method development.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Information on sample, define separation goals</td>
<td>Determine method and separation goals</td>
<td>Gather/generate background information – obtain physico-chemical properties</td>
</tr>
<tr>
<td>2</td>
<td>Need for special HPLC procedure, sample pre-treatment, etc.</td>
<td>Gather sample and analyte information</td>
<td>Determine if special handling/treatment of sample is needed</td>
</tr>
<tr>
<td>3</td>
<td>Choose detector and detector settings</td>
<td>Initial method development</td>
<td>From physico-chemical properties select detector $\lambda_{\text{max}}$</td>
</tr>
<tr>
<td></td>
<td>Choose LC method; preliminary run; estimate best separation conditions</td>
<td></td>
<td>Select LC mode and perform initial runs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Guessimate separation parameters/isocratic or gradient mode</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Perform forced degradation experiments to challenge method</td>
</tr>
<tr>
<td>4</td>
<td>Optimise separation conditions</td>
<td>Method fine-tuning and optimisation</td>
<td>Optimise separation conditions; tweak $R_s$ equation parameters</td>
</tr>
<tr>
<td></td>
<td>Check for problems or requirements for special procedure</td>
<td></td>
<td>Summarise methodology. Finalise documentation.</td>
</tr>
<tr>
<td>5</td>
<td>Validate method for release to routine laboratory</td>
<td>Method validation</td>
<td>Validate method/transfer to control laboratory</td>
</tr>
</tbody>
</table>

$\lambda_{\text{max}}$ - wavelength of maximum UV absorbance, $R_s$ - resolution

Hong and Shah (2007:333) state that as an alternative to developing a totally new method an existing method can be optimised. This may not always be optimal and it
is preferable to develop a new method (Snyder et al., 1997:403; Hong & Shah, 2007:342).

With previous stability studies conducted on API's formulated in the Pheroid™ delivery system, the HPLC methods did not prove to be suitable for the whole duration of the testing period. For that reason the strategy followed in this study was more focused on the evaluation of an existing method and the possible optimisation thereof, in an attempt to determine where the pitfalls are. The methods used are discussed under section 3.3.

The following sections elaborate on the five steps of method development as illustrated in Table 1.1, and important considerations applicable to this study.

1.4 SEPARATION GOALS

The first step of the method development strategy is to establish the separation goals for the method. The separation goals define the expectations and regulatory requirements for the final method. As soon as the method has reached these goals the development phase is complete and validation can commence. Aspects to consider when setting the separation goals include:

- The purpose of the method (quantitative, qualitative or preparative).
- The type of sample matrix, and if the method will be used for more than one matrix.
- Acceptance criteria and requirements for sensitivity, resolution, retention, repeatability, accuracy, linearity, efficiency and peak symmetry.
- The number of samples to be analysed at the same time.
- The cost and frequency of analyses.
- Experience and available equipment (Snyder et al., 1997:5; Dong, 2006:196; Hong & Shah, 2007:334).

The methods used to determine the API content in Pheroid™ formulations are quantitative in nature. For quantitative methods the following characteristics are recommended:

- a capacity factor ($k'$) > 2,
- resolution ($R_s$) ≥ 1.5 – 2,
• repeatability (%RSD) ≤ 1 % for five or more replicates,
• tailing factor (T) ≤ 2,
• efficiency (N) > 2000 plates; and
• a separation time of preferably 5-30 min. (< 60 min. for complex samples like Pheroid™ formulations) (Dong, 2006:196; Hong & Shah, 2007:370).

The above recommended characteristics are the main focus point of method optimisation (sec. 1.12) and are also evaluated during method validation (sec. 1.13).

Since interference of the Pheroid™ delivery system with the API during HPLC analysis were suspected by Cassim (2007:159) and Kühn (2008:70), this study’s main concern was to improve the resolution between peaks as well as the repeatability of peak area values.

1.5 BACKGROUND INFORMATION NEEDED

As demonstrated in Table 1.2, known sample and analyte information can be applied to determine the appropriate sample preparation and handling procedures, as well as the initial chromatographic conditions. The time spent to develop a new method can be dramatically decreased when sufficient information about the analyte and sample is available (Hong & Shah, 2007:334).

Sufficient background information was available for the API’s previously formulated in the Pheroid™ delivery system. Conversely what is known about the matrix, namely the Pheroid™ delivery system, is limited.
Table 1.2: Useful information concerning the analyte and sample [table adjusted from Dong (2006:197)].

<table>
<thead>
<tr>
<th>Sample</th>
<th>Information</th>
<th>Application of information</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complexity of sample – number of components</td>
<td>Deciding between gradient and isocratic elution (sec. 1.9.2)</td>
</tr>
<tr>
<td></td>
<td>Concentration range of analytes</td>
<td>Selection of the detector and detector settings (sec. 1.7)</td>
</tr>
<tr>
<td></td>
<td>Nature of sample matrix: solvent, fillers, etc.</td>
<td>Sample preparation (sec. 1.6)</td>
</tr>
<tr>
<td></td>
<td>Chemical structure and molecular weight</td>
<td>Selection of the detector (sec. 1.7), chromatographic mode (sec. 1.8), and mobile phase composition (sec. 1.9.3 – 1.9.5)</td>
</tr>
<tr>
<td></td>
<td>pKₐ</td>
<td>Determination of mobile phase pH and possible ion-pairing (sec. 1.9.5)</td>
</tr>
<tr>
<td>Analyte(s)</td>
<td>Solubility in solvents: (Hong &amp; Shah, 2007:334)</td>
<td>Sample preparation (sec. 1.6) and selection of the organic solvent (sec. 1.9.4)</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
<td>Organic</td>
</tr>
<tr>
<td></td>
<td>Water Buffers</td>
<td>Ethanol/methanol</td>
</tr>
<tr>
<td></td>
<td>0.1N HCl</td>
<td>Chloroform</td>
</tr>
<tr>
<td></td>
<td>0.1N NaOH</td>
<td>Cyclohexane</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acetonitrile</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td></td>
<td>Chromophore, maximum absorbance wavelength (λₘₐₓ)</td>
<td>Type of detector and detector settings (sec. 1.7)</td>
</tr>
<tr>
<td></td>
<td>Chiral centres, isomers</td>
<td>A specific mode of chromatography will be needed (sec. 1.8)</td>
</tr>
<tr>
<td></td>
<td>Stability and toxicity</td>
<td>Indicates whether special treatment and handling procedures will be necessary. Will aid sample preparation (sec. 1.6)</td>
</tr>
<tr>
<td>Others</td>
<td>Availability and purity of reference standards</td>
<td></td>
</tr>
</tbody>
</table>

1.6 SAMPLE PREPARATION

Since not all samples are suitable for direct injection, but needs to be dissolved, extracted, converted, diluted, buffered, or contains interferences; sample preparation usually precedes HPLC analysis (Snyder et al., 1997:6). The purpose of sample preparation is to produce a sample free of possible interferences ensuring accurate quantification of the API, that will not damage the HPLC equipment and is compatible with the HPLC method, and to enhance detection (Snyder et al., 1997:101; Hong & Shah, 2007:340). In addition to this, Smith (2003:5) explains that sample preparation
aims to make the analytical method more robust, reproducible and independent of the sample matrix.

The product of sample preparation, one suitable for injection into the HPLC system, should be a clear solution free of any insoluble particles. Depending on the sample type different sample treatments (e.g. dilution, sonication, shaking, filtration, liquid or solid phase extraction, evaporation, reconstitution, heating or cooling, and derivatisation) can be applied to achieve this (Hong & Shah, 2007:342). More than one treatment may be needed before the sample is ready for injection. It is ideal to perform a minimum of treatments as each step introduces possible errors that may influence precision (Snyder et al., 1997:102).

The sample solvent plays a significant role in the sample preparation step. It should be compatible with the HPLC system and appropriate in terms of the solubility and stability of the analyte, as well as other compounds present in the sample. Hong and Shah (2007:342) recommend that the final dilution should be done with the mobile phase before injection to prevent any peak distortions.

For Pheroid™- based drug products sample preparation mainly included dilution with 100% methanol followed by sonication or shaking and then filtration (Cassim, 2007:56; Kühn, 2008:48; Pretorius, 2008:45). This also served as the starting point, in terms of sample preparation, for this study.

1.7 CHOOSING A DETECTOR AND DETECTOR SETTINGS

The separation of an analyte from impurities and other compounds present in a given sample would be fruitless and in vain if no procedure was in place to establish whether separation has indeed occurred, and to provide information concerning the analyte’s concentration. By tracking an intrinsic property of the analyte, the HPLC detector responds to the analyte as it elutes from the column, fulfilling this role (Dong, 2006:87).

Thompson and LoBrutto (2007:654) define the ideal HPLC detector as:

- **Highly universal**

HPLC detectors can be distinguished as either universal (responding to all analytes) or selective (responding to analytes that possess a specific physico-chemical property) (Thompson & LoBrutto, 2007:654).
• **Highly sensitive**

Sensitivity differs from detector to detector. It is expressed as a ratio of the detector's response to the analyte's concentration (LabHouse, 1999:42). Factors like the flow cell's dimensions and the energy source employed also play a role in the detector's sensitivity. The baseline noise is used as specification of sensitivity (Dong, 2006:88). It refers to the short-term baseline instabilities caused by stray light and electronic interference of the detector (Snyder et al., 1997:71; LabHouse, 1999:41). A reduction in the effect of the baseline noise, by increasing the analyte concentration (keeping in mind linearity issues), or implementing a wavelength (with UV detection) that will provide a maximum signal, will enhance sensitivity (Snyder et al., 1997:73).

• **Linear over a broad concentration range**

Detector linearity is described by Beer’s Law (Dong, 2006:87):

\[
\text{Absorbance (A)} = \text{molar absorptivity (}\varepsilon\text{)} \times \text{pathlength (b)} \times \text{concentration (c)}
\]

It states that the concentration of the analyte in the flow cell is directly proportional to the absorbance. At very high concentrations this linear relationship may however deteriorate (LabHouse, 1999:29). The linear working range is dependent upon the detector as well as the analyte, and should be determined for a specific analyte to ensure the acquisition of accurate results (FDA, 1994:11).

• **Unaffected by possible changes in temperature and mobile phase composition**

Some detectors require that the mobile phase needs to be constant in composition and are therefore not compatible with gradient elution. Variations in temperature influence the refractive index of the mobile phase. The pH and polarity of the mobile phase may compromise the fluorescence of compounds (Thompson & LoBrutto, 2007:654).

Based on the analyte's physico-chemical properties a suitable HPLC detector can be chosen (LoBrutto, 2007:367). Table 1.3 contains some of the most commonly used detectors with HPLC and gives an indication of how the respective detectors can be applied.
Table 1.3: Common HPLC detectors and their attributes (Dong, 2006:88).

<table>
<thead>
<tr>
<th>Detector</th>
<th>Analytes/attributes</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV/Vis absorbance (UV/Vis)</td>
<td>Selective: compounds with UV chromophores</td>
<td>ng – pg</td>
</tr>
<tr>
<td>Photo Diode Array (PDA)</td>
<td>Selective: same as UV/Vis detectors, also provides UV spectra</td>
<td>ng – pg</td>
</tr>
<tr>
<td>Fluorescence (FL)</td>
<td>Very selective: compounds with native fluorescence or fluorescent tags</td>
<td>fg – pg</td>
</tr>
<tr>
<td>Refractive Index (RI)</td>
<td>Universal: polymers, sugars, triglycerides, organic acids, excipients; not compatible with gradient analysis</td>
<td>0.1 – 10 µg</td>
</tr>
<tr>
<td>Mass Spectrometry (MS)</td>
<td>Both universal and selective, structural identification; very sensitive and specific</td>
<td>ng – pg, pg – fg</td>
</tr>
</tbody>
</table>

The PDA detector is a popular detector in the pharmaceutical industry and recommended for HPLC method development. It functions as a UV/Vis detector, but is able to provide UV absorbance spectra that are helpful in identifying and tracking peaks (Dong, 2006:91). Although this type of detector is not considered universal, it is satisfactorily sensitive, linear over a broad range, and rugged towards temperature and mobile phase changes (Thompson & LoBrutto, 2007:654). The PDA detector was also indicated by the methods used in this study.

The following UV detector settings should be considered:

- **The detection wavelength**
  
The appropriate wavelength is one that provides more or less similar UV absorbance for the different compounds of interest in the sample, maximum detection sensitivity and sufficient transparency for the mobile phase (Snyder et al., 1997:63). This can be determined by overlaying the UV spectra of the different compounds and considering the UV cut-off values for the organic solvent and mobile phase additives (Snyder et al., 1997:66).

- **Spectral bandwidth**
  
The spectral bandwidth is linked to both the detector’s sensitivity and linearity. Increasing the bandwidth will improve sensitivity, but the linear range of the detector becomes smaller. Typical values ranges from 5-8 nm when one wavelength is selected (Dong, 2006:89).
1.8 SELECTION OF THE CHROMATOGRAPHIC MODE

Several theories exist as to how compounds are separated from each other through chromatography. Kazakevich (2007:39,40,54) discusses three models for analyte retention namely partitioning, adsorption and a combined model that includes both partitioning and adsorption.

With the partitioning model, the analyte is distributed in a mobile phase which is constantly moving over a stationary phase. The analyte partitions between the mobile and stationary phase due to its differing affinity for these phases and the presence of an instant dynamic equilibrium (Kazakevich, 2007:39).

The adsorption model considers the stationary phase to be an interface instead of a separate phase as with the partitioning-model, where adsorption of the analyte takes place due to surface forces (Kazakevich, 2007:40).

The partitioning-adsorption model suggests that the analyte’s retention depends on two processes. Firstly the organic solvent in the mobile phase adsorbs to the surface of the bonded phase forming a layer of a certain thickness (one monolayer for methanol and five for acetonitrile), into which the analyte partitions from the free flowing mobile phase. Secondly adsorption of the analyte onto the bonded phase takes place (Kazakevich, 2007:54).

With all of these models it is clear that some sort of interaction takes place on a molecular scale that plays a role in the separation process. HPLC can be divided into four main types based on the dominant molecular interactions that they implement (Kazakevich & LoBrutto, 2007a:10):

- **Normal phase chromatography (NPC): polar interactions**
  
  Analytes with differing polarity are separated on a polar stationary phase (silica/alumina), and non-polar solvents (hexane, heptane, etc.) adjusted with a polar organic solvent (methanol, ethanol or iso-propanol) as the mobile phase. The polar organic solvent also interacts with the stationary phase and thus competes with the analyte. Aqueous solvents cannot be used. Analyte retention increases with increasing polarity of the analyte. NPC is useful when the analyte is very hydrophobic and not suitable for reversed phase separation.
• **Reversed phase chromatography (RPC): dispersive interactions**

The silica stationary phase is chemically modified to be hydrophobic. Polar solvents (methanol, acetonitrile, THF) are implemented as mobile phase. An aqueous phase can be included which makes ion-suppression and ion-pairing possible. The more hydrophobic the analyte, the longer it is retained on the stationary phase. Due to the presence of an aqueous phase, RPC can be used for most analytes in the pharmaceutical industry.

• **Ion-exchange chromatography (IEC): ionic interactions**

Cationic ($\text{SO}_3^-$, $\text{CO}_2^-$) or anionic (quaternary or tertiary amines) exchange groups on a polymeric base act as the stationary phase. The mobile phase is free from organic solvents and consists out of buffer solutions. Ionic compounds interact with the ion-exchange groups and are eluted by varying the pH or salt concentration. IEC is used for the separation of ions, isomers and biomolecules.

• **Size-exclusion chromatography (SEC): no specific interactions between analyte and stationary phase**

A polystyrene resin with pores uniform in size, fulfill the role of stationary phase. The mobile phase (toluene, THF) functions only as a carrier, but does adsorb to the stationary phase in the event that it still has regions where molecular interactions can take place. Separation is based on the molecular size of the analyte only. The smaller the molecular size of the analyte, the more accessible the pores, the longer its retention in the stationary phase. SEC is applied for macromolecules, such as polymers.

Considering the above, the choice of HPLC mode is based upon the polarity and size of the analyte (Dong, 2006:199). The typical API is defined by Hong and Shah (2007:335) in terms of:

- size: smaller than 1000 daltons; and
- polarity: soluble in either water (ionic or non-ionic compounds) or an organic solvent (polar or non-polar compounds).

Compounds that fit the above description can be separated by reversed phase HPLC (RP-HPLC) alone or coupled with ion-pairing. RP-HPLC is the most popular and
versatile HPLC method, and used for the majority of HPLC analyses in the pharmaceutical industry (Hong & Shah, 2007:335). It is also the recommended starting point in the method development process (LabHouse, 1999:97).

The popularity of RP-HPLC can be attributed to the employment of dispersive forces. RP-HPLC has a high discriminating power due to the low background energy of the weak dispersive interactions. Small differences in the molecular interactions can thus be distinguished making it possible to separate closely related compounds (Kazakevich & LoBrutto, 2007a:12). In addition to this equilibration times are shorter which is ideal for method development and gradient elution. Columns also have longer lifetimes since they are more resistant toward irreversible bonding of components to the stationary phase (LabHouse, 1999:76).

The fact that an aqueous phase can be incorporated into the mobile phase, accounts for RP-HPLC's versatility. The retention of an analyte is mainly determined by its partitioning between the mobile and stationary phases. With RP-HPLC, however, the mobile phase is not just a carrier, but provides room for secondary equilibriums that can also influence the analyte's retention in the column (e.g. ionisation control, ion-pairing and solvation) (LoBrutto & Kazakevich, 2007:140). Therefore, as already mentioned, both water-based and organic samples can be analysed by means of RP-HPLC.

1.9 CHOOSING THE EXPERIMENTAL CONDITIONS FOR RP-HPLC

The determination of the experimental conditions can be either empirical or computerised as explained in section 1.3. What is known about the analyte and existing methods provides useful information to establish the initial chromatographic conditions.

Hong and Shah (2007:343) give recommendations for initial parameters based on the nature of the analyte (neutral, ionic-acidic, ionic-basic). The parameters for all three types of analyte seem to be generally the same, differing only in terms of the pH and the mobile phase modifier (see Table 1.4). Values of the chromatographic variables for this study, was dependent on the existing methods used, and can be found under section 3.3. These values agree more or less with the recommendations in Table 1.4.
Table 1.4: Recommended initial parameters for analyses (Hong & Shah, 2007:343).

<table>
<thead>
<tr>
<th>Chromatographic variables</th>
<th>Neutral, ionic-acidic, ionic-basic Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COLUMN</strong></td>
<td></td>
</tr>
<tr>
<td>Dimension</td>
<td>25 cm x 0.46 cm</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>C\textsubscript{18} or C\textsubscript{8}</td>
</tr>
<tr>
<td>Particle size</td>
<td>10 (\mu)m or 5 (\mu)m</td>
</tr>
<tr>
<td><strong>MOBILE PHASE</strong></td>
<td></td>
</tr>
<tr>
<td>Solvents A and B</td>
<td>Buffer-acetonitrile</td>
</tr>
<tr>
<td>% B (organic) isocratic</td>
<td>50%</td>
</tr>
<tr>
<td>% B (organic) gradient</td>
<td>20% - 80%</td>
</tr>
<tr>
<td>Buffer</td>
<td>Phosphate</td>
</tr>
<tr>
<td>Type</td>
<td>50 mM</td>
</tr>
<tr>
<td>Concentration</td>
<td></td>
</tr>
<tr>
<td>(pH)</td>
<td></td>
</tr>
<tr>
<td>Neutral</td>
<td>3.0</td>
</tr>
<tr>
<td>Ionic-acidic (gradient)</td>
<td>3.0 &amp; 7.5 (gradient)</td>
</tr>
<tr>
<td>Ionic-basic (gradient)</td>
<td>3.0 &amp; 7.5 (gradient)</td>
</tr>
<tr>
<td>Modifier</td>
<td>10 mM TEA, and 1% acetic acid if needed</td>
</tr>
<tr>
<td></td>
<td>1% acetic acid</td>
</tr>
<tr>
<td></td>
<td>25 mM TEA</td>
</tr>
<tr>
<td><strong>FLOW RATE</strong></td>
<td>1.5 – 2.0 mL/min</td>
</tr>
<tr>
<td><strong>TEMPERATURE</strong></td>
<td>Ambient to 35°C</td>
</tr>
<tr>
<td><strong>SAMPLE SIZE</strong></td>
<td></td>
</tr>
<tr>
<td>Volume</td>
<td>10 (\mu)L – 25 (\mu)L</td>
</tr>
<tr>
<td>Mass</td>
<td>&lt;100 (\mu)g</td>
</tr>
</tbody>
</table>

The next few sections will elaborate more on these chromatographic variables.

1.9.1 The column

Consisting out of a stainless steel tube filled with the stationary phase, the column represents the part of the HPLC system where a sample mixture is separated into its respective compounds (Dong, 2006:48). It can be classified as a packed, monolithic or capillary column (Kazakevich & LoBrutto, 2007b:113).

This stationary phase contained in the column consists out of a base material (e.g. silica) that has been chemically modified by the addition of a bonded phase (e.g. C\textsubscript{18}). The types and characteristics of these two constituting parts are given in Table 1.5.

Apart from the stationary phase's integral role in the column's efficiency, as indicated by Table 1.5, the column's dimensions also carry some weight. Column efficiency, sensitivity, analysis time, loading capacity as well as operating parameters like the
maximum flow rate and resulting back pressure, are dependent on the column length and the internal diameter (Dong, 2006:51).

**Table 1.5: A summary of the two constituents of the reversed phase stationary phase and their characteristics [compiled from Kazakevich and LoBrutto (2007b:75-115)].**

<table>
<thead>
<tr>
<th>Types</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silica, hybrid silica, high-purity silica (Dong, 2006:58)</td>
<td><strong>Support Base material</strong></td>
</tr>
<tr>
<td>Types</td>
<td></td>
</tr>
<tr>
<td>Zirconia</td>
<td></td>
</tr>
<tr>
<td>Alumina</td>
<td></td>
</tr>
<tr>
<td>Polymers</td>
<td></td>
</tr>
<tr>
<td>Types</td>
<td></td>
</tr>
<tr>
<td><strong>Type: porous, non-porous, monolithic</strong></td>
<td>Porous support provides a larger surface area.</td>
</tr>
<tr>
<td><strong>Shape</strong></td>
<td>Spherical particles render higher column efficiency.</td>
</tr>
<tr>
<td><strong>Particle size and particle size distribution</strong></td>
<td>Smaller particle sizes (&lt; 3 µm) and particle size distributions provide better column efficiency, but may yield higher backpressures.</td>
</tr>
<tr>
<td><strong>Surface area</strong></td>
<td>A higher surface area increases the density of the bonded phase, increasing analyte retention, and ultimately the column's efficiency. Uniformity of the surface is also important.</td>
</tr>
<tr>
<td><strong>Pore size</strong></td>
<td>An increase in pore size will increase the surface area.</td>
</tr>
<tr>
<td><strong>Surface chemistry</strong></td>
<td>Stability towards mechanical stress as well as elevated temperature and pH, depends on the surface chemistry. This can be altered by chemical modification. For silica base materials the presence of residual silanol groups and metal ions determines the surface chemistry. High pH mobile phases activate silanol groups and metal ions increase their activity, reducing silica's hydrolytic stability. The separation of basic analytes is mostly affected by the activity of silanol groups. End-capping is performed to reduce the effects of residual silanols. High-purity silica aims to reduce the presence of metal ions (Dong, 2006:58).</td>
</tr>
</tbody>
</table>

**Bonded phase**

<table>
<thead>
<tr>
<th>Types</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkyl (C1-C18, C18)</td>
<td></td>
</tr>
<tr>
<td>Phenyl</td>
<td></td>
</tr>
<tr>
<td>Amino</td>
<td></td>
</tr>
<tr>
<td>Cyano</td>
<td></td>
</tr>
<tr>
<td>Polar-embedded</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.5 (cont.): A summary of the two constituents of the reversed phase stationary phase and their characteristics [compiled from Kazakevich and LoBrutto (2007b:75-115)].

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bonding density</td>
<td>It determines the hydrophobicity of the column, to what extent the residual silanols are shielded and the stability of the stationary phase towards hydrolysis. The bonding density is usually indicated by carbon content on the column's Certificate Of Analysis (COA).</td>
</tr>
<tr>
<td>Surface chemistry</td>
<td>The bonded phases differ in terms of interactions with the analyte, with resultant differences in selectivity between columns for some analytes. Hydrophobicity of the bonded phase and thus analyte retention, increases with increasing chain length for the alkyl groups (Snyder et al., 1997:193).</td>
</tr>
<tr>
<td>Surface coverage</td>
<td>The size of the bonded phase ligand and thus steric hindrance, dictates the surface coverage (Snyder et al., 1997:213).</td>
</tr>
<tr>
<td>Methylene selectivity</td>
<td>Refers to the ability of a bonded phase to separate closely related compounds. It can be used to compare different columns.</td>
</tr>
</tbody>
</table>

Snyder et al. (1997:205) have listed requirements that should be investigated when determining the applicability of a specific column:

- Plate number (N) – a measure of column efficiency which is a property of the column.
- Peak asymmetry – this is especially important in the case of basic analytes.
- Selectivity ($\alpha$).
- Back pressure – the pressure drop depends on the column length, mobile phase viscosity, the column dead time and particle diameter.
- Retention ($k$) reproducibility.
- Surface coverage of the bonded phase.
- Stability towards elevated temperatures and pH.

Due to their porosity, rigidity and the reproducibility of synthesis, the most commonly used columns are silica-based. The bonded phases that are most widely applied are of the alkyl-type (Kazakevich & LoBrutto, 2007b:86,101). For the development of assay methods the recommended starting point is C18 or C8 high-purity silica-based columns, with 3 or 5 μm sized particles, an internal diameter of 3 – 4.6 mm and length of 15 – 25 cm (Dong, 2005:199).
1.9.2 Isocratic versus Gradient elution

Isocratic and gradient elution differ from each other in terms of the percentage organic solvent that moves through the column at any given time. The percentage organic solvent in the mobile phase increases during a gradient run, while it remains constant with isocratic analysis (Snyder et al., 1997:365).

The choice between gradient and isocratic separation is dependent on the complexity of the sample, the availability of the appropriate equipment and the detector that will be used. Both elution methods have their advantages which are evident from the following comparison between isocratic and gradient separations:

- Column equilibration and interactions between analyte, mobile and stationary phase remain constant under isocratic conditions, but vary with gradient elution. This makes isocratic separations more reproducible.

- Gradient elution can separate complex samples more effectively than isocratic separations.

- Late eluting peaks tend to broaden with isocratic separation, while gradient elution can produce narrower peaks throughout the whole run.

- Varying the percentage organic solvent may damage the column if it has not been manufactured to withstand such stress (LoBrutto, 2007:381).

It is recommended that if possible, gradient elution should be used for initial exploratory runs, especially if the sample is unknown. This will provide insight concerning the nature of the compounds present in the sample, and assist in choosing the appropriate mobile phase conditions for isocratic separation (Snyder et al., 1997:359).

1.9.3 Mobile phase

The mobile phase used for RP-HPLC separations usually consists of an organic solvent and an aqueous phase. It is a very powerful tool which controls the retention of the analyte as well as the selectivity of the separation, and can be readily adjusted during method development and optimisation (Dong, 2006:205).
1.9.4 The organic solvent

Acetonitrile and methanol are the polar organic solvents mostly applied in RP-HPLC. Other solvents include tetrahydrofuran (THF), iso-propanol, dimethyl sulfoxide (DMSO) and ethanol. They are only used in small amounts due to their UV absorbance, higher viscosity and health risks (LoBrutto & Kazakevich, 2007:145).

Methanol, acetonitrile and THF differ from each other in terms of their polarity, solvent strength and interactions with the stationary phase. They can be arranged in order of increasing solvent strength and decreasing polarity: methanol – acetonitrile – THF. The organic solvent type and concentration, collectively determine the solvent strength of the mobile phase (Snyder et al., 1997:239).

Variation of the solvent type and its concentration affects the selectivity and retention of compounds in a sample mixture. It is an effective way of improving the resolution between critical pairs (Snyder et al., 1997:254).

The following should be considered when choosing the appropriate organic solvent (LoBrutto & Kazakevich, 2007:145):

- **Solubility of the analyte/sample**
  
  The analyte/sample must be soluble in the mobile phase to avoid damage to the column.

- **UV transparency of the organic solvent**
  
  A low UV cut-off is desired to ensure that the solvent does not absorb at the UV detection wavelength of the analyte, causing interference. The UV cut-off value for acetonitrile and methanol is 190 nm and 205 nm, respectively.

- **The possible addition of buffers or ion-pairing agents**
  
  Methanol provides better solubility than acetonitrile for these components. The precipitation of salts like the phosphates, may damage the HPLC system.

- **Compatibility between solvents and compatibility with the HPLC system**
  
- **Viscosity of the organic solvent**
  
  Solvents with high viscosity levels will increase back pressure in the column and thus low flow rates will have to be used, consequently prolonging the run
time. Acetonitrile-water mixtures have a viscosity 2.5 times lower than methanol (LoBrutto, 2007:380).

Both acetonitrile (Cassim, 2007:55; Pretorius, 2008:38,44; Kühn, 2008:47,49,50) and methanol (Kühn, 2008:48) have been used as organic solvent in gradient and isocratic separations of Pheroid™- based samples. Acetonitrile has been implemented to a greater extent than methanol. Acetonitrile was also used in this study.

1.9.5 The aqueous phase

The aqueous phase acts as the weak solvent of the mobile phase in RP-HPLC, as mentioned in section 1.9.3. It provides room for further improvement of selectivity via secondary equilibriums. The main role players in these secondary equilibriums are the mobile phase pH, the buffer, ion-pairing agents and other mobile phase additives.

1.9.5.1 The mobile phase pH

Mobile phase pH should be determined and adjusted in the aqueous phase, before addition of the organic solvent (Snyder et al., 1997:296). The appropriate mobile phase pH is dependent upon the column packing material, buffer pK_a, the ionic nature of the analyte as well as the type of organic solvent to be added and its concentration.

Firstly, as mentioned in section 1.9.1, the silanol activity of the column's packing material increases at higher pH values and may lead to poor peak shapes and reproducibility especially for basic analytes (Snyder et al., 1997:311). This has restricted the use of alkaline mobile phases (pH > 6-8). The recent development of more stable packing materials has, however, extended the working pH range from 2-10 (Dong, 2006:33).

Secondly, to ensure optimum buffer capacity, and a method that is more robust towards small pH changes, the pH of the aqueous phase should be adjusted to be in close proximity of the buffer pK_a. The buffering range is usually within ± 1.5 pH units from the buffer pK_a and should preferably be controlled over a range of pK_a ±1 (Snyder et al., 1997:297).

The third factor refers to the ability of some analytes (weak acids and bases) to exist in more than one form depending on the pH:
The ionisation profile of a weak acid (blue curve) as illustrated by Figure 1.1, is as follow: 2 pH units above the pK\textsubscript{a}-value the acid occurs 99% in its ionized form [A\textsuperscript{-}], and will be mainly neutral [HA] at 2 pH units below the pK\textsubscript{a}-value (LoBrutto & Kazakevich, 2007:163).

For a weak base (red curve) the converse is true, being neutral [B] at a pH higher than the pK\textsubscript{a}, and ionized [BH\textsuperscript{+}] at a lower pH (LoBrutto & Kazakevich, 2007:162).

Since these two forms (neutral and ionised) differ in their retention on the RP-HPLC stationary phase, it is of great importance to perform analyses at a pH that will ensure the analyte is predominantly in one ionisation state. The pH is recommended to be at least 1.5 – 2.0 pH units from the analyte’s pK\textsubscript{a} (Hong & Shah, 2007:344; LoBrutto & Kazakevich, 2007:161).

Lastly, the addition of an organic solvent produces a shift in the pH, causing the mobile phase (hydro-organic mixture) pH to be different from the pH of the aqueous phase. It is also true for the analyte’s pK\textsubscript{a}. This change in the pH differs with the type of organic solvent (e.g. methanol versus acetonitrile) added, and its concentration. Possible shifts in the mobile phase pH should be kept in mind when developing a method where pH control is vital for successful separation and quantification (LoBrutto & Kazakevich, 2007:158,171).

Figure 1.1: The correlation between retention and pH for basic and acidic compounds (Adjusted from Sorbtech, 2009).
LoBrutto (2007:375) considers the alteration in mobile phase pH to be "one of the greatest tools" at the hands of the analyst. Snyder et al. (1997:407) suggests that the pH should only be adjusted after other parameters have been experimented with, since it may jeopardise method ruggedness. They also state that a pH of 2-3 will bridge problems related to silanol activity, and ensure that both acids and bases are in one ionisation state.

1.9.5.2 The buffer

A buffering system is added to the mobile phase for the purpose of ionisation control (Dong, 2006:31). This makes it possible to analyse not only neutral, but also ionic compounds like weak acids and bases with RP-HPLC. The buffer components also play a role in reducing the effect of residual silanol groups through interaction with them (Snyder et al., 1997:311).

When deciding upon a buffer the following should be considered (Hong & Shah, 2007:347):

- **Buffer capacity which depends on the pH, buffer pKₐ and the buffer concentration**

  The desired aqueous pH should be within ±1.0 pH-units of the pKₐ of the buffer as mentioned in section 1.9.5.1. Phosphate buffers have three pKₐ values (2.1, 7.2, and 12.3) and thus three different buffering ranges (<3.1, 6.2-8.2 and 11.3-13.3 respectively) (Snyder et al., 1997:299). Concentrations between 25 and 50 mM provide sufficient buffer capacity (Snyder et al., 1997:407). The use of buffer concentrations <10 mM and >100 mM, is not recommended (LoBrutto, 2007:379).

- **UV absorbance of the buffering system**

  Transparency is important to prevent interference with the analyte peak. A buffer with a UV cut-off below the working wavelength should be chosen. Phosphate buffers do not absorb UV radiation above 200 nm (Snyder et al., 1997:299).

- **Solubility, stability and possible interactions of the buffer components with the analyte**
Solubility in the presence of the organic solvent and other mobile phase additives should be established. Methanol generally provides better solubility for buffer components than acetonitrile. In addition to this potassium salts are more soluble than their sodium counterparts (Snyder et al., 1997:300,312).

1.9.5.3 **Ion-pairing agents and other mobile phase additives**

Ion-pair chromatography differs from RP-HPLC in terms of the addition of ion-pairing agents to the mobile phase. This mode is usually implemented when RP-HPLC is not able to yield sufficient separation for ionic samples. Analytes with pK_a values < 2 (acids) or >8 (bases), may need ion-pairing HPLC (Snyder et al., 1997:317).

As mentioned the mobile phase used during ion-pairing HPLC is very similar to that of RP-HPLC. The objective of pH control, however, is different from RP-HPLC. With ion-pairing HPLC the pH is controlled to ensure complete ionisation of the analyte. Ion-pairing agents consist out of one or more alkyl chains which have affinity for the hydrophobic stationary phase, and a negative (cationic) or positively (anionic) charged group that is able to interact with the ionized analyte. This collectively then increases the retention of the hydrophilic analyte, improving the selectivity of the separation. Anionic agents (e.g. hexane sulfonate) are implemented for basic analytes (cationic solutes), and cationic agents (e.g. tetrabutylammonium) for acidic analytes (anionic solutes) (Lab House, 1999:88).

The inclusion of an ion-pairing agent complicates the HPLC method by introducing additional parameters that needs to be controlled, and may compromise the robustness of the method. Solubility in mobile phases containing organic solvents other than methanol may be a problem, since methanol provides better solubility. Temperature also plays a more pronounced role in RP-HPLC coupled with ion-pairing than in RP-HPLC alone. In addition to this, ion-pairing agents prolong the column equilibration times. For these reasons their use should be restricted to cases when no other means will provide sufficient separation of ionic analytes (Snyder et al., 1997:407; LabHouse, 1999:90). It was also not implemented during this study.

Another mobile phase additive, namely triethylamine (TEA), functions as a competing base. It interacts with the residual silanol groups, reducing the peak tailing of basic analytes. As with the ion-pairing agents, its use should be a last resort (Snyder et al., 1997:407).
1.9.6 **Flow rate and Temperature**

When choosing the flow rate, the column dimensions should be considered to ensure the least amount of back pressure. The flow rate is seldom adjusted to improve resolution during isocratic separations, but plays a significant role in gradient elution (Dong, 2006:206).

Similar to the flow rate, temperature can also influence retention and selectivity. It is, however, more effective to vary the mobile phase composition in order to improve the selectivity and retention of analytes (Snyder *et al.*, 1997:242). The column’s stability toward elevated temperatures, as mentioned in Table 1.5, should be taken into account when adjusting the temperature. It is also recommended that the temperature is controlled for separations especially during method development and validation (Hong & Shah, 2007:350).

Neither the flow rate nor the temperature was varied for the isocratic methods used in this study.

1.10 **FORCED DEGRADATION OR STRESS TESTING**

The purpose of forced degradation/stress studies is to provide a means of establishing the selectivity of the analytical method, for the API in the presence of degradation products, impurities and excipients (FDA, 2000:11). It is required for registration purposes (FDA, 2000:11). Hong and Shah (2007:338) recommend that stress studies are performed early in the method development process. Stress testing is described by Dong (2006:204) as part of method optimisation.

According to the ICH (2003:2) forced degradation/stress testing should establish the effects of temperature and humidity, as well as the oxidation, photolysis and hydrolysis of the drug substance. For the drug product, tests in terms of photolysis are recommended (ICH, 2003:6).

Detailed guidelines and specific conditions for the execution of such studies are not given by the regulatory authorities. It is therefore left to the discretion of the analyst (Hong & Shah, 2007:338). A group of representatives, known as the Impurity Profiling Group (IPG), established that a universal set of conditions does not exist for stress testing (Klick *et al.*, 2005:56). A condition that produces relevant degradation for one API may not be suitable for another. Severe conditions may render irrelevant
products which can unnecessarily complicate method development. Klick et al. (2005:56) believes that using less severe testing conditions, at the cost of prolonging the testing period, will provide the best results. The IPG also found that formal accelerated stability studies are accurate in predicting the long-term storage conditions and impurity profiles. Comparing the results of stress testing with formal stability studies should thus be done (Klick et al., 2005:56,64).

Forced degradation studies were not performed per se, instead the samples were subjected to elevated temperatures and humidity as required for stability testing, to challenge the methods.

1.11 PEAK PURITY

A homogeneous peak is important to ensure accurate quantification of an analyte peak under all conditions. This can be determined through a variety of methods including ratiograms, spectral overlay when using a PDA detector and parallel detectors. It is recommended that the PDA detector should not be used on its own to demonstrate peak purity, but rather in conjunction with mass spectrometry (Snyder et al., 1997:78; LoBrutto, 2007:368).

1.12 METHOD OPTIMISATION

The method optimisation step is included in the development strategy to ensure that the method reaches the goals that were set in terms of resolution, precision and sensitivity, before proceeding with method validation (Dong, 2006:204).

As mentioned in section 1.8, RP-HPLC has opened the door for secondary equilibriums that can influence the retention of an analyte and consequently its resolution from other peaks.

The resolution equation \( R = 0.25 \times N^{0.5} \times (\alpha - 1) \times (k'/k'^{+}+1) \), shows that three main factors play a role in the resolution of adjacent peaks (Hong & Shah, 2007:344). These three factors are efficiency \((N)\), selectivity \((\alpha)\) and the capacity factor \((k')\):

- The capacity/retention factor \((k')\) provides information concerning the analyte's retention on the stationary phase relative to the time it resides in the mobile phase. This retention is dependent upon the stationary phase, the nature of the analyte and its interaction with the stationary phase, the mobile phase strength and the temperature (Dong, 2006:19).
Selectivity refers to the ability of a chromatographic method to separate different analytes from each other. Selectivity is calculated by the ratio of the capacity factors of two analytes. The factors that influence the analytes' retention in the column will thus most likely also affect the selectivity of the separation. The nature of the analyte and the stationary phase are however the primary determinants of selectivity. Mobile phase composition and temperature will affect selectivity if it changes the nature of the analyte (Kazakevich & LoBrutto, 2007a:18).

Efficiency provides a measure of the degree of band broadening, and is measured by the theoretical plate number (N). The efficiency of a separation is mostly dependent upon the column (particle size and uniformity of packing material) (Kazakevich & LoBrutto, 2007a:19). Narrow peaks and the separation of several compounds within a short period of time are important attributes of an efficient column (Dong, 2006:21). Choosing an efficient column should be done at the beginning of the developmental process.

Both selectivity and efficiency play an important role in providing sufficient resolution between peaks and symmetrical Gaussian peak shapes. Selectivity produces bigger changes in resolution when adjusted, compared to efficiency. Therefore choosing the appropriate stationary and mobile phase is of vital importance. It is more practical and efficient to first attempt to adjust the parameters that affect selectivity during method optimisation (Kazakevich & LoBrutto, 2007a:22).

Dong (2006:205) listed the parameters that can be adjusted to improve separations:

- Mobile phase parameters (e.g. percentage organic solvent, solvent type, buffer type and concentration, and the pH).
- Operating parameters (e.g. flow rate, temperature, gradient range and gradient time).
- Column (e.g. the bonded phase, length, column diameter and particle size).
- Detector setting and sample amount.

Although existing methods were implemented with optimisation in mind, the evaluation of these methods' performances formed the larger part of this study. This
study experimented with some of the mobile phase parameters as discussed in Chapter 4.

1.13 METHOD VALIDATION

Validation is the final step in the method development process. It provides a means of ensuring that the analytical method meets the specified requirements set for its intended purpose (ICH, 2005:1; Hong & Shah, 2007:354). Method development should thus be approached with validation requirements in mind.

Before the validation process can commence a protocol is required. Hong and Shah (2007:354) listed the following as components of such a protocol: detailed test procedure, experimental design, elements of validation, the acceptance criteria, reference to related methods and approval by management.

Regulatory authorities (e.g. ICH and FDA) as well as pharmacopoeia (e.g. USP) have provided guidelines against which analytical performance parameters should be tested to demonstrate the validity of a specific method (Hong & Shah, 2007:356).

The ICH (2005:1) distinguish between analytical procedures for identification, assay of impurities, limit tests for impurities and the assay of the API or any other component of interest.

The analytical performance parameters to be tested to validate an assay for a specific API are listed by the ICH (2005:3) as follows:

- **Accuracy**
  
  Accuracy gives an indication of how true the obtained value is. The closer the experimental value to the true/reference value, the more accurate the method. Recovery of a spiked placebo or comparison with a reference standard can be implemented to demonstrate the accuracy of a method (Dong, 2006:232).

- **Precision**
  
  The distribution of a series of test results around their average determines the precision of the method. It is expressed in terms of %RSD (Hong & Shah, 2007:361). Precision can be determined on different levels:
- **Repeatability** (Intra-assay precision)
  This is a measure of the ability of the method to yield reproducible results for analyses done in one laboratory by one analyst, on one set of instrumentation, within one day (Dong, 2006:234).

- **Intermediate Precision** (Ruggedness)
  This is a measure of the ability of the method to yield reproducible results for analyses done in the same laboratory by different analysts, on more than one set of instrumentation, executed on different days (Dong, 2006:234).

- **Specificity**
  This refers to the ability of the method to distinguish the analyte from any degradants, excipients and impurities that might be present in the sample. Degradation studies are usually performed to test method specificity. A placebo of the sample is also analysed to check for any possible interferences (Dong, 2006:230). If specificity of a method is poor it can be supplemented with one that is specific (Hong & Shah, 2007:363).

- **Linearity**
  The linearity of a method is based upon its ability to produce results that are proportional to the concentration of the analyte in the sample over a certain range. Beer's law plays an important role in the linear range of detection. The linear range is dependent on the compound analysed and the type of detector used (Hong & Shah, 2007:366).

- **Range**
  The range of a method provides upper and lower concentration limits within which the method will be precise, accurate and linear (Hong & Shah, 2007:267).

Aside from these parameters, system suitability specifications and tests are important to ensure that the HPLC system performs optimally, producing accurate and precise results (FDA, 1994:21). Other information that should also be included in the validation documentation is the stability of analytical sample preparations, the results
of stress studies, as well as the identification and characterization of impurities (FDA, 2000:10).

1.14 CONCLUSION

HPLC introduces a magnitude of possibilities in terms of parameters that can be adjusted to analyse almost any pharmaceutical sample. With the right adjustments, the successful use of this analytical procedure in the stability testing of Pharoid™-based drug products, would therefore be possible. In the chapters to follow this possibility will be discussed in greater detail.
2.1 INTRODUCTION

Patented as a novel delivery system able to improve the absorption and efficacy of several medicines, the Pheroid™ delivery system has captured the interest of researchers over the past few years (Grobler et al., 2008:284). The improvement of the efficacy and safety of drug substances used in the treatment of infective diseases like tuberculosis, HIV and AIDS, as well as malaria, were put under the magnifying glass. With in vitro, in vivo and transdermal efficacy studies yielding promising results, a lot of time and effort have been invested in the development of Pheroid™-based drug products.

With particle sizes usually ranging between 200 nm and 2 μm, Grobler et al. (2008:284) define this novel delivery system as being “a colloidal system containing unique stable lipid-based submicron and micron-sized structures, called Pheroids™, which are uniformly distributed in a continuous phase that may be adapted to the indication”. Pheroids™ differing in size, morphology and function can be formulated by changing the composition and varying the manufacturing parameters (Grobler et al., 2008:283). The continuous phase can also be adjusted to enhance drug solubility and stability (Grobler et al., 2004:12).

The Pheroid™ delivery system shares characteristics with other lipid-based delivery systems including liposomes, emulsions, micro-emulsions, polymeric and macromolecular microspheres (Grobler et al., 2008:287). It does, however, differ from the above mentioned delivery systems in some aspects which provide the Pheroid™ delivery system with important advantages.

2.2 PHEROID™ COMPONENTS AND ADVANTAGES

Pheroids™ are formed by a self-assembly process which resembles that of micro-emulsions, but without the usually necessary lyophilisation or hydration of lipids. These structures possess a lipid bilayer which consists mainly of ethylated and pegylated polyunsaturated fatty acids like omega-3 and -6, but excluding arachidonic
acid (Grobler et al., 2008:288). In addition to this a gas, namely nitrous oxide, is found in association with the fatty acids, and provides the colloid with another dimension which accounts for this delivery system’s unique characteristics.

Studies have shown that nitrous oxide and the fatty acid components are equally important for the stability and efficacy of Pheroid™ formulations (Grobler et al., 2008:290). Nitrous oxide contributes to the miscibility of fatty acids in the continuous phase and plays a very significant role in the self-assembly process of the Pheroids™. Nitrous oxide’s hydro and lipophilic nature, combined with the unsaturated fatty acids, gives this system its polyphilic characteristic which improves the absorption of drug molecules with differing solubility across cell membranes (Grobler et al., 2008:290). With the use of fatty acids in the absence of phospholipids and cholesterol, the movement of drug molecules out of the vascular system is enabled by the increased fluidity and elasticity of Pheroids™ compared to that of liposomes (Grobler et al., 2004:7).

Other advantages, key to the Pheroid™ delivery system, as summarised by Grobler et al. (2004:3) include:

- an increase in therapeutic efficacy and the delivery rate of actives;
- a reduction in the time to onset of action, cytotoxicity, the minimum effective concentration and drug resistance;
- the targeting of specific areas for treatment;
- lack of immunological responses;
- penetration of most barriers in the body and cells; and
- the ability to transfer genes to cell nuclei.

2.3 THE DESIGN AND DIFFERENT TYPES OF PHEROIDS™

The basic Pheroid™ is a vesicular structure with a diameter of 200 - 440 nm. Its size, morphology and function can be adjusted, making this delivery system highly versatile (Grobler et al., 2008:283). Sizes are determined mainly by the ratio, saturation and modification state of the fatty acids used. Other aspects which influence the functional and structural characteristics of the Pheroid™ include:
the addition of non-fatty acids (e.g. phospholipids and cholesterol), cryoprotectants or charge-inducing agents; and

changes in the hydration medium, preparation method or character and concentration of the active ingredients (Grobler et al., 2008:291).

According to size and structure, the following types of Pheroids™ have been described:

i. Lipid-bilayer vesicles with mean diameters in the nano range.

ii. Pheroid™ microsponges with sizes between 1.5 - 5 μm.

iii. Reservoirs and depots that can range in sizes from 5 - 100 μm (Grobler et al., 2008:286; Padayachee, 2008:10).

Apart from the manipulation of the Pheroid™ structures, the continuous phase also allows room for adjustment. Pheroids™ are usually formed upon the addition of gassed water to the oily base containing the fatty acids (Padayachee, 2008:11). Grobler et al. (2004:12) introduced the pro-Pheroid concept to improve the stability, solubility and bioavailability of certain drug substances. The pro-Pheroid is formed by the gassing of the oily phase, without the addition of an aqueous phase as is the case with Pheroid™ formation. The difference between the Pheroid™ and the pro-Pheroid is therefore the presence and absence of an aqueous phase, respectively. The pro-Pheroid acts as a precursor to the Pheroid™ since Pheroids™ are formed when the pro-Pheroid is introduced to the aqueous environment of the stomach (Padayachee, 2008:11).

2.4 **CONCLUSION**

Considering its polyphilic nature and consequent versatility, the Pheroid™ delivery system holds great potential to eradicate or improve formulation problems in the pharmaceutical industry. It will therefore be beneficial to continue developing this novel delivery system and the analytical methods used to establish the stability of Pheroid™-based drug products.
CHAPTER 3
EVALUATION OF THE HPLC ANALYSIS AND STABILITY OF
THE PHEROID™ DELIVERY SYSTEM

3.1 INTRODUCTION

Regulatory authorities require that the stability of a drug product must be established for registration purposes. For this reason Pheroid™-based drug products have not only been subjected to efficacy studies, but also to stability testing. Such stability studies were performed by Cassim (2007:126) and Kühn (2008:61) who formulated combinations of anti-retroviral drug substances into the Pheroid™ and the pro-Pheroid. Pretorius (2008:50) also focused on anti-malarial Pheroid™-based drug products.

The HPLC analysis of most of these formulations yielded inconclusive results with only a few being successful. Previous stability studies concluded that the stability of the delivery system itself and compatibility of the API’s with the delivery system may be in question, or that the HPLC method used was not suitable for the analysis of Pheroid™-based drug products (Cassim, 2007:156; Kühn, 2008:74; Pretorius, 2008:61). The problem is therefore multi-faceted.

In an attempt to determine what the contributing factor for these poor results were, HPLC method performance and product stability were evaluated. The conditions, under which previous HPLC analyses were performed, were recreated by subjecting the Pheroid™ delivery system to the same storage conditions used for previous stability studies. Two existing HPLC methods were implemented and evaluated in terms of their performance.

In addition to HPLC analysis, particle size analysis and Confocal Laser Scanning Microscopy (CLSM) were also performed to support the HPLC results and provide information on the overall stability of the Pheroid™ delivery system.

The aim for this study can be summarised as follows:

1. To determine if the Pheroid™ delivery system changes significantly over time at accelerated storage conditions, and how these changes influence the HPLC analysis.
2. To determine the effect of the anti-oxidant tert-butylhydroquinone (TBHQ) on the stability and HPLC analysis of the Pheroid™ delivery system.

3.2 PHEROID™ FORMULATIONS

Four Pheroid™ microsponges formulations were prepared within the formulation facility of the Pharmaceutics department of the North-West University, Potchefstroom. As illustrated in Table 3.1, two variables were implemented:

1. The addition of TBHQ.
2. The method of analysis.

TBHQ was included in two of the four batches. The other specific ingredients used during formulation are kept confidential under agreement. A previous study recommended the use of TBHQ for possible improvement of the stability of Pheroid™ formulations (Cassim, 2007:156). Künn (2008:68) performed stability studies on abacavir entrapped in the pro-Pheroid containing TBHQ. The results Künn (2008:68) obtained for abacavir in that formulation were highly variable and therefore THBQ's influence on the stability of the formulation could not be concluded.

Our initial goal was to formulate both of the anti-malarial drugs, mefloquine and artemesunate, in the Pheroid™ delivery system to create a combination therapy drug product. Since it was not clear what the role of the matrix was in the variable results obtained in previous studies, no active pharmaceutical ingredients (API’s) were included in the batches listed in Table 3.1. The HPLC analyses were performed on these Pheroid™ formulations using existing methods for mefloquine and artemesunate to identify possible chromatographic interferences caused by the matrix.

Table 3.1: Details of batches Pheroid™ microsponges prepared.

<table>
<thead>
<tr>
<th>BATCH NUMBER</th>
<th>TBHQ</th>
<th>METHOD OF ANALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>S08016 (1)</td>
<td>Yes</td>
<td>Mefloquine – Method 1</td>
</tr>
<tr>
<td>S08017 (2)</td>
<td>No</td>
<td>Mefloquine – Method 1</td>
</tr>
<tr>
<td>S08018 (3)</td>
<td>Yes</td>
<td>Artesunate – Method 2</td>
</tr>
<tr>
<td>S08019 (4)</td>
<td>No</td>
<td>Artesunate – Method 2</td>
</tr>
</tbody>
</table>
3.2.1 Storage conditions

Four containers were kept at each of the storage conditions stated in the ICH guidelines (2003:8) for stability testing:

- 5°C – [C1]
- 25°C + 60% RH – [C2]
- 30°C + 65% RH – [C3]
- 40°C + 75% RH – [C4]

Tests were performed after 15, 30, 60 and 90 days of storage.

3.3 HPLC ANALYSIS

3.3.1 Origin of the methods

Mefloquine – Method 1:

This method originated from the method developed by Bergqvist et al. (1991:170) for the purpose of separating mefloquine from other antimalarials in plasma. Pretorius (2008:44) optimised it for the quantitation of mefloquine in Pheroid™-based samples. The method provided by Pretorius (2008:44) was used. However, in an effort to keep the method as simple as possible, we reverted back to RP-HPLC without ion-pairing. Thus, no hexane sulphonic acid (HSA) was used during the HPLC analyses.

Artesunate – Method 2:

The method as described in the International Pharmacopoeia, was used (Ph.Int., 2008).

3.3.2 Apparatus

Shimadzu Prominence (Shimadzu, Japan) series HPLC equipped with:

- A quaternary pump – LC-20AD.
- An auto sampler – SIL-20AC.
- A diode array detector – SPD-M20A.
- Data acquisition and analysis software – Shimadzu LC solution.
3.3.3 Chromatographic conditions

Table 3.2: The initial chromatographic conditions for analyses.

<table>
<thead>
<tr>
<th>Column</th>
<th>Neoflaxine - Method 1</th>
<th>Artesunate - Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Luna C18 (2) 150 x 4.6 mm, 5 µm [Phenomenex]</td>
<td>Luna C18 (2) 150 x 4.6 mm, 5 µm [Phenomenex]</td>
</tr>
<tr>
<td>Mobile phase</td>
<td>0.1 M potassium dihydrogen phosphate (KH$_2$PO$_4$) buffer and acetonitrile mixed in the ratio 52:48. The buffer is prepared by dissolving 13.61 g KH$_2$PO$_4$ in 1000 ml Milli Q water. pH is adjusted with 1 M NaOH and concentrated orthophosphoric acid to 3.5 ± 0.05.</td>
<td>0.01 M potassium dihydrogen phosphate (KH$_2$PO$_4$) buffer and acetonitrile mixed in the ratio 50:50. The buffer is prepared by dissolving 1.36 g KH$_2$PO$_4$ in 1000 ml Milli Q water. pH is adjusted with 1 M NaOH and concentrated orthophosphoric acid to 3.0 ± 0.05.</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min.</td>
<td>0.6 ml/min.</td>
</tr>
<tr>
<td>Temperature</td>
<td>Not controlled</td>
<td>Controlled at 30°C</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>Detection</td>
<td>UV detection at 229 nm</td>
<td>UV detection at 216 nm</td>
</tr>
<tr>
<td>Stop time</td>
<td>20 min.</td>
<td>20 min.</td>
</tr>
</tbody>
</table>

Due to changes in the chromatography, it became necessary to increase the runtime during the study.

3.3.4 Sample preparation

Methanol was used as sample solvent in previous studies as mentioned in section 1.6. It was pre-determined that Pheroid™ microsponges produce a clear solution in methanol (HPLC gradient grade) when diluted in the ratio 1:20. Samples were prepared in triplicate for each storage condition [C1, C2, C3, C4] and filtered through a membrane filter (Millipore, 33 mm Millex®-HV syringe filter unit, pore size of 0.45 µm) before injection onto the HPLC system.

3.3.5 Results and discussion

3.3.5.1 Evaluation of chromatography

Some of the detected peaks are relatively large. At first glance the chromatogram thus appears to be acceptable as seen in Figure 3.1. All of the peaks seem fully resolved and with a baseline that is quite stable. The results were still variable even though the errors that occurred were corrected (see section 3.3.5.2). Figure 3.2 indicates peaks that have very small areas, but are not classified as baseline noise.
and could possibly influence peak purity and quantitation. The chromatograms were therefore magnified concentrating on the baseline of the first 20 min.

Figure 3.1: Full scale chromatogram of batch 1 [C1] at 15 days.

Figure 3.2: Magnified version of the chromatogram in Figure 3.1.

Aside from the peak formed by TBHQ, the peak areas of three other peaks related to the Pheroid™ delivery system (matrix peaks), were also quantified with every batch analysis.

Table 3.3: Quantified peaks and their retention times for both methods.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Retention time – Method 1</th>
<th>Retention time – Method 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBHQ</td>
<td>≈ 4.1 min.</td>
<td>≈ 6.2 min.</td>
</tr>
<tr>
<td>1 (matrix peak)</td>
<td>≈ 3.0 min.</td>
<td>≈ 4.7 min.</td>
</tr>
<tr>
<td>2 (matrix peak)</td>
<td>≈ 5.3 min.</td>
<td>≈ 8.1 min.</td>
</tr>
<tr>
<td>3 (matrix peak)</td>
<td>≈ 10.8 min.</td>
<td>≈ 16.4 min.</td>
</tr>
</tbody>
</table>
To ensure that the same peaks were quantified with each analysis, the peaks were identified not only on the basis of their approximate retention times, but also according to their UV absorbance spectra as determined with the PDA detector (see Figure 3.3 – 3.6).

![UV absorbance spectrum of peak 1.](image1)

**Figure 3.3:** UV absorbance spectrum of peak 1.

![UV absorbance spectrum of peak 2.](image2)

**Figure 3.4:** The UV absorbance spectrum of peak 2.

In an attempt to determine whether the appropriate wavelength can improve the quantitation of the peaks of interest, two additional wavelengths (255 and 291 nm) were evaluated. The wavelength at 255 nm, represents the UV absorbance maxima of peaks 1 and 2 (Figure 3.3 & 3.4), and 291 nm that of peak 3 and THBQ (Figure 3.5 & 3.6).
Figure 3.5: The UV absorbance spectrum of peak 3.

Figure 3.6: The UV absorbance spectrum of TBHQ.

Observations were made in accordance with the original methods and thus stated in terms of 216 nm (batch 1 & 2) and 229 nm (batch 3 & 4). It will be indicated when reference is made to the two additional wavelengths (225 & 291 nm).

3.3.5.2 Median peak area and repeatability values for peaks 1, 2, 3 and TBHQ

- A batch analysis consisted out of 6 runs in total (duplicate runs of three samples). Highly variable results within batch analyses made it difficult to eliminate values as outliers. It was only determined close to the end of the study that this variation may have been due to sample preparation and autosampling errors. It was decided to rather use the median instead of the mean of each data set as the representative value.
Samples stored for 90 days yielded acceptable repeatability. Even though instrumentation errors were no longer a problem, this adequate repeatability was unexpected since the Pheroid™'s solubility in methanol decreased (section 3.4.2) and more peaks were detected which could cause possible interference. Since the initial analysis of batches 1 and 2 were faulty, were considered the chromatogram closest to the initial and served as reference for the analyses to follow. This was implemented for all four batches.

Table 3.4: The median peak areas of TBHQ for batches 1 and 3.

<table>
<thead>
<tr>
<th>BATCH</th>
<th>Storage condition</th>
<th>15 days (Area &amp; %RSD)</th>
<th>30 days (Area &amp; %RSD)</th>
<th>60 days (Area &amp; %RSD)</th>
<th>90 days (Area &amp; %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5°C</td>
<td>896605 (12.0)</td>
<td>972223 (9.6)</td>
<td>810617 (14.9)</td>
<td>929282 (17.1)</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>1104073 (4.7)</td>
<td>1068766 (6.6)</td>
<td>1139482 (5.1)</td>
<td>983327 (26.9)</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>947308 (27.2)</td>
<td>1070604 (15.7)</td>
<td>960381 (33.8)</td>
<td>1163314 (0.59)</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>1098484 (7.6)</td>
<td>1057172 (16.4)</td>
<td>759407 (10.1)</td>
<td>209519 (20.5)</td>
</tr>
<tr>
<td>2</td>
<td>5°C</td>
<td>1944770 (3.4)</td>
<td>1874174 (11.7)</td>
<td>2270970 (9.9)</td>
<td>2256267 (2.9)</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>2253583 (4.1)</td>
<td>2179134 (6.9)</td>
<td>2324344 (1.5)</td>
<td>2316476 (3.4)</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>1887768 (28.8)</td>
<td>2094187 (9.9)</td>
<td>2129038 (3.5)</td>
<td>2144654 (4.9)</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>1787400 (32.7)</td>
<td>1673269 (13.2)</td>
<td>1773568 (3.6)</td>
<td>2209703 (1.73)</td>
</tr>
</tbody>
</table>

Table 3.5: The median peak areas for peak 1 of the Pheroid™ microsponges.

<table>
<thead>
<tr>
<th>BATCH</th>
<th>Storage condition</th>
<th>15 days (Area &amp; %RSD)</th>
<th>30 days (Area &amp; %RSD)</th>
<th>60 days (Area &amp; %RSD)</th>
<th>90 days (Area &amp; %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5°C</td>
<td>4108628 (12.2)</td>
<td>3669290 (49.7)</td>
<td>4262059 (14.6)</td>
<td>4326808 (1.81)</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>4966579 (4.6)</td>
<td>4786335 (7.1)</td>
<td>5853188 (5.5)</td>
<td>5776896 (25.7)</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>4143209 (27.7)</td>
<td>4850414 (15.7)</td>
<td>4774106 (35.0)</td>
<td>5853407 (1.11)</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>4791278 (7.8)</td>
<td>4932810 (16.6)</td>
<td>4933068 (12.4)</td>
<td>5805233 (2.9)</td>
</tr>
<tr>
<td>2</td>
<td>5°C</td>
<td>4823045 (41.0)</td>
<td>4969607 (5.8)</td>
<td>5416293 (12.8)</td>
<td>5937560 (2.6)</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>4118585 (27.9)</td>
<td>5112540 (35.2)</td>
<td>5469944 (4.4)</td>
<td>--- --- ---</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>5165352 (14.1)</td>
<td>4944601 (8.0)</td>
<td>5532468 (1.92)</td>
<td>--- --- ---</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>5185433 (12.1)</td>
<td>4694945 (7.9)</td>
<td>5360345 (8.6)</td>
<td>--- --- ---</td>
</tr>
<tr>
<td>3</td>
<td>5°C</td>
<td>26931836 (4.7)</td>
<td>26374705 (15.6)</td>
<td>29928192 (12.4)</td>
<td>29772404 (3.7)</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>28630193 (5.3)</td>
<td>28936868 (5.5)</td>
<td>30025310 (0.87)</td>
<td>28913004 (2.6)</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>24436556 (33.7)</td>
<td>26238137 (12.8)</td>
<td>29804826 (1.4)</td>
<td>29857071 (2.6)</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>20754698 (37.9)</td>
<td>25347879 (17.1)</td>
<td>30229701 (5.9)</td>
<td>29621158 (1.92)</td>
</tr>
<tr>
<td>4</td>
<td>5°C</td>
<td>2571088 (8.5)</td>
<td>25854827 (10.4)</td>
<td>--- --- ---</td>
<td>--- --- ---</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>27074812 (20.4)</td>
<td>26733530 (11.6)</td>
<td>--- --- ---</td>
<td>--- --- ---</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>13816477 (44.5)</td>
<td>24473725 (2.0)</td>
<td>--- --- ---</td>
<td>--- --- ---</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>26428234 (8.5)</td>
<td>24624761 (10.3)</td>
<td>--- --- ---</td>
<td>--- --- ---</td>
</tr>
</tbody>
</table>
Table 3.6: The median peak areas for peak 2 of the Pheroid™ microsponges.

<table>
<thead>
<tr>
<th>BATCH</th>
<th>Storage condition</th>
<th>15 days (Area &amp; %RSD)</th>
<th>30 days (Area &amp; %RSD)</th>
<th>60 days (Area &amp; %RSD)</th>
<th>90 days (Area &amp; %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5°C</td>
<td>165473 12.2</td>
<td>180225 9.9</td>
<td>193497 14.9</td>
<td>217839 24.6</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>190064 4.9</td>
<td>183939 6.6</td>
<td>226997 5.4</td>
<td>233889 26.6</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>157312 28.3</td>
<td>190327 16.0</td>
<td>186137 34.9</td>
<td>233229 1.36</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>183142 7.8</td>
<td>199995 17.1</td>
<td>187280 12.2</td>
<td>212186 4.2</td>
</tr>
<tr>
<td>2</td>
<td>5°C</td>
<td><strong>178189</strong> 41.8</td>
<td><strong>188378</strong> 5.7</td>
<td><strong>208557</strong> 12.8</td>
<td><strong>225210</strong> 2.6</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>157277 28.2</td>
<td>200885 35.6</td>
<td>214843 4.2</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>197339 14.0</td>
<td>195661 8.1</td>
<td>223894 2.2</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>203068 12.4</td>
<td>187168 7.8</td>
<td>229781 8.2</td>
<td>---</td>
</tr>
<tr>
<td>3</td>
<td>5°C</td>
<td><strong>885570</strong> 4.0</td>
<td><strong>860388</strong> 13.0</td>
<td><strong>91894</strong> 10.4</td>
<td><strong>985011</strong> 3.7</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>944478 4.8</td>
<td>958300 5.6</td>
<td><strong>1000059</strong> 0.87</td>
<td>972331 2.3</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>813140 30.8</td>
<td>870040 10.6</td>
<td>995064 1.17</td>
<td>996280 2.4</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>725024 35.2</td>
<td>842222 14.6</td>
<td>1023651 5.4</td>
<td><strong>1022534</strong> 1.84</td>
</tr>
<tr>
<td>4</td>
<td>5°C</td>
<td><strong>862099</strong> 7.0</td>
<td>954645 8.9</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>913101 16.6</td>
<td>903920 10.1</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>508180 40.6</td>
<td>946486 2.2</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>889899 7.9</td>
<td>954971 11.0</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 3.7: The median peak areas for peak 3 of the Pheroid™ microsponges.

<table>
<thead>
<tr>
<th>BATCH</th>
<th>Storage condition</th>
<th>15 days (Area &amp; %RSD)</th>
<th>30 days (Area &amp; %RSD)</th>
<th>60 days (Area &amp; %RSD)</th>
<th>90 days (Area &amp; %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5°C</td>
<td>268836 17.7</td>
<td>287354 10.1</td>
<td>301766 15.5</td>
<td>339585 1.93</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>283906 4.7</td>
<td>272771 6.6</td>
<td>329710 5.4</td>
<td>322654 26.2</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>260964 28.7</td>
<td>277859 16.2</td>
<td>273067 35.1</td>
<td>332459 1.02</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>273868 7.8</td>
<td>290903 17.2</td>
<td>298035 13.2</td>
<td>357546 0.76</td>
</tr>
<tr>
<td>3</td>
<td>5°C</td>
<td><strong>507763</strong> 3.8</td>
<td>482073 13.1</td>
<td>566992 10.4</td>
<td>557609 3.5</td>
</tr>
<tr>
<td></td>
<td>25°C + 60% RH</td>
<td>539017 4.7</td>
<td>576720 15.6</td>
<td><strong>569602</strong> 1.1</td>
<td>548293 2.9</td>
</tr>
<tr>
<td></td>
<td>30°C + 65% RH</td>
<td>457063 30.7</td>
<td>485167 10.7</td>
<td><strong>566858</strong> 1.07</td>
<td>561435 2.2</td>
</tr>
<tr>
<td></td>
<td>40°C + 75% RH</td>
<td>417829 35.5</td>
<td>474733 14.4</td>
<td>583378 5.5</td>
<td><strong>572452</strong> 1.50</td>
</tr>
</tbody>
</table>

Our investigation focused on the storage condition that yielded the most changes, namely C4. As mentioned earlier, the chromatogram of C1 at 15 days for each batch served as the reference for that specific batch (Figure 3.7 – 3.10).
With TBHQ (Figure 3.7 and 3.8):

- The number of detectable peaks for batch 1 increased with time, while batch 3 did not show as much change. For batch 1 at 90 days the greatest part of the baseline presented with peaks as shown in Figure 3.7B. If an API was present it would have been subjected to the influences of interfering peaks.

- Only peak 3 seemed to be resolved from the other peaks. Peak 1, 2 and TBHQ proved not to be entirely pure. This became more apparent over time (Figure 3.7A and B), and also in some cases at other wavelengths (Figure 3.7C and D), where the co-eluting peak is presented as a shoulder.

Peak 2 have a higher UV absorbance at 216 and 229 nm compared to 291 nm. At 291 nm the co-eluting peak could be observed more clearly (Figure 3.7D).

- The peak areas for all four peaks were larger with the separations done by Method 2 (batch 3) compared to that obtained with Method 1 (batch 1). For peak 1 and 2 this difference in peak area may be a result of the significant differences in UV absorbance at 216 and 229 nm (Figure 3.3 and 3.4). The UV absorbance spectra of peak 3 and TBHQ (Figure 3.5 and 3.6) do not show such significant difference in absorbance between 216 and 229 nm, but the peak areas obtained suggest that these two compounds do have a slightly higher UV absorbance at 216 nm.

Figure 3.7: Representative chromatograms for batch 1.
Figure 3.7 (cont.): Representative chromatograms for batch 1.
Figure 3.8: Representative chromatograms for batch 3.

Without TBHQ (Figure 3.9 and 3.10):

- The formation of new peaks for batch 2 and 4 was not so distinct when compared to the formation of peaks in batch 1 and 3. The peaks also increased in size with time, but were still very small.

- As with the observation made for batch 1 and 3, there is a difference between the number of detectable peaks and the peak areas of the four peaks of interest obtained with Method 1 (batch 2) compared to that of Method 2 (batch 4). This may indicate that the HPLC method played a role.

- Only peaks 1 and 2 could be quantified. There was a peak with the same retention time as peak 3 (≈ 16.8 min for Method 1; ≈ 16.4 min for Method 2), but it had a very small peak area and a UV spectrum that did not correspond to that of peak 3 quantified in batch 1 and 3. It was therefore not considered to be the same peak and was not quantified.
• Neither peak 1 nor 2 was pure. A small peak also eluted where TBHQ would have eluted. This may in part explain why the results in some cases tend to be more variable for TBHQ than for the other peaks in the same batch analysis.

A: Reference chromatogram
C1 at 15 days (229 nm)

B: Final chromatogram
C4 at 60 days (229 nm)

C: Additional wavelength
C1 at 15 days (255 nm)

Figure 3.9: Representative chromatograms for batch 2.
Figure 3.9 (cont.): Representative chromatograms for batch 2.

Figure 3.10: Representative chromatograms for batch 4.
To summarise the above findings:

- Both the presence of TBHQ and the HPLC method used influenced the results. TBHQ-formulations yielded more peaks within the first 20 min., with both methods. When taking the slower flow rate of Method 2 into account, more peaks were detected for both formulations with Method 1 compared to Method 2. The differing detection wavelengths influenced the peak areas, which were bigger for Method 2 than Method 1.
- Peak purity is questionable when considering the additional wavelengths (Figure 3.7C - D, Figure 3.9C - D).
- Later eluting peaks formed and the runtimes had to be adjusted.

3.3.6 Conclusion

Firstly the Pheroid™'s chromatography changes when exposed to elevated temperature and humidity. This indicates the need for a method that will be robust towards changes in the matrix.

Secondly results suggest that incompatibilities may exist between the Pheroid™ delivery system and TBHQ. This should in future be considered and investigated by the formulation team, before the inclusion of TBHQ in Pheroid™ formulations.

Lastly the HPLC method seems to have played a role in the poor results. The methods employed lacked selectivity for the peaks that were of interest. The conditions implemented by these two methods were not suitable. Careful consideration of the detection wavelength, mobile phase composition (percentage and type of organic solvent, buffering systems, ion-pairing, etc.) and the sample preparation is necessary. Gradient elution and/or more complex sample preparation techniques should be considered due to the complexity of the Pheroid™ samples.

3.4 PHYSICAL PROPERTIES

The Pheroid™ delivery system has been classified as a colloidal system. Being an oil-in-water dispersion it can further be distinguished as an emulsion. A stable emulsion is defined by Attwood (2002:97) as “a system in which the globules retain their initial character and remain uniformly distributed throughout the continuous phase".
Attwood (2002:97) notes that the instability of emulsions can be due to:

- **Breaking/cracking** – it denotes the destruction of the interfacial film and is a more serious type of instability.
- **Phase inversion** – when an o/w (oil-in-water) emulsion becomes a w/o (water-in-oil) system, it depends on the phase volume ratio and the alteration of the hydrophile-lipophile balance of the emulsifying agent.
- **Flocculation** – it occurs due to attractive and repulsive forces between the globules, redistribution is possible, but the risk of coalescence is increased.
- **Creaming** – is caused by a difference in density between the dispersed and continuous phase, as with flocculation redistribution is possible and the risk of coalescence is increased.

3.4.1 **Colour and appearance**

*With TBHQ (batch 1 and 3):*

- The colour of the formulations changed from white (initial) to light yellow to bright yellow with time at elevated temperature and humidity.
- The samples stored at C1 and C2 remained uniform in distribution for the duration of three months, with no definite signs of instability.
- The first signs of instability were noted at 15 days for C3 and C4 samples. It presented as creaming, seeing that redistribution was possible.
- It seemed as though the yellow component concentrated against the glass just below the creamed portion.

*Without TBHQ (batch 2 and 4):*

- The colour changed from white (initial) to light yellow. The bright yellow colour that was visible for batch 1 and 3 was not observed for batch 2 and 4.
- Physical instability was not as prominent. The formulations seemed to remain mostly homogeneous. The concentration of the yellow component against the glass at 90 days, gave the impression that there might be some sort of instability.
3.4.2 Solubility in methanol

As the study progressed it was noticed that the Pheroid™ - methanol solutions, prepared during sample preparation, became turbid. It was determined that higher ratios of methanol were needed to produce clear solutions. In addition to this, it was found that when methanol was substituted with alcohols like ethanol and isopropanol, they were able to yield clear solutions when methanol failed to do so.

With TBHQ (batch 1 & 3):

- The loss in solubility could only be observed when comparing the Pheroid™-methanol solution against a methanol control solution.
- C1 samples remained apparently soluble throughout the three months.
- C2, C3 and C4 samples rendered clear solutions up until 30 days. After 30 days the solutions became turbid.

Without TBHQ (batch 2 & 4):

- The turbidity of methanol solutions was more apparent for the formulations without TBHQ than for the batches that did contain TBHQ.
- C1 samples also remained seemingly soluble for the duration of three months.
- C2 samples produced clear solutions for the first 60 days.
- C3 and C4 samples rendered clear solutions at 15 days. After 15 days the solutions became turbid.
- Filtration seemed to reduce the turbidity to some extent. However, not all of the solutions were clear after filtration which made them unsuitable for injection onto the HPLC system.

3.4.3 Conclusion

Cassim (2007:133) reported the yellowing of the Pheroid™ formulations and suggested that it may have been due to oxidation. She recommended the possible inclusion of another antioxidant like TBHQ.

Conversely, the inclusion of TBHQ did not improve the discolouration, in fact it lead to colour changes. The formulations without TBHQ proved to be more stable towards colour changes. A possible explanation for the discolouration is that the colour
change was caused by some sort of incompatibility with the Pheroid™ delivery system. Both TBHQ and the API's used by Cassim (2007:30), seemed to have interacted in the same way with the Pheroid™ delivery system, which lead to the yellowing of the formulations.

There also seemed to be a trade-off between the emulsion's physical stability and its solubility in methanol. Emulsion instability such as creaming, was observed with TBHQ-containing formulations, but maintained their solubility in methanol to a greater extent. The formulations without TBHQ seemed to be physically more stable, but rendered a more pronounced loss of solubility in methanol.

The observation that alcohols with a higher hydrocarbon-content than methanol were better solvents, may indicate that in the absence of TBHQ, degradation products were formed that were more non-polar.

3.5 PARTICLE SIZE ANALYSIS

3.5.1 Apparatus and procedure

A Malvern Mastersizer Micro (Malvern Instruments Ltd., Malvern, Worcestershire, UK) was used to determine the particle sizes of the Pheroid™ formulations. The principle of particle size determination is based on laser diffraction (Malvern instruments Ltd., 2010).

The Standard Operating Procedure (SOP): Pheroid™ vesicles using Hydro 2000 MU (ALL/QC/EQP 2.1), was followed. Measurements were executed as follows:

- The instrument was switched on half an hour before use in order to warm up and stabilise the laser.
- 800 ml of distilled water was pumped through the system at 2500 rpm, ensuring no air bubbles are present (Uys, 2006:48).
- The instrument determined the electronic and optical background.
- The sample bottle was shaken and an amount sufficient to produce 10-30% of laser obscuration was added to the distilled water.
- Samples were measured in duplicate with a few seconds delay between measurements.
• Refractive index (RI):
  o 1.458 - emulsion droplets. The method used by Uys (2006:49) implemented a RI of 1.4564.
  o 1.330 - distilled water.
• Absorption of the emulsion droplets was set at 0.1.

3.5.2 Results

Monthly particle size analyses were done. The particle size distributions obtained can be grouped into the following categories:

• Normal distribution.
• Distribution skewed to the right – contained a small number of large particles.
• Distribution skewed to the left – contained a small number of small particles.
• Two peaks – particle sizes were more or less equally distributed around two imaginary lines unlike the one line of a normal distribution.

Refer to Figure 3.11 for the representative histogram of each category. The colour-coding of Table 3.8 and Figure 3.11 serves as a link between each individual value and the four categories. The median values of the particle size distributions are given in the Table 3.8.

The following observations were made:

• TBHQ-containing formulations (batch 1 and 3) yielded mostly submicron particles with small, normal particle size distributions, while the formulations without TBHQ (batch 2 and 4) had larger particles and particle size distributions skewed to the right which indicate a small number of large particles.
• With TBHQ-formulations (batch 1 and 3) larger particles tend to form over time and at elevated temperatures, while the particle size distributions of the formulations without TBHQ (batch 2 and 4) indicates the presence of a few particles larger than 1 μm right from the beginning. Since microsponges are between 0.5 – 1.5 μm in size, the presence of bigger particles could either indicate the formation of microsponges or the coalescence of oil droplets. By means of CLSM the source of the larger particles can be established.
Table 3.8: Median particle size (µm) \([d(0.5)]\) of the Pheroid™ formulations.

<table>
<thead>
<tr>
<th>BATCH</th>
<th>Initial</th>
<th>Storage condition</th>
<th>15 days</th>
<th>30 days</th>
<th>60 days</th>
<th>90 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.193</td>
<td>5°C</td>
<td>0.185</td>
<td>0.184</td>
<td>0.184</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C + 60% RH</td>
<td>0.265</td>
<td>0.324</td>
<td>0.194</td>
<td>1.679</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C + 65% RH</td>
<td>0.294</td>
<td>0.356</td>
<td>0.379</td>
<td>0.203</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40°C + 75% RH</td>
<td>0.302</td>
<td>0.338</td>
<td>0.188</td>
<td>0.269</td>
</tr>
<tr>
<td>2</td>
<td>0.224</td>
<td>5°C</td>
<td>0.230</td>
<td>0.225</td>
<td>0.226</td>
<td>0.223</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C + 60% RH</td>
<td>0.226</td>
<td>0.234</td>
<td>0.225</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C + 65% RH</td>
<td>0.210</td>
<td>0.222</td>
<td>0.240</td>
<td>No result</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40°C + 75% RH</td>
<td>0.185</td>
<td>0.184</td>
<td>2.442</td>
<td>0.186</td>
</tr>
<tr>
<td>3</td>
<td>0.185</td>
<td>5°C</td>
<td>0.259</td>
<td>0.338</td>
<td>0.250</td>
<td>0.411</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C + 60% RH</td>
<td>0.309</td>
<td>0.353</td>
<td>0.269</td>
<td>0.404</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C + 65% RH</td>
<td>0.308</td>
<td>0.325</td>
<td>0.256</td>
<td>0.433</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40°C + 75% RH</td>
<td>0.303</td>
<td>0.577</td>
<td>1.084</td>
<td>1.244</td>
</tr>
<tr>
<td>4</td>
<td>0.225</td>
<td>5°C</td>
<td>0.229</td>
<td>0.221</td>
<td>0.213</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25°C + 60% RH</td>
<td>0.225</td>
<td>0.235</td>
<td>0.214</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30°C + 65% RH</td>
<td>0.221</td>
<td>0.212</td>
<td>0.206</td>
<td>0.221</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40°C + 75% RH</td>
<td>0.216</td>
<td>1.454</td>
<td>1.890</td>
<td>5.390</td>
</tr>
</tbody>
</table>

A: Normal distribution

B: Skewed to the right

Figure 3.11: Representative histograms for the particle size distributions of the Pheroid™ formulations.
Without TBHQ (batch 2 and 4) some of the particles became extremely large at elevated temperature which may indicate a loss of integrity and droplet fusion, this should also be confirmed by CLSM. TBHQ may thus provide better stability in terms of particle size and integrity. The results for batch 2 and 4 are, however, more stable at the first three storage conditions for the duration of the study, compared to that of batch 1 and 3 which contained TBHQ.

### 3.5.3 Conclusion

TBHQ plays a definite role in the particle size and particle size distribution of the Pheroid™ droplets. In order to decide which formulation was more optimal and stable, the integrity of the particles was investigated by means of CLSM.

In section 3.6 the results obtained for CLSM will be discussed.
3.6 CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

3.6.1 Apparatus and procedure

For the determination of droplet integrity a Confocal Laser Scanning Microscope (CLSM) (Nikon D-eclipse C1 si, with a violet diode laser [400-405 nm], a He/Ne laser [543 nm] and an Argon ion laser [457-514 nm]) was used. A 60 x oil objective and immersion oil for microscopy (50 cc, Type A, Nikon, Japan) was implemented.

The slide was prepared as follows:

• 1 μl of Nile Red (Molecular Probes, Inc., U.S.A.) was added to 50 μl of the Pheroid™ sample. It was then vortexed and left to stain for ± 15 min in a dark cupboard.

• 20 μl of the incubated sample was transferred to a glass slide and covered with a cover slip.

CLSM was performed with a He/Ne laser, which excited the Nile Red at 540 nm. Images were captured between 530 nm and 680 nm, in which the Nile Red’s emission spectra can be observed.

Monthly analyses were done.

3.6.2 Results

In the section 3.5 the particle size and particle size distribution of the Pheroid™ formulations, were investigated. In some cases the formation of larger particles was noted. Microsponges are known to be relatively large particles that can act as depots. In order to establish whether these larger particles are in fact microsponges and not the result of coalescence, CLSM was implemented. The following observations were made:

• Microsponges could not be identified with confidence in TBHQ-formulations (batch 1 and 3). In many cases the particles were too small to see if they were indeed microsponges. It is more likely that the bigger particles that formed were products of coalescence than microsponges. Particle integrity was sufficient for those particles that did not cling to the glass.

• For the formulations without TBHQ (batch 2 and 4) the presence of microsponges could be established with greater confidence. In the sample
analysed for batch 4, C4 at 90 days, a depot was present (Figure 3.12). With these formulations the microsponges were not destroyed by long-term exposure to elevated temperature and humidity, but seemed to increase in size.

- Affinity for glass and the formation of colonies.
  - From 30 days onward the Pheroid™ particles seemed to form colonies against the glass. This was not restricted to only the TBHQ-containing formulations, but could be seen in all four batches.

- The formation of colonies increases the risk of coalescence and the affinity to the glass bottle can potentially jeopardise dosage uniformity.

Figure 3.12: CLSM image of the microsponge depot that was present in the sample analysed for batch 4 [C4] at 90 days.

- Unidentified objects.
  - There were a few cases where objects were detected under the light microscope. It is not clear what the source of these objects were, but it may have been:
    - Crystals – No API was present, but it could have been the precipitate of one of the Pheroid™ components.
Glass/contaminant – Bottles were rinsed with distilled water and autoclaved prior to packaging. The manufacturing process was carried out under sterile conditions. It is, however, possible that a contaminant could have entered the sample bottle at any time before analysis during handling.

3.6.3 Conclusion

TBHQ may have influenced the size and structure of the Pheroids™ and possibly their stability towards coalescence. It may therefore not be suitable for the formulation of microsponges.

3.7 SUMMARY AND CONCLUSION

The following conclusions were made after analysis of the Pheroid™ formulations:

- Exposure to elevated temperature and humidity produced chromatographic changes in the Pheroid™ formulations. More peaks were detected and the run times had to be increased.

- Sample preparation was compromised due the Pheroid™ formulations' loss of solubility in the sample solvent (methanol). TBHQ managed to preserve the Pheroid™ formulations' methanol solubility to some extent.

- TBHQ supposedly compromised the physical stability of these formulations. It affected the particle size and particle size distributions as well as the structure of the Pheroid™.

With the occurrence of changes in the Pheroid™ due to exposure to temperature and humidity, and consequent changes in chromatography, a HPLC method will have to be adjusted at every testing interval. This can, however, be costly and impractical from a manufacturing point of view.

Since the Pheroid™ delivery system is still in its developmental stage, it may be beneficial to explore other alternatives for the analysis thereof. HPLC could still be an option if the effect of the Pheroid™ matrix is eliminated. Procedures to extract the API from the Pheroid™ formulation like solid phase extraction or liquid-liquid extraction should be considered. It is recommended that attention is given to the sample preparation before attempting to develop a HPLC method.
Furthermore, the observation made in terms of the discolouration could indicate potential incompatibilities, degradation of the Pheroid™ components, or an ineffective preservative system. From a formulation development point of view, each component should be evaluated in terms of compatibility and stability in the formulation.
4.1 INTRODUCTION

In the previous chapter the Pheroid™ samples were subjected to elevated temperature and humidity for a certain period of time. It was found that the chromatography changed over the three month period.

Furthermore, the HPLC methods used were deemed not suitable and needed some adjustments. In this chapter we explore alternatives for certain chromatographic parameters according to the observations made after the exposure of the Pheroid™ and pro-Pheroid, to elevated temperature and humidity. The aim was to highlight possible pitfalls and to suggest a general approach for the development of HPLC methods for Pheroid™-based samples.

The difference between the formulations used, lie with the presence of an API (mefloquine HCl). No API was added to the Pheroid™ formulations evaluated in Chapter 3, while formulations described in section 4.2.1, contained an API. Due to solubility problems of the API (mefloquine HCl), a pro-Pheroid rather than a Pheroid™ formulation was used in the experiments discussed in the sections to follow.

One of the methods described to analyse the Pheroid™ microsponges, namely Method 1, an existing method for the analysis of mefloquine, was used. In the absence of computer software like DryLab®, a trial-and-error approach was followed to optimise this method.

4.2 EXPOSURE TO ELEVATED TEMPERATURE AND HUMIDITY

It was noted in Chapter 3 that the Pheroid™ delivery system had undergone changes when exposed to elevated temperature and humidity. Since these changes are important in terms of the performance of the HPLC method, the pro-Pheroid samples used were also subjected to elevated temperature and humidity. The storage condition, namely C4 (40°C + 75% RH), rendered the most changes for the
Pheroid™ microsponges and were applied for the pro-Pheroid formulations described in section 4.2.1.

4.2.1 pro-Pheroid formulations

The pro-Pheroid contains no aqueous phase in contrast to the Pheroid™. With mefloquine being only slightly soluble in water (Lim, 1985:170), the pro-Pheroid was therefore better suited as a carrier than the Pheroid™. With the evaluation of the HPLC analysis of Pheroid™-based samples, as described in Chapter 3, Pheroid™ microsponges were used. However, in this part of the study, pro-Pheroid vesicles were used. These two formulations differ in terms of the globule size and structure, and more importantly the nature of the continuous phase. Refer to section 2.3 for further details.

Two batches pro-Pheroid vesicles were prepared for method development purposes:

- P09011 (batch 5) – containing 2 mg/ml of mefloquine hydrochloride.
  - When experimenting with different concentrations prior to manufacturing, no visible sediment was noted when 2 mg/ml of mefloquine was mixed with the pro-Pheroid delivery system. However, during manufacturing, mefloquine produced sediment. The formulation had to be stirred for a few hours in order to obtain a uniform suspension.

- P09012 (batch 6) – the control batch, containing no API.
  - Some of the compounds within the pro-Pheroid system did not remain suspended, but formed a “layer” at the bottom. This was only noticed after manufacturing, when packaging was almost complete. Care was given to make sure that both samples, with and without “layers”, were analysed.

Packaging was the same as for the Pheroid™ microsponges.

4.2.2 Storage conditions

These formulations were stored at 40°C + 75% RH only. Tests were performed after 30, 60 and 90 days of storage.
4.2.3 Chromatographic conditions for Method 1a

Method 1 described under section 3.3 was used for the monthly HPLC analyses. Certain alterations were made after consideration of the results obtained with the HPLC analyses of the Pheroid™ microsponges. The adjusted version of Method 1 will be referred to as Method 1a. Changes were made in terms of the stop time and detection wavelength.

Table 4.1: The chromatographic conditions for the control Method 1a.

<table>
<thead>
<tr>
<th>Column</th>
<th>Luna C18 (2) 150 x 4.6 mm, 5 μm [Phenomenex]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mobile phase</td>
<td>0.1 M potassium dihydrogen phosphate (KH₂PO₄) buffer and acetonitrile mixed in the ratio 52:48. The buffer is prepared by dissolving 13.61 g KH₂PO₄ in 1000 ml Milli Q water. pH is adjusted with 1 M NaOH and concentrated orthophosphoric acid to 3.5 ± 0.05.</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 ml/min.</td>
</tr>
<tr>
<td>Injection volume</td>
<td>20 μl</td>
</tr>
<tr>
<td>Detection</td>
<td>UV detection at 229 nm and 280 nm (280 nm represents the other UV absorption maxima of mefloquine), bandwidth = 4 nm</td>
</tr>
<tr>
<td>Retention time</td>
<td>≈ 2.3 min. for mefloquine HCl at both wavelengths</td>
</tr>
<tr>
<td>Stop time</td>
<td>55 min. (adjusted after completion of the Pheroid™ microsponges study)</td>
</tr>
</tbody>
</table>

4.2.4 Integration parameters

Methanol caused the formation of negative peaks which influenced the quantitation of peak areas, especially the areas of small peaks. For the time interval, 0 – 3 min., negative peaks were rejected during integration. Detector response was measured in terms of peak area.

4.2.5 Sample preparation

Methanol remained the sample solvent. Pro-Pheroid formulations are more concentrated in Pheroid™ components than Pheroid™ formulations, due to the absence of a water phase. In order to obtain more or less the same amount of Pheroid™ components that were present after dilution of the Pheroid™ microsponges with methanol, some adjustment to the dilution ratio had to be made. The dilution ratio of 1:20, as described in section 3.3.4, could not be implemented directly for the pro-Pheroid vesicles, and was changed to a 1:50 ratio.
4.2.6 Results and discussion

Mefloquine was poorly resolved from an adjacent peak at both 30 and 60 days. With the initial and 90 days analyses this interfering peak was absent. The interfering peak was also observed for the mefloquine control sample at 229 nm (Figure 4.1) and 280 nm. Since this control sample was prepared from the raw material used in the formulation of batch 5, and not a primary reference standard, the possibility of impurities exists. The investigation conducted to find the source, will be described in section 4.3.

![Representative chromatogram of the spilt peaks obtained for the mefloquine control sample (229 nm).](image)

Figure 4.1: Representative chromatogram of the spilt peaks obtained for the mefloquine control sample (229 nm).

The number of detectable peaks greater than 10 000 AU increased with time. The control formulation (batch 6), which contained no API, yielded more peaks than the mefloquine-containing formulation (batch 5) at both wavelengths (Figure 4.2 and 4.3).

Some overlapping peaks became better resolved over time as the selectivity for these peaks seemed to change.

The results for mefloquine in terms of peak area appeared to be variable. Average peak areas increased in some instances and decreased in others (Table 4.2).

<table>
<thead>
<tr>
<th>Wavelength</th>
<th>Initial (Area &amp; %RSD)</th>
<th>30 days (Area &amp; %RSD)</th>
<th>60 days (Area &amp; %RSD)</th>
<th>90 days (Area &amp; %RSD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>229 nm</td>
<td>1717746 7.2</td>
<td>882816 8.1</td>
<td>385366 19.2</td>
<td>785894 21.6</td>
</tr>
<tr>
<td>280 nm</td>
<td>430683 13.5</td>
<td>217335 7.8</td>
<td>153827 7.7</td>
<td>222006 30.6</td>
</tr>
</tbody>
</table>
Results obtained indicate that the wavelength 280 nm is not superior to 229 nm, this is unexpected since at 280 nm less peaks could be detected where mefloquine would have eluted. Mefloquine’s UV absorbance, however, is also lower at 280 nm. The effect of interfering peaks appeared to be less, but this might not necessarily have been the case.

In addition to these observations, it was also noticed that the baseline of the pro-Pheroid – containing samples, became unstable after a number of consecutive runs. Figure 4.2 illustrates the first pro-Pheroid run in the initial batch analysis, while Figure 4.3 illustrates the fourth run in the same batch. This observed instability fits the
description of long-term noise as given by Snyder et al. (1997:647), and may be an indication that not all of the compounds are eluted from the column within the given run time. The mobile phase may therefore be unsuitable or the run time is not long enough. In section 4.4 the selection of a suitable organic solvent for Pheroid™-based samples, will be discussed.

4.3 MEfloquINE PEAK ANOMALIES

As mentioned in the previous section mefloquine eluted as split peaks. The size of the extra peak varied, being more prominent in some cases than others. This peak anomaly was present with both the analyses of the pro-Pheroid – mefloquine formulation (batch 5) and the mefloquine raw material.

Possible reasons for the observed split peaks were:

1. Impurities present in the mefloquine raw material, co-elute with mefloquine.
2. The sample solvent and mobile phase are not of equal strength. The sample solvent (100% methanol) contains a higher percentage of organic solvent than the mobile phase (48% acetonitrile), which leads to split peaks. Literature suggests that samples should be prepared with the mobile phase or a solvent that is equal in strength. Alternatively the injection volume can be decreased to reduce the appearance of split peaks (Dong, 2006:253).

Experiments were conducted to determine whether the split peaks could be attributed to impurities or to the sample solvent being too strong.

4.3.1 Results and discussion

4.3.1.1 The possibility of impurities in the mefloquine raw material being the causative factor

Two samples were prepared and analysed in triplicate according to Method 1a:

1. Mefloquine raw material dissolved in 100% methanol (Figure 4.4).
2. A primary mefloquine reference standard dissolved in 100% methanol (Figure 4.5).

Both the mefloquine raw material and reference standard produced the same results.
Interference from impurities was eliminated as being the reason for the apparent split peaks.

Figure 4.4: Representative chromatogram of the spilt peaks obtained for the mefloquine raw material sample (229 nm).

Figure 4.5: Representative chromatogram of the spilt peaks obtained for the mefloquine primary reference standard sample (229 nm).

4.3.1.2 The possibility that the sample solvent strength is not compatible with the mobile phase strength

Pro-Pheroids are insoluble in water. Dissolving samples in the mobile phase, which contains an aqueous phase, is not feasible. One mefloquine sample was prepared with 100% methanol and analysed by means of Method 1a. Only the injection volume was varied, ranging from 5 – 30 μl. Duplicate runs were performed for each injection volume (5 μl, 10 μl, 15 μl, 20 μl, 25 μl and 30 μl).
• Injection volumes 5 – 15 μl rendered no visible signs of peak anomalies. The mefloquine peak is baseline resolved from solvent peaks (Figure 4.6).

• At 20 μl split peaks appear to be absent, but baseline resolution of mefloquine and solvent peaks are no longer present (Figure 4.7).

• When injection volume is increased to 25 μl (Figure 4.8) and 30 μl, mefloquine elutes as split peaks.

Considering the above findings, 15 μl would therefore provide better repeatability than 20 μl. The injection volume should, however, not be too small as this could jeopardise accuracy.

Figure 4.6: Representative chromatogram obtained for a mefloquine sample when a 15 μl injection volume was implemented (229 nm).

Figure 4.7: Representative chromatogram obtained for a mefloquine sample when a 20 μl injection volume was implemented (229 nm).
To test the statement that 15 µl will provide better repeatability:

A sample of the batch 5 formulation, was analysed in triplicate according to Method 1a by injecting 15 µl instead of 20 µl. No split peaks were visible. Repeatability of peak area was sufficient at 280 nm (0.45%), but not at 229 nm. The pro-Pheroids have higher UV absorbance at 229 nm than 250 nm; more peaks are detected that have retention times in close proximity to that of mefloquine, which could have influenced the results at 229 nm.

4.3.1.3 **Co-elution of mefloquine peaks with sample solvent peaks**

According to methanol's UV cut-off (205 nm) it was unlikely that interfering peaks of the sample solvent, was the reason for split peaks at 229 nm and 280 nm. However, after the injection of a 100% methanol sample onto the column, peaks that elute close within the vicinity of where the mefloquine peak usually appears, were indeed observed at both 229 nm (Figure 4.9) and 280 nm. This also strengthens the assumption that the sample solvent at that specific concentration is not compatible with the mobile phase. Since the effect of methanol could be minimised by the adjustment of the injection volume, no further investigations concerning co-eluting peaks were performed.
4.4 **THE ORGANIC SOLVENT**

Varying the solvent strength and type are usually attempted first, before considering other parameters, when optimising a method. The reasons being that it can be easily adjusted and have a powerful influence on the separation's selectivity and retention (Snyder *et al.*, 1997:242,254). Due to acetonitrile's low UV cut-off and viscosity, this organic solvent is often the recommended starting point (Snyder *et al.*, 1997:240).

The HPLC methods used in previous studies implemented acetonitrile to a great extent. Acetonitrile was also used in the methods that were focused on in this study. Methanol is, however, preferred for the separation of ionic samples which are also hydrophobic and require high percentages of the organic solvent, since methanol provides better solubility for mobile phase additives such as the buffer components and ion-pairing agents (Snyder *et al.*, 1997:307). Most of the API’s formulated into the Pheroid™ and pro-Pheroid delivery systems are ionic (e.g. mefloquine, abacavir sulphate, lamivudine, nevirapine). The presence of oil-based components accounts for the hydrophobicity of the samples. Methanol thus may be the better option.

The suspected presence of late eluting compounds, as mentioned in section 4.2.6, also questions the suitability of the organic solvent (acetonitrile) used and its concentration (48%). Due to the excessive retention of some of the Pheroid™ components, gradient elution may be a better option as opposed to isocratic elution. The implementation of an initial gradient separation can prove to be very
advantageous to determine whether isocratic analysis is feasible before a lot of time and money is spent on a method that will not be effective.

Considering all of the above, experiments were carried out to compare the effectiveness of acetonitrile and methanol as organic solvent for the separation of Pheroid™-based samples.

For isocratic analyses the percentage of acetonitrile used was 50%. This is close to the acetonitrile content of Method 1a (48%), and since a buffering system is required by the ionic API's, much higher concentrations would not be feasible. According to scales provided in literature (Snyder et al., 1997:426), 60% methanol would be more or less equal in strength to 50% acetonitrile. The methanol mobile phase therefore contained 60% methanol. It should be noted that no buffer was added to the mobile phases prepared in these experiments and that the samples analysed were of batch 6 (only pro-Pheroids, no API present).

Isocratic analyses were performed with two different mobile phases with the following content:

- Mobile phase 1 – 50% acetonitrile
- Mobile phase 2 – 60% methanol

Linear gradient elution, 5 to 100% of the organic in 15 min. (t = 5 - 20 min.) and with a 20 min. hold of 100% organic at the end of the gradient, was also performed to determine the appropriate isocratic conditions if applicable. The column was equilibrated with 5% organic solvent for 5 min. at the beginning, and for 10 min. after the gradient.

Chromatographic conditions of Method 1a was implemented with the exception of the mobile phase and the injection volume. 15 µL was used instead of the 20 µL specified by Method 1a.

4.4.1 Results and discussion

Methanol and acetonitrile were compared based on their performances in both isocratic and gradient separations.
4.4.1.1  *Isocratic separation*

With both acetonitrile and methanol separations, long-term baseline noise were observed after a number of consecutive runs (Figure 4.10 and 4.12). A C18 column was used as described by Method 1a. When the C18 column was substituted by a C8 column, the presence of late eluting compounds became more apparent (Figure 4.11 and 4.13). From Figure 4.13 and 4.14 it is clear that the suspected late eluting compounds do not absorb UV light significantly at 280 nm compared to 229 nm.

![Chromatogram 4.10](image)

*Figure 4.10: Chromatogram of the separation of a batch 6 sample with 60 % methanol on a C18 column 229 nm (4th consecutive run).*

![Chromatogram 4.11](image)

*Figure 4.11: Chromatogram of the separation of a batch 6 sample with 60 % methanol on a C8 column at 229 nm (4th consecutive run).*
Figure 4.12: Chromatogram of the separation of a batch 6 sample with 50 % acetonitrile on a C18 column at 229 nm (4th consecutive run).

Figure 4.13: Chromatogram of the separation of a batch 6 sample with 50 % acetonitrile on a C8 column at 229 nm (4th consecutive run).

Figure 4.14: The chromatogram given in Figure 4.13 at 280 nm.
Acetonitrile tends to show signs of late eluting compounds earlier than methanol. Although acetonitrile is a better solvent for some of the Pheroid™ components, it was also unable to elute all of the compounds in one run. The baseline of acetonitrile separations was more unstable than with methanol. With acetonitrile separations the peak which eluted at the solvent front, increased in size with each consecutive run (Figure 4.15 and 4.16). This was only noticed at 229 nm and not at 280 nm, and coincides with the UV absorbance of the late eluting compounds as stated earlier. In regard to the increasing size of some peaks for acetonitrile separations, the chromatograms obtained for methanol seemed to be more reproducible than that of acetonitrile.

Figure 4.15: Chromatogram of the separation of a batch 6 sample with 50 % acetonitrile on a C18 column at 229 nm (1st run).

Figure 4.16: Chromatogram of the separation of a batch 6 sample with 50 % acetonitrile on a C18 column at 229 nm (last run).
Higher percentages of both acetonitrile and methanol are therefore needed to elute all of the sample components within the given runtime. This complicates the separation of API's that are dependent upon ion-pairing or ion-suppression. The development of a gradient separation method may thus be the best place to start for the analysis of Pheroid™- based drug products.

4.4.1.2 Gradient separation

With both acetonitrile (Figure 4.17) and methanol (Figure 4.18), most of the peaks only eluted when the gradient reached the 100% organic solvent mark (at \( t = 20 \) min.). This was observed at both of the wavelengths (229 nm and 280 nm).

![Figure 4.17: Chromatogram representative of an acetonitrile gradient separation for a batch 6 sample at 280 nm.](image1)

![Figure 4.18: Chromatogram representative of a methanol gradient separation for a batch 6 sample at 280 nm.](image2)
In contrast to what was found with isocratic analyses, methanol eluted the last peak earlier than acetonitrile. Considering this and the fact that methanol was able to yield more reproducible chromatograms as stated in section 4.4.1.1, it may be an indication that with the sample solvent and chromatographic conditions used, methanol is superior to acetonitrile.

It is clear that most of the compounds are highly retained and not suitable for separation with reversed phase columns. Non-aqueous reversed phase (NARP) or normal phase separation will be more suited for this type of compounds (Snyder et al., 1997:416). As mentioned in section 4.4.1.1, the use of 100% organic mobile phases is not feasible for a lot of the API's analysed. Snyder et al. (1997:416) also suggest the use of a solvent stronger than acetonitrile like THF or the use of more polar columns such as cyano.

4.5 pH AND THE BUFFER CONCENTRATION

As with the organic solvent, the pH also has a pronounced effect on the selectivity as well as the retention, but of ionic compounds, and is considered to be a very valuable parameter for the separation of ionic compounds (LoBrutto, 2007:375). Poor ionisation control of ionisable compounds may lead to variable results and poor peak shapes.

As discussed in section 1.9.5.1, the pH should be adjusted with the pK\textsubscript{a} of the analyte in mind. Since the chromatograms of pro-Pheroid and Pheroid\textsuperscript{TM} formulations yields a lot of peaks that elute close to the API, variation in the retention of these matrix peaks are expected to influence the quantitative determination of the API. pH screening experiments were therefore conducted to determine the Pheroid\textsuperscript{TM} components' response to changes in the pH.

LoBrutto and Kazakevich (2007:180) recommend that five different pH values should be used to determine the pK\textsubscript{a} of an analyte chromatographically. Six pH values were selected of which five were within ±1 pH unit from the buffer's pK\textsubscript{a}. Refer to section 1.9.5.2 for the pK\textsubscript{a} values and buffering ranges of the phosphate buffers. The sixth pH (8.64) was close to the aqueous pK\textsubscript{a} of mefloquine (8.60) as given by Lim (1985:170), but was still within ±1.5 pH units of the buffer's pK\textsubscript{a}. The pH values used were 2.29, 2.95, 6.37, 7.20, 7.99 and 8.64.
Methanol was implemented as organic solvent, and its concentration kept constant at 60%. A phosphate buffer with a concentration of 50 mM, as recommended by Snyder et al. (1997:407), was prepared. The C18 column recommended by Method 1a was used, but the injection volume was again adjusted to 15 μl.

The pro-Pheroid samples analysed were of batch 6 (containing no API). A primary mefloquine standard was also analysed at the different pH conditions.

4.5.1 Results and discussion

The chromatograms obtained for the pro-Pheroid samples, at the first four pH values, were similar. At pH 7.99 and 8.64 some changes in selectivity were observed (Figure 4.19 – 4.21). These changes in selectivity could be observed at both 229 nm and 280 nm. The retention of the late eluting compounds was not affected by the variation in pH.

The Pheroid™ components therefore seem to be neutral at the normally acidic conditions used in reversed phase separations, and unlikely to have contributed to possible variation in the quantitative results obtained for mefloquine. Considering the above, when implementing more alkaline pH values the effect thereof on the Pheroid™ components should be determined and taken into account.

Figure 4.19: Chromatogram of a batch 6 sample separated with a mobile phase with an aqueous pH of 2.95 (229 nm).
With the suspected incompatibility mentioned in section 4.3.1.3, the following observation was made when comparing two buffer concentrations. A comparison was made between a 10 mM and a 100 mM phosphate buffer to assist in the decision of an appropriate concentration. Results show that lowering the buffer concentration had an effect, similar to that of the smaller injection volumes, on the size of the apparent sample solvent peaks. The incompatibility of the sample solvent with the mobile phase may thus be related to the buffering components in the mobile phase.
4.6 SUMMARY AND CONCLUSION

To summarise the above observations:

- Problems with compatibility of the sample solvent with the HPLC system were encountered. It was determined that the sample solvent is too strong and lead to the formation of split peaks. The appearance of split peaks was reduced by lowering the injection volume.

- Due to the long-term noise observed after a number of consecutive pro-Pheroid—sample runs, the presence of hydrophobic late eluting compounds was suspected. It was confirmed by the implementation of a less non-polar column (C8) and gradient separations.

- With the comparison of two wavelengths (229 nm and 280 nm), it was found that more peaks are detected at a shorter wavelength (229 nm). The effect of the apparent late eluting compounds was also observed at 229 nm, being basically absent at 280 nm.

- Both acetonitrile and methanol deemed not to be fit for the elution of all of the Pheroid™ components within an acceptable amount of time, with isocratic as well as gradient separations.

- The pH did not seem to influence the retention of most of the Pheroid™ components at the pH conditions usually implemented. Late eluting compounds also remained unaffected. At alkaline conditions the effect of pH on certain Pheroid™ components should be kept in mind.

In conclusion, reversed phase HPLC may not be suitable for the analysis of pro-Pheroid or Pheroid™-based samples, not in the form that these samples have been introduced to the HPLC system, namely only diluted in methanol.

After experimenting with some of the key chromatographic parameters, it became clear that the Pheroid™ delivery system is too complex and complicates HPLC analysis. Due to this complexity, the implementation of a more universal detector, such as Mass Spectrometry (MS), would only further complicate analyses. Other separation strategies given for very hydrophobic compounds will also be difficult to apply to the analysis of Pheroid™-based drug products which usually contains ionic API's.
HPLC, however, remains a very valuable analytical technique used in stability testing. It is thus recommended that a more appropriate sample preparation procedure should be developed, one that will eliminate the effects of the Pheroid™ components.
Stability is one of the main concerns in the development of a drug product. Regulatory authorities require that analytical proof of such stability be provided for registration purposes.

The Pheroid™ delivery system holds incredible potential as a pharmaceutical drug carrier (Chapter 2). Proof of its stability in formulation with API's has, however, not been established. Stability studies have been conducted on Pheroid™ formulations aimed at the treatment of diseases such as HIV/AIDS, tuberculosis and malaria. Only a very few of these studies confirmed the stability of such formulations. Analysts concluded that the Pheroid™ delivery system itself is unstable, or the HPLC methods employed to analyse the Pheroid™/pro-Pheroid - based samples are not suitable.

The stability of the Pheroid™ delivery system together with the HPLC analysis thereof was evaluated (Chapter 3). TBHQ was included into two of the four Pheroid™ microsponge formulations. Formulations were subjected to accelerated stability testing conditions and analysed by means of HPLC according to existing methods. In addition to this the physical stability of these formulations were also monitored through particle size analysis, CLSM and visual evaluations.

To supplement this evaluation of the HPLC methods used for the analysis of Pheroid™- based samples, experiments were conducted where some of the key chromatographic parameters were adjusted (Chapter 4). Pro-Pheroid formulations, instead of Pheroid™ formulations, were used to accommodate the inclusion of an API (mefloquine HCl) that is not soluble in water. The parameters that were subjected to change included the organic solvent, isocratic versus gradient separation, pH and detection wavelength.

The presence of the anti-oxidant TBHQ, affected the physical stability of the emulsion as well as the particle size and particle size distributions of the Pheroids™. Discolouration of the formulations which contained TBHQ may indicate potential incompatibilities, degradation of the Pheroid™ components, or an ineffective
preservative system. From a formulation development point of view, each component should thus be evaluated in terms of compatibility and stability in the formulation.

Furthermore, it was found that the Pheroid™/pro-Pheroid delivery system underwent changes when subjected to elevated temperature and humidity, which influenced the HPLC analyses. These changes also lead to a decrease in the solubility of the Pheroid™ formulations in the sample solvent (methanol), and consequently compromised sample preparation.

It was concluded that due to these changes in the chromatography of the Pheroid™/pro-Pheroid - based samples, the HPLC method will have to be adjusted at every testing interval, which is both impractical and costly.

It was also found that the Pheroid™/pro-Pheroid - based samples are too complex and hydrophobic for analysis with reversed phase HPLC. Thus, preparing a sample only through dilution of the Pheroid™/pro-Pheroid formulation with methanol, is not optimal. This sample preparation procedure introduces a complex mixture of compounds to the reversed phase column which causes interferences with the quantitation of analyte peaks, and can not be easily eluted from the column. Other HPLC modes recommended for very hydrophobic samples, like normal phase or non-aqueous reversed phase, are not feasible for the analysis of most API's which are ionic compounds. More suitable alternative methods should therefore be explored.

HPLC, however, remains a very valuable analytical technique used in stability testing. In order to continue using HPLC for stability testing purposes of Pheroid™ based drug products, attention should be given to the development of a sample preparation procedure that will eliminate the effect of the Pheroid™ matrix. Procedures to extract the API like solid phase extraction or liquid-liquid extraction should be considered.
REFERENCES


FDA see FOOD AND DRUG ADMINISTRATION.


ICH see INTERNATIONAL CONFERENCE ON HARMONIZATION.


Ph.Int. see INTERNATIONAL PHARMACOPOEIA.


Poster presented at the 5th International Conference on Pharmaceutical and Pharmacological Sciences.
HPLC method development for the Pheroid™ delivery system

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Introduction and Background

Stability plays an important role in the development of a new drug product. HPLC is considered a stability indicating method of analysis. It is widely used in the pharmaceutical industry for the quantification of small organic molecules.

Previous stability studies conducted on Pheroid™-based drug products, indicated problems with generating reliable stability data. The methods used to analyze these formulations were validated for the API only. Results were inconclusive in most cases and the need developed for a HPLC method that takes the Pheroid™-delivery system into account.

Objectives

The purpose of this study was to determine how the changes, that occur in the Pheroid™-delivery system, under accelerated storage conditions, influence HPLC analysis.

Method

Pheroid™ microsponges, with no API, were prepared and stored for a period of three months at 5°C (C1), 22°C+60%RH (C2), 30°C+85%RH (C3) and 40°C+75%RH (C4). Monthly HPLC analyses were done, using an existing method formulæquent.

Results and Discussion

Two peaks were identified to track possible changes in peak area. With time, increased temperature and humidity, the peak areas showed a slight increase, but no definite signs of degradation.

- The number of detectable peaks however, seemed to increase, indicating possible degradation or chemical interactions. Physical signs of instability, like discoloration and creaming, were noted.

![Average number of detectable peaks > 10000AU](image)

Fig. 2: The average number of peaks with areas greater than 10000AU detected at each storage condition, determined after 15, 30, 60 and 90 days.

- A loss in solvility of the Pheroid™ microsponges in the sample solvent, namely methanol, developed as the study progressed. Consequently, not all of the samples could be analysed by means of HPLC.
- Acetonitrile, which constituted the organic portion of the mobile phase, also weren't able to act as a suitable sample solvent. Ethanol and isopropanol rendered clear solutions when methanol failed to do so, which lead to the belief that the degradation products may be more lipophilic than the initial Pheroid™ microsponges.
- This is supported by the fact that longer runtimes became necessary to elute all the detectable peaks.

- An answer for the apparent increase in peak area, could not be given during this study. Using an organic solvent as mobile phase, in which the sample compounds are not soluble, is impractical.
- A study dedicated to finding the appropriate HPLC conditions when analysing Pheroid™-based samples is recommended.

- Stability of the Pheroid™-based delivery system may also be in question when considering the results obtained. Further investigation of the Pheroid™ formula is recommended.

References