SIMULTANEOUS DETERMINATION OF PHOSPHODIESTERASE-5 (PDE-5) INHIBITORS AND THEIR ANALOGUES IN SEXUAL ENHANCEMENT PRODUCTS

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ABSTRACT

Adulteration of herbal health supplements (HHSs) with phosphodiesterase-5 (PDE-5) inhibitors and their analogues is becoming a worldwide problem. These HHSs are marketed for increasing sexual performance, in men. In legal medical practice, PDE-5 inhibitors are only used to treat patients with erectile dysfunction (ED), but these adulterated HHSs are also being utilised by men without ED for recreational purposes. Screening the presence of these adulterants is important to protect the safety of consumers. However, the complexity in managing this adulteration problem involve many different aspects including the ever increasing number of new analogues, the budget restriction in obtaining the reference standards for these analogues and the method needed to simultaneously detect all PDE-5 inhibitors and their analogues.

In order to develop the capability to produce in-house standards, ten sildenafil analogues were synthesised. The purity of the synthesised standards was above 90%. These synthesised standards together with fifty-one commercial reference standards of PDE-5 inhibitors and their analogues were then used to build a LCMS spectral library. A LCMS IT-TOF method was developed and validated for simultaneous determination of the sixty-one standards.

The determination method was applied to investigate adulterated HHSs sold in the Malaysian market. Sixty-two products that claim to enhance men's sexual health were sampled between April 2014 and April 2016. These products included unregistered products seized by the Pharmacy Enforcement Division of the Ministry of Health (n = 39), products sent to the National Pharmaceutical Regulatory Agency for pre-registration testing (n = 9) and products investigated under the post-registration market surveillance programme (n = 14). The products were tested against the in-house spectral

library consisting of 61 PDE-5 inhibitors and analogues using the validated LCMS IT-TOF method. Thirty-two (82%) of the unregistered products and two (14%) of the registered products were found to be adulterated with at least one PDE-5 inhibitor or analogue, while none of the pre-registration products contained the adulterants of our interest. A total of 16 different adulterants were detected and 36% of the adulterated products contained a mixture of two or more adulterants. Two of the adulterants, which were not included in the spectral library, were identified as acetil acid and xanthoantrafil using the formula predictor software included in the LCMS IT-TOF system. In conclusion, this study has demonstrated that the adulteration of unregistered herbal products in the Malaysian market is an alarming issue that needs to be urgently addressed by the relevant authorities.

ABSTRAK

Makanan tambahan kesihatan herba yang dicampurpalsu dengan perencat phosphodiesterase-5 (PDE-5) dan analog-analog berkaitan membawa kepada permasalahan sejagat. Makanan tambahan kesihatan herba ini dipasarkan untuk meningkatkan prestasi seksual lelaki. Di dalam praktis perubatan, perencat PDE-5 hanya digunakan untuk merawat penyakit mati pucuk. Walaubagaimanapun, makanan tambahan kesihatan herba yang dicemari bahan campurpalsu ini turut digunapakai oleh lelaki yang tidak menghidap mati pucuk untuk tujuan rekreasi. Demi melindungi keselamatan pengguna. saringan untuk mengesan bahan campurpalsu ini adalah penting. Walau bagaimanapun, kerumitan dalam menguruskan permasalahan campurpalsu ini melibatkan pelbagai aspek termasuk jumlah analog yang semakin meningkat, kekangan kewangan dalam mendapatkan piawai rujukan analog dan kaedah yang diperlukan untuk mengesan semua perencat PDE-5 dan analog secara serentak.

Dalam usaha untuk membangunkan keupayaan bagi menghasilkan piawai rujukan "in-house", sepuluh analog sildenafil telah disintesis. Ketulenan piawai rujukan yang disintesis adalah melebihi 90%. Seterusnya, analog sildenafil yang telah disintesis ini dan lima puluh satu piawai rujukan komersial untuk perencat PDE-5 dan analog berkaitan telah digunakan untuk membina sebuah perpustakaan spektrum LCMS. Satu kaedah LCMS IT-TOF telah dibangunkan dan divalidasi untuk mengesan enam puluh satu piawai rujukan ini secara serentak.

Kaedah pengesanan LCMS ini telah digunakan untuk mengesan bahan campurpalsu di dalam makanan tambahan kesihatan herba yang dijual di pasaran Malaysia. Enam puluh dua produk yang dijual dengan indikasi untuk meningkatkan kesihatan seksual lelaki telah disampel di antara April 2014 dan April 2016. Produk-produk ini termasuk produk tidak berdaftar yang dirampas oleh Bahagian Penguatkuasa Farmasi

Kementerian Kesihatan (n = 39), produk dihantar ke Agensi Regulatori Farmasi Negara untuk ujian pra-pendaftaran (n = 9) dan produk disiasat di bawah program pasca pendaftaran pengawasan pasaran (n = 14). Produk-produk tersebut telah diuji berdasarkan perpustakaan spektrum "in-house" dengan menggunakan tatacara LCMS IT-TOF yang telah dibangunkan. Tiga puluh dua (82%) daripada produk yang tidak berdaftar dan dua (14%) daripada produk berdaftar telah didapati dicemari dengan sekurang-kurangnya satu perencat PDE-5 atau analog berkaitan, manakala tiada satu pun daripada produk-produk pra-pendaftaran yang dikesan mengandungi bahan campurpalsu. Sebanyak 16 jenis bahan campurpalsu berbeza telah dikesan dan 36% daripada produk-produk dicemari mengandungi campuran dua atau lebih bahan campurpalsu. Dua daripada bahan campurpalsu, yang tidak tersenarai di dalam perpustakaan spektrum, telah dikenal pasti sebagai asid acetil dan xanthoanthrafil dengan menggunakan perisian peramal formula yang terdapat di dalam sistem LCMS IT-TOF tersebut. Kesimpulannya, kajian ini telah menunjukkan bahawa pencemaran produk makanan tambahan kesihatan herba yang tidak berdaftar dalam pasaran Malaysia adalah satu isu yang membimbangkan dan perlu segera ditangani oleh pihak berkuasa yang berkenaan.

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LIST OF SYMBOLS AND ABBREVIATIONS

DCA	:	Drug Control Authority
ED	:	Erectile Dysfunction
GCMS	:	Gas Chromatography Mass Spectrometry
HHSs	:	Herbal Health Supplements
HPLC	:	High Performance Liquid Chromatography
IR	:	Infrared
IT -TOF	:	Ion Trap Time of Flight
LCMS	:	Liquid Chromatography Mass Spectrometry
LOD	:	Limit of Detection
MOH	:	Malaysia Ministry of Health
MS	:	Mass Spectrometry
NIR	:	Near Infrared
NMR	:	Nuclear Magnetic Resonance
NPRA	:	National Pharmaceutical Regulatory Agency
PDE-5	:	Phosphodiesterase-5
Q- TOF	:	Quadrupole Time of Flight
TLC	:	Thin Layer Chromatography
USFDA	:	United States Food and Drug Administration
UV	:	Ultraviolet

CHAPTER 1:

INTRODUCTION

In the last decade, the popularity of herbal health supplements (HHSs) has been increasing worldwide (Justa Neves & Caldas, 2015; Skalicka-Woźniak, Georgiev, & Orhan, 2016). One contributory factor is that these supplements are considered to be made of only natural ingredients and therefore should be safer than prescription medicines. Another factor might be an embarrassment of the consumers over their health status, leading them to choose HHSs instead of seeking for professional medical help (Campbell et al., 2013; Rocha, Amaral, & Oliveira, 2016).

However, intentional adulterations of HHSs in order to boost sales and increase product performances have widely appeared in previous studies. Adulterations in these supplements intended for male enhancement or sexual stimulation are amongst the highest being reported. Phosphodiesterase-5 (PDE-5) inhibitors are commonly the type of adulterant found in these types of supplements (Damiano et al., 2014; ElAgouri, ElAmrawy, ElYazbi, Eshra, & Nounou, 2015; Rocha et al., 2016; Vaclavik, Krynitsky, & Rader, 2014). Malaysia, a multicultural country with a rich heritage in herbal preparations, is also facing the same issue (Pharmaceutical Services Division, 2013).

PDE-5 inhibitors are used therapeutically to treat erectile dysfunction (ED) (Steers, 2002). The first synthetic PDE-5 inhibitors licensed by the United States Food and Drug Administration (FDA) was sildenafil, vardenafil and tadalafil. Up until now, four additional PDE-5 inhibitors have been approved in different countries, namely udenafil, mirodenafil, lodenafil and avanafil (Patel et al., 2014; U.S Food And Drug Administration (FDA), 2012). The chemical structures of these approved PDE-5 inhibitors are presented in Fig. 2.1.

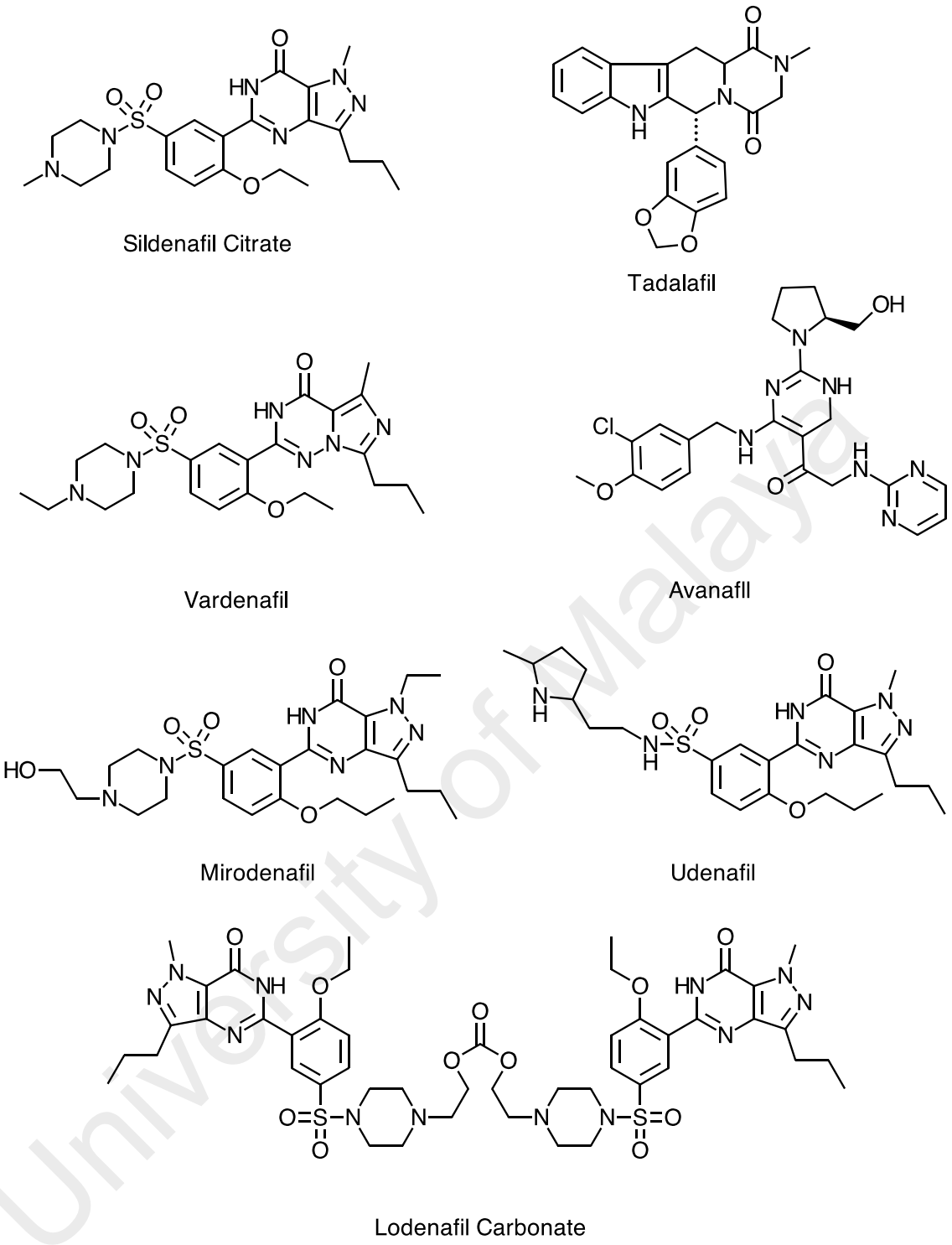

Figure 1.1 Structure of approved PDE-5 inhibitors

HHSs may not only be adulterated with approved PDE-5 inhibitors but also unapproved analogues. Such analogues are obtained through chemical modification of

the parent compounds and so may differ in terms of potency, side effects and toxicity (Venhuis & de Kaste, 2012). Hence continuous monitoring of HHSs by the drug regulatory authorities is necessary as adulteration poses a significant threat to the health of the general public (Ng, Law, Cheung, Ng, & Choi, 2010).

Detection of PDE-5 inhibitors and their analogues in HHSs presents a major challenge to regulatory authorities, as they are complex matrices, which differ greatly from one another. Furthermore, the existence of new and exotic analogues (Patel et al., 2014) adds to the difficulties since general laboratory methods with limited reference standards are usually not adequate to detect them. In addition, purchasing of the steadily increasing number of reference standards for these unapproved analogues is becoming a financial burden to regulatory authorities. In Malaysia, HHSs are required to undergo a series of tests, including testing for adulterants, before they can be registered with the Drug Control Authority of Malaysia for legitimate sale (National Pharmaceutical Regulatory Agency, 2015b). However, adulterated unregistered HHSs are still widely available in the Malaysian market (Jamia, 2006; Pharmaceutical Services Division, 2013).

Currently, the most dominant technique used in the characterisation of PDE-5 inhibitors and its analogues is Liquid Chromatography Mass Spectrometry (LCMS). The sensitivity of MS allows direct investigation of trace levels of adulterants, which eliminates the need for extensive sample preparation procedure (Singh et al., 2009). LCMS is also the principal technique used in the characterisation of unknown analogues (Patel et al., 2014).

This research study was designed to strengthen and further explore the usage of LCMS in analysing sample HHSs from the Malaysian market, which are adulterated with PDE-5 inhibitors and/or their analogues. The specific objectives of this study were:

i. To synthesise ten representative examples of sildenafil analogues

ii. To build a LCMS library database consisting of 61 PDE-5 inhibitors and their analogues

iii. To develop and validate a LCMS method for simultaneous determination of the of 61 PDE-5 inhibitors and their analogues

iv. To screen for adulterant of PDE-5 inhibitors and their analogues in herbal health supplements (HHSs) marketed in Malaysia

CHAPTER 2:

LITERATURE REVIEW

2.1 Herbal health supplements (HHSs)

Supplements are commonly defined as non-drug products that may contain natural ingredients such as herbs, vitamins, minerals or amino acids which are intended for increasing nutritional value to the diet (US Food & Drug Administration, 2015). In Malaysia, under the Sale of Drugs Act 1952, HHSs or traditional medicines are defined as any product, which consists solely of one or more naturally occurring substances of a plant, animal or mineral, or its parts, either in unextracted or crude extract form, and a homeopathic medicine (Legal Research Board, 2014). HHSs are usually presented in pharmaceutical dosage forms, such as capsule, pills, powders, liquids, ointments and others. Health supplements may also be presented in the form of food such as tea bags and drinks (Jamia, 2006; National Pharmaceutical Regulatory Agency, 2015b).

In the last decades, the usage of HHSs has been increasing worldwide (Justa Neves & Caldas, 2015; Rocha et al., 2016). A study conducted by the National Health and Nutrition Examination Survey between 2007-2010, reported that about half of the United States adult population (49%) uses one or more HHSs for a variety of reasons (Bailey, Gahche, Miller, Thomas, & Dwyer, 2013). One of the main reasons is due to the many side effects related to synthetic drugs, thus making people opt for these natural HHSs (Egan, Hodgkins, Shepherd, Timotijevic, & Raats, 2011; Skalicka-Woźniak et al., 2016).

In concordance with the increasing popularity of HHSs, Malaysia government have imposed the Control of Drugs and Cosmetics Regulation 1984 in 1992, in which all HHSs which are manufactured, imported and sold in the country must be registered with the Drug Control Authority (DCA) (Jamia, 2006). Each registered product will be

given a specific product registration number. On the other hand, registration of food will be made based on food-drug interphase classification. Food products that contain less than 80% of food based ingredients and more than 20% active ingredients of natural products shall need to register with the DCA (National Pharmaceutical Regulatory Agency, 2015a).

The type of HHSs offered in the market varies according to its intended purposes. However, most often, the HHSs are labelled or indicated to promote general health. Among the most popular type of HHSs are those for weight loss, increasing sports performance and sexual stimulation (Rocha et al., 2016). In this study, the focus is solely created for sexual stimulation HHSs.

The tremendous popularity of HHSs as being 'natural' and 'healthier' is due to the common belief that they are only formulated with naturally occurring ingredients such as plant extracts. However, this may not necessarily be true. Question regarding HHSs safety matter to the consumers has very much been an issue especially in the aspect of adulteration (Rocha et al., 2016; Skalicka-Woźniak et al., 2016).

HHSs marketed for sexual stimulation are very well known as a promising market, especially for men with erectile dysfunction (ED). However, adulteration of these HHSs has always been an issue and often reported in previous studies. The resulting consequences of such adulteration may impose danger to the consumers.

ED is defined as a consistent inability to achieve an erect penis sufficiently for fulfilling sexual performance (Wright, 2006). A survey called Massachusetts male aging study which was conducted from 1987-1989, reported that the prevalence of ED increases with age (Feldman, Goldstein, Hatzichristou, Krane, & McKinlay, 1994). According to the United States Food and Drug Administration (US FDA), it is

estimated that 30 million men are affected by ED in the United States (U.S Food And Drug Administration (FDA), 2012). A study conducted in 1999, reported that in the year of 1995, Asia has the highest prevalence of ED, about 86.9 million men as compared to the total estimation of over 152 million men with ED worldwide. The study reported that ED is likely to continue growing to approximately 322 million men worldwide, in the year of 2025, with Asia still occupying the largest number (Aytac, McKinlay, & Krane, 1999).

During the early years, the treatment of ED was complicated due to the limited understanding of the whole erection process. Some of the early treatments include penile implants and vacuum devices. However, the discovery of oral PDE-5 inhibitors has completely revolutionised the management of ED (Alwaal, Al-Mannie, & Carrier, 2011). These ED drugs work by inhibiting PDE-5 enzyme from degrading cyclic guanosine monophosphate (cGMP). Increased level of cGMP will lead to smooth muscle relaxation, increase blood flow to the penis and hence an erection will occur (Singh et al., 2009; Skalicka-Woźniak et al., 2016).

Cyclic nucleotide PDE-5 inhibitors become the recommended first line treatment for ED in most men, specifically those without any contraindication to their use. The first FDA approved synthetic PDE-5 inhibitors is sildenafil citrate (Viagra) by Pfizer in 1998. Two other PDE-5 inhibitors, vardenafil hydrochloride (Levitra) and tadalafil (Cialis) (Zou, Oh, Hou, Low, & Koh, 2006) were then approved by FDA in 2003. In recent years, four new PDE-5 inhibitors have been developed and approved in different countries. Udenafil (Zydena) was found to be an effective and well-tolerated treatment for ED by a study conducted in Korea (Paick et al., 2008). Udenafil is currently available in Korea, Russia and Malaysia. Mirodenafil (Mvix) has been approved in South Korea since 2007.Lodenafil (Helleva) is a PDE-5 inhibitor that has been

developed and approved in Brazil. The most recent PDE-5 inhibitor approved by FDA is avanafil (Stendra) in April 2012, which are reported to be fast acting and highly selective (Alwaal et al., 2011; Codevilla, dos Santos Castilhos, & Bergold, 2013; DeNoon, 2012; Gratz, Flurer, & Wolnik, 2004; Patel et al., 2014).

PDE-5 inhibitors oral agents have been reported to be safe, effective and easy to administer (Eardley et al., 2010; Hatzimouratidis & Hatzichristou, 2007). Legitimate prescription is required to use these drugs in many countries (Ng et al., 2010; Rocha et al., 2016).

The success of PDE-5 inhibitors in treating ED was increasing tremendously ever since it was introduced into the market. Men without ED are also using PDE-5 inhibitors for recreational purposes, along with alcohol and other drugs (Bechara, Casabe', De Bonis, Helien, & Bertolino, 2010; Shaeer, 2012). The sale of sildenafil oral tablet reached more than 400 million US dollars during its first quarter on the US market (Keith, 2000). This proves that the ED drugs sales are profitable and promising.

2.1.1 Analogues of PDE-5 inhibitors

Due to the success of synthetic PDE-5 inhibitors drugs, more and more HHSs have been made available in the market with claims of treating ED and improving men's sexual life 'naturally' with only natural ingredients. These HHSs are being marketed as 'safe' alternatives for any consumers including those with cardiac problems, despite the fact that PDE-5 inhibitors are contraindicated with organic nitrates. Another factor contributing to the popularity of these HHSs is the exaggerated marketing health claims through advertisement, which most likely are not scientifically proven. ED itself is usually related to emotional distress and shame, which may lead the patient to self-treatment with these HHSs. However, more often not, from previous studies and reports, most of these illicit HHSs have been found to be intentionally formulated with

synthetic PDE-5 inhibitors and their structurally modified analogues (Campbell et al., 2013; Gilard et al., 2015; Patel et al., 2014; Rocha et al., 2016; Venhuis & de Kaste, 2012).

Analogues are compounds that resemble the parent structure of approved PDE-5 inhibitors but have minor modification in certain parts. According to Venhuis & de Kaste (2012), almost all known analogues are those that have been previously disclosed in the patent literature, which are publicly accessible. This opens the doors to the creation of an unlimited number of analogues. Recent studies have revealed that there are more than 50 unapproved analogues of PDE-5 inhibitors reported as adulterants and the number is still increasing (Patel et al., 2014).

2.1.1.1 Sildenafil analogues

The first reported adulterant was homosildenafil, a sildenafil analogue which was detected in a functional food beverage in Korea in 2003 (Shin, Hong, Kim, Lee, & Jeoung, 2003). The following year, two more sildenafil analogues were reported, namely acetildenafil and hydroxyhomosildenafil (Blok-Tip et al., 2004). The structure of acetildenafil is different compared to other analogues, in that it has an acetyl group in place of the sulphonyl group of sildenafil. Subsequently, more acetyl-type analogues were discovered, such as hydroxyacetildenafil and noracetildenafil (Hou, Zou, Low, Chan, & Koh, 2006; Reepmeyer & Woodruff, 2007). In recent years, new and more exotic sildenafil analogues have been discovered, for example, thiohomosildenafil, thiodimethylsildenafil, dithiodesmethylcarbodenafil, propoxyphenylthiohydroxy-homosildenafil and propoxyphenylsildenafil. Thio analogues involve changing of the oxygen substituent of the pyrimidine ring to sulphur, whereas propoxy analogues involve exchange of the ethoxy substituent on the phenyl ring with a propoxy

substituent (Alp, Coskun, & Goker, 2013; Balayssac, Gilard, Zedde, Martino, & Malet-Martino, 2012; Ge, Li, Koh, & Low, 2011; Zou, Hou, Oh, Chong, et al., 2008).

Nevertheless, the most common adulterants reported are sildenafil and its analogues. The reason is that these analogues can be synthesised from a sildenafil intermediate, which is readily available and cheap. The synthetic route is also straightforward and freely available from patent literature (Bell & Terrett, 1993; Patel et al., 2014; Venhuis & de Kaste, 2012)

2.1.1.2 Tadalafil analogues

Tadalafil and its analogues are the second most reported adulterant, the reason being that the synthesis of tadalafil and its analogues requires the use of piperonal, which is essential for their pharmacological activity (Daugan et al., 2003; Venhuis & de Kaste, 2012). However, piperonal is closely monitored as it is also used in the production of ecstasy, thus limiting its availability (Bohn, Bohn, & Blaschke, 1993).

Aminotadalafil was the first analogue of tadalafil to be discovered (Gratz et al., 2004; Zou, Hou, Low, & Koh, 2006). Patel et al. (2014) claims that up to 2014, only nine types of tadalafil analogues have been reported in adulterated HHSs. However, recently, there has been an increase in the number of reports on new type of tadalafil analogues in adulterated HHSs. Among the latest type of tadalafil analogues being reported are 2-hydroxyethylnortadalafil (Kern, Nickum, Flurer, Toomey, & Litzau, 2014), diethylaminopretadalafil (Zhang, Yu, Wu, & Li, 2014), *trans*-bisprehomotadalafil (Lee, Kim, Mandava, et al., 2015), homotadalafil (Lee, Kim, Noh, et al., 2015), cyclopentyltadalafil, *trans*-cyclopentyltadalafil, *trans*-bisprecyclopentyltadalafil (Lee, Mandava, Baek, & Lee, 2015) and *N*-cyclopentylnortadalafil (Xu, Kee, Ge, Low, & Koh, 2016).

2.1.1.3 Vardenafil analogues

Vardenafil and its analogues are the least common type of analogues reported as adulterants. This might be due to the fact that they lack of any significant pharmacological advantages over sildenafil analogues (Venhuis & de Kaste, 2012). Among the few vardenafil analogues reported are acetylvardenafil and hydroxythiovardenafil (Jankovics, Lohner, Darcsi, Németh-Palotás, & Béni, 2013; Lee, Kim, Jang, Kwon, & Lee, 2011).

2.1.2 Cases of HHSs adulteration: Prevalence, danger and law

The growing trend of intentional adulteration of PDE-5 inhibitors and their analogues in HHSs is a worldwide concern. United States Food and Drug Administration (FDA) reported that from 2007 till 2014, out of 572 adulterated supplements found in the country, 41.6% of them were in the sexual enhancement category (Justa Neves & Caldas, 2015). Pfizer, the licensed manufacturer of sildenafil have conducted a study in 2013 on 91 HHSs around USA which claim to treat ED naturally and revealed that 81% were found to be adulterated (Campbell et al., 2013). A report of illegal sexual stimulation products seized in the Netherlands from 2007-2010, showed that, out of 538 products, 98% of them were adulterated with PDE-5 inhibitors and their analogues (National Institute for Public Health and the Environment, 2011). Sixty-one percent out of 150 HHSs sampled in France between March 2011 and March 2014 were reported to be adulterated (Gilard et al., 2015). Singapore conducted raids in two of its red-light districts in 2008 and out of 175 illegal sexual enhancement HHSs seized, 70% of them were found to be adulterated with sildenafil (Low et al., 2009). Statistics from Malaysia Ministry of Health (MOH) showed that from 2012 until June 2013, 46% of the HHSs tested for adulteration were adulterated with PDE-5 inhibitors and their analogues (Mohammad, 2013).

Such adulteration is a significant danger to the society. Approved PDE-5 inhibitors have been documented to produce side effects such as headache, flushing, nasal congestion, dyspepsia and back pain. Severe decrease in blood pressure and syncope may also be experienced by consumers as a result of negative interaction between PDE-5 inhibitors and co-administered nitrates or α-blockers (Gur et al., 2013). Therefore, health risks are posed to patients with cardiovascular diseases when they take these illegal adulterated HHSs together with nitrates (Patel et al., 2014). Analogues of PDE-5 inhibitors may produce a higher pharmacological and toxicological risk to the consumer because their safety and toxicity profile are mostly unknown. Another aspect that may harm the consumer is related to the quality of the HHSs, as their production is not controlled (Justa Neves & Caldas, 2015; Rocha et al., 2016). A study conducted by Keizers, Wiegard and Venhuis (2016) on illegal active raw material of sildenafil disclosed that the quality of the compound itself is not at par with European Pharmacopoeia standards, putting an increase in the risk of side effects and overdosing.

Adverse events have been reported following consumption of adulterated HHSs. In 2008, fatal cases following consumption of illegal erectile dysfunction HHSs were reported in Singapore. The HHSs were found to contain varying ratios of PDE-5 inhibitors and glibenclamide (Kao, 2009; Low et al., 2009). Recent reports have revealed that the same HHSs are still available in the market (Kuramoto, Yabe, Kurose, & Seino, 2015). A 28-year-old man in Hong Kong suffered from ataxia after taking an acetildenafil adulterated HHSs (Poon, Lam, Lai, Chan, & Mak, 2007). As for Malaysia, a product called Maca Tongkat Ali, which was adulterated with tadalafil, was reported to cause myalgia, joint stiffness and pain in the limbs. Another product, Vimax Capsule, which was adulterated with sildenafil, was reported to cause dizziness and eye pain after consumption (Basmiah, 2013).

In Malaysia, PDE-5 inhibitors and their analogues are listed as a Group B poison under the First Schedule of poison list, controlled under Poison Act 1952 (Legal Research Board, 2014). According to the regulation, Group B poisons are only to be sold and supplied by authorised persons such as licensed pharmacists and registered medical practitioners in concordance with their professional work needs in providing medical treatment. Any unauthorised person who is in possession of these poisons for illegal matters is punishable under this Act.

Malaysia MOH has a Pharmacy Enforcement Division, which is specifically assigned to conduct investigation and raids of registered and unregistered medicines, including HHSs in premises, supplier, store, manufacturer and the country entry point. The Pharmacy Enforcement Division has joined forces with more than 100 countries around the world in operation Pangea, which is coordinated by Interpol to combat fake and illegal medicines (Noor, 2016). Products suspected of being adulterated, substandard and counterfeit worth up to almost 37 million Ringgit Malaysia were seized throughout 2015 (Bahagian Perkhidmatan Farmasi, 2015). For adulteration cases, the seized products are then subjected for laboratory test to detect any illegal compounds.

One of the main institutions that perform tests for adulterant in Malaysia is the National Pharmaceutical Regulatory Agency (NPRA). In order to cope with the increase of adulterated samples, NPRA has improved its test method from time to time. However, dealing with complex matrices and the existence of so many PDE-5 inhibitors analogues has presented great challenges to NPRA. This study was developed to reduce or curb these difficulties by improving existing methods in the laboratory.

2.2 Techniques used for detecting adulteration

The choice of techniques employed in detecting PDE-5 inhibitors and their analogues in adulterated HHSs depends on the complexity of the HHSs matrices and sensitivity of

the equipment to detect a wide range of adulterants. Singh et al. (2009) have reviewed four different types of techniques in detail; thin-layer chromatography (TLC), ultraviolet (UV) spectroscopy, high-performance liquid chromatography (HPLC) and liquid chromatography mass spectrometry (LCMS). Patel et al. (2014) have made a more extensive review of more than 30 techniques, though not all the techniques are frequently used in the detection of PDE-5 inhibitors adulteration in HHSs. The techniques could be classified into three different large groups, which are, the chromatographic methods, spectroscopic methods and mass spectrometry (Rocha et al., 2016).

2.2.1 Chromatographic methods

TLC has been used as a preliminary testing of known PDE-5 inhibitors and their analogues in products. Kumasaka et al. (2008) have used TLC as a preliminary tool to detect a new analogue, xanthoantrafil. A characteristic yellowish spot was detected from the analysis and used as an indicator (Kumasaka, Kawahara, Doi, Kojima, & Goda, 2008). Cai et al. (2010), demonstrated the use of TLC in identifying PDE-5 inhibitors and their analogues in 36 HHSs. Eight were found to be adulterated, and TLC screening was made against 8 known reference standards. The authors summarised that TLC is a simple technique for rapid identification of PDE-5 inhibitors and their analogues in HHSs (Cai et al., 2010). It is clearly known that TLC method is easy and cheap but the existence of known reference standards is mandatory and furthermore, it has very low sensitivity. This makes TLC method less favourable for screening adulterants, especially new analogues, when reference standards are not available (Patel et al., 2014; Rocha et al., 2016).

HPLC has been used extensively in the detection of PDE-5 inhibitors or their analogues. The first analogue ever reported, homosildenafil, proposed the use of HPLC

in its detection (Shin et al., 2003). HPLC works by matching relative retention time between reference standards and the compound of interest in the HHSs (Singh et al., 2009). Fejos et al. (2014) demonstrated the use of HPLC with UV detection for simultaneous determination of sildenafil, tadalafil, vardenafil and 11 analogues (Fejos, Neumajer, Beni, & Jankovics, 2014). HPLC is easy to use with high resolution and selectivity (Deconinck, Sacré, Courselle, & De Beer, 2013). Although HPLC can be used as a good identification tool, its usage is still limited to the availability of reference standards (Patel et al., 2014).

2.2.2 Spectroscopic methods

Raman spectroscopy is usually used in combination with infrared (IR) and near infrared (NIR) for detection of known analogues. A substantial library of reference materials is required to perform screening using these techniques (Venhuis & de Kaste, 2012).

On the other hand, nuclear magnetic resonance (NMR) is considered as one of the powerful tools to elucidate known and unknown analogues of PDE-5 inhibitors. The technique may require simpler sample preparation but usually requires a large quantity of sample and is known to be less sensitive as compared to mass spectrometry (MS) (Patel et al., 2014). Mustazza et al. (2014) characterised 16 sildenafil analogues using NMR. In another study, 150 dietary supplements marketed for sexual enhancement in France were analysed using NMR for detection and quantification of adulterant (Gilard et al., 2015).

2.2.3 Mass spectrometry (MS)

With the advancement of technology, several different types of MS detectors have been made available, such as triple quadrupole, orbitrap, ion trap (IT) and time of flight (TOF). MS is extensively used in hyphenated techniques such as gas chromatography

mass-spectrometry (GCMS) and LCMS. Tandem MS where two or more MS detectors are combined together is widely used in current studies (Patel et al., 2014; Rocha et al., 2016). Among the available tandem mass spectrometry are quadrupole-orbitrap (Q-orbitrap), quadrupole-TOF (Q-TOF) and IT-TOF (Lee, Ji, Park, & Chung, 2015; Roh et al., 2011; Shi et al., 2014)

GCMS is considered to be a rapid, sensitive and accurate technique but there have been a very limited number of studies that have employed the use of GCMS in the detection of PDE-5 inhibitors and their analogues adulteration in HHSs. The reason is due to the thermal instability of PDE-5 inhibitors and their analogues (Patel et al., 2014). In 2009, a group of researchers from Malaysia disclosed a method for detection of sildenafil, vardenafil and tadalafil using GCMS. Their method was employed to screen 25 HHSs, among which 11 were found to be adulterated (Man, Nor, Lajis, & Harn, 2009). The same group further explored the usage of GCMS by analysing 3 different sildenafil analogues in 2011 (Man, Noor, & Lajis, 2011). In the latest study, they showed the use of GCMS triple quadrupole in analysing sildenafil and 5 of its analogues (Mokhtar et al., 2016).

The most dominant hyphenated technique in the characterisation of adulterants is LCMS. It has been proven to provide the highest sensitivity and can separate target compounds in complex matrices without the need of extensive sample preparation. The fragmentation patterns of known PDE-5 inhibitors and their analogues provide a very good tool for detecting adulterants in HHSs (Singh et al., 2009; Venhuis & de Kaste, 2012). An extensive number of studies have employed the usage of LCMS for screening both known and unknown analogues of PDE-5 inhibitors and also for structural elucidation of new unknown analogues.

One study reported the characterisation of three sildenafil analogues, hydroxyhomosildenafil, homosildenafil and acetildenafil in HHSs by LCMS (Blok-Tip et al., 2004). Noracetildenafil were first detected and elucidated by a few techniques including LCMS (Reepmeyer & Woodruff, 2007). Propoxyphenyl isobutyl aidenafil was structurally elucidated using high-resolution orbitrap mass spectrometry (Kee et al., 2014).

LCMS has also been used for simultaneous determination of PDE-5 inhibitors and their analogues. Zou et al. (2006) reported the method of simultaneous determination of 3 PDE-5 inhibitors and 3 sildenafil analogues using liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS). LC-ESI-MS/MS has also been used by Lee et al. (2013) to simultaneously determine 38 PDE-5 inhibitors and their analogues. A study employing the usage of LTQ Orbitrap was reported to simultaneously determine 71 PDE-5 inhibitors and their analogues in one run (Lebel, Gagnon, Furtos, & Waldron, 2014).

The extensive advantages of LCMS in detecting adulterants in HHSs have led us to choose this technique. LCMS IT-TOF available in NPRA has been utilised for the purpose of this work. IT-TOF detector enables MS^n data measurement with high resolution and mass accuracy.

CHAPTER 3:

SYNTHESIS OF SILDENAFIL ANALOGUES

3.1 Introduction

This chapter describes the synthesis and purification of ten different sildenafil analogues. These compounds include propoxy, ethoxy and thio analogues with variations in the sulfonamide moiety and an acetyl analogue.

The synthetic route of most currently known analogues has been revealed previously in the patent literature. During drug discovery, which is the first stage of drug development, quite a number of compounds will be synthesised and tested to obtain a compound with optimal activity. This is what exactly happened during the development of sildenafil. Structural variations were made to the parent compound to get the final product, which is sildenafil and the rest of the compounds, known as analogues, were discarded after clinical evaluation. Morpholino-acetildenafil, homosildenafil and hydroxyhomosildenafil were among the analogues competing with sildenafil to be the active ingredient of Viagra (Bell & Terrett, 1993; Venhuis & de Kaste, 2012).

The existence of new and exotic analogues as adulterants and purchasing of the steadily increasing number of reference standards for these unapproved analogues is becoming a financial burden to regulatory authorities. In order to develop the capability to produce additional PDE-5 inhibitor standards for the ever-increasing number of new analogues being used as adulterants, ten known sildenafil analogues were synthesised in-house (with numbering following alphabetical order as shown in Table 4.1), namely dimethylsildenafil (**13**), *N*-desmethylacetildenafil (**30**), *N*-desmethylsildenafil (**31**), propoxyphenylhydroxyhomosildenafil (**42**), propoxyphenylsildenafil (**43**), propoxyphenylthiodimethylsildenafil (**44**), propoxyphenylthiohomosildenafil (**45**), propoxythio-*N*-desmethylsildenafil (**48**), thio-*N*-desmethylsildenafil (**54**) and

thiodimethylsildenafil (**55**). The synthesised compounds were solely used for research and validation purposes only.

3.2 Experimental

3.2.1 Synthetic Route

Synthesis was performed in accordance with the procedures in the patent literature (Bell, Brown, & Terrett, 1995; Bell & Terrett, 1993; Kim et al., 2004). Structural variations to produce many different analogues are possible with this synthetic route, which is presented in Fig. 3.1.

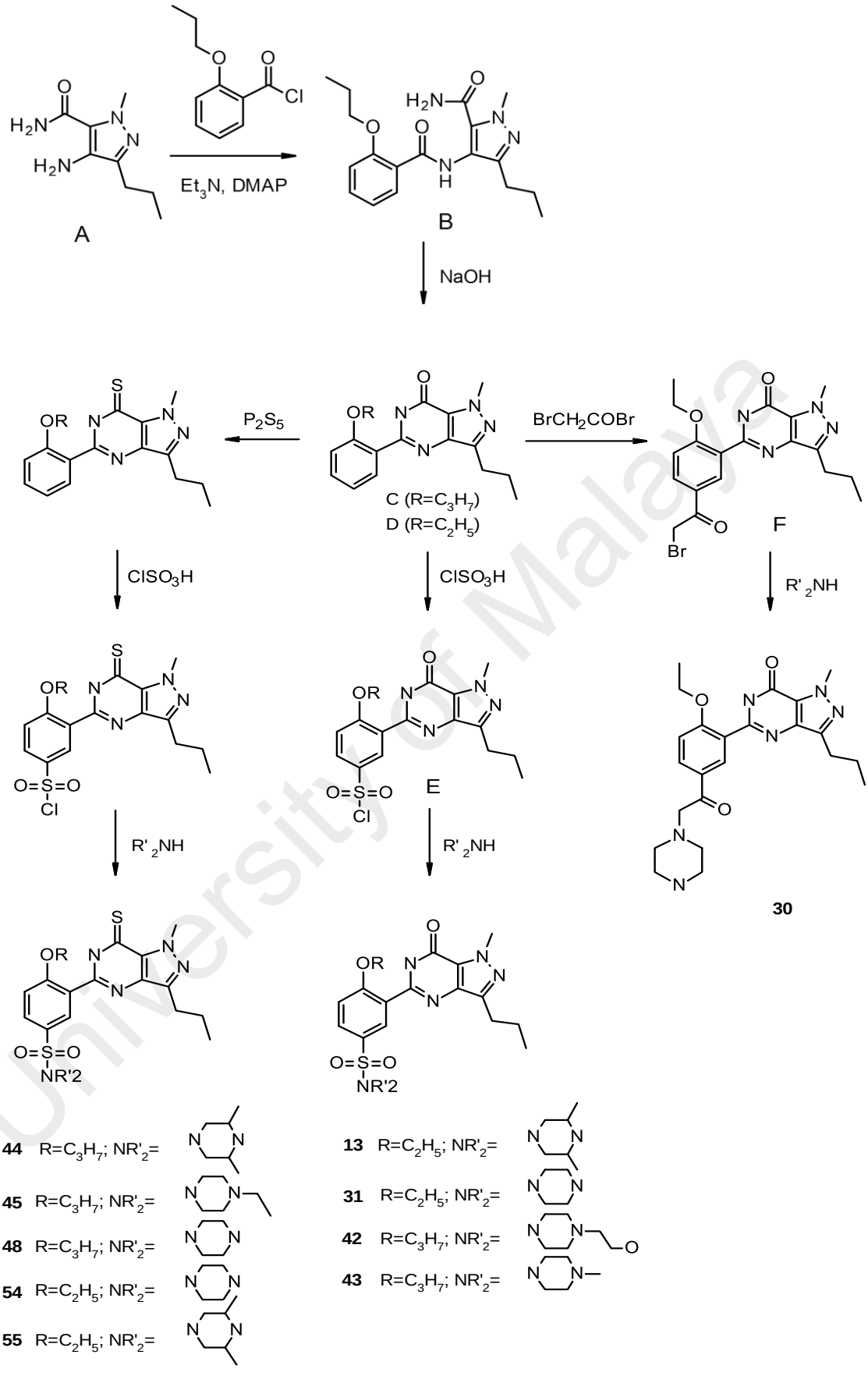

Figure 3.1 Synthetic route for synthesis of sildenafil analogues

3.2.2 Chemicals

The following amines were used; piperazine, 1-methylpiperazine, 1-ethylpiperazine, 1-(2-hydroxyethyl) piperazine), 2,6-dimethylpiperazine and sildenafil intermediates, 4-(2-propoxybenzamido)-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide and 5-(2-ethoxyphenyl)-1-methyl-3-*n*-propyl-1,6-dihydro-7*H*-pyrazolo[4,3-*d*]-7-pyrimidinone. All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chemicals and solvents used were all of reagent grade and were used without further purification.

3.2.3 Method

3.2.3.1 Propoxy analogues

Step 1: Acylation of compound **A**, 4-(2-propoxybenzamido)-1-methyl-3-propyl-1*H*-pyrazole-5-carboxamide (900 mg), was conducted by stirring compound **A** with dichloromethane, triethylamine and 4-dimethylaminopyridine at room temperature for 10 to 20 minutes. 2-n-propoxybenzoic chloride was then slowly poured into the mixture and the resulting solution was stirred overnight at 0°C. The solution was then extracted with dichloromethane and dilute hydrochloric acid (1M), dried with sodium sulphate and evaporated under vacuum. Purification was performed on the crude product by column chromatography (dichloromethane: methanol, 9:1).

Step 2: Cyclisation of compound **B** was achieved by refluxing a mixture of compound **B**, sodium hydroxide, 30% hydrogen peroxide, water and ethanol at 90°C for 3 hours. The resulting solution was neutralised with 2N hydrochloric acid, extracted with dichloromethane, dried with sodium sulphate and evaporated under vacuum. Purification was performed on the crude product by column chromatography (hexane: ethyl acetate, 1:1).

Step 3: Portionwise addition of the carboxamide compound **C** into chlorosulphonic acid under stirring condition at 0°C, yielded 4-propoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*–pyrazolo [4,3-d]pyrimidin-5-yl)benzene-1-sulfonyl chloride (compound **E**). The solution was stirred overnight and further extracted with dichloromethane: methanol (9:1) and washed with ice water before being dried with sodium sulphate and evaporated under vacuum.

Step 4: A mixture of compound **E** and an appropriate amine in anhydrous ethanol was stirred for 1 to 2 hours at room temperature. The solution was then dried with sodium sulphate and evaporated under vacuum. The crude product was purified by column chromatography (dichloromethane: methanol, 9:1). 1-(2-hydroxyethyl) piperazine and 1-methylpiperazine were used to produce propoxyphenylhydroxyhomosildenafil (**42**) and propoxyphenylsildenafil (**43**), respectively.

3.2.3.2 Ethoxy analogues

The synthetic route to the ethoxy analogues, followed the same chlorosulphonylation and alkylation steps 3 and 4 in section 3.2.3.1, but starting with the commercially available compound 5-(2-ethoxyphenyl)-1-methyl-3-n-propyl-1,6-dihydro-1*H*-pyrazole[4,3-d]-7-pyrimidinone (Compound **D**) (100 mg). Piperazine and 2,6-dimethylpiperazine were used to produce dimethylsildenafil (**13**) and *N*-desmethylsildenafil (**31**), respectively.

3.2.3.3 Thio analogues

The respective compounds **C** and **D** were refluxed with phosphorus pentasulphide in toluene for one hour. The resulting solution was left to dry overnight then extracted with dichloromethane and washed with sodium hydroxide solution before being dried with sodium sulphate and evaporated under vacuum. Thio analogues were obtained by the

same chlorosulphonylation and alkylation steps 3 and 4 in section 3.2.3.1. Piperazine, 2,6-dimethylpiperazine and 1-ethylpiperazine were used to produce propoxyphenylthiodimethylsildenafil (**44**), propoxyphenylthiohomosildenafil (**45**), propoxythio-*N*-desmethylsildenafil (**48**), thio-*N*-desmethylsildenafil (**54**) and thiodimethylsildenafil (**55**).

3.2.3.4 Acetyl analogues

Chloroacetylation was perfomed by adding aluminium chloride portionwise to compound **D (100 mg)** and bromoacetylbromide in dichloromethane at 0°C. The mixture was stirred overnight, extracted with ice water and dichloromethane and then dried and evaporated in vacuum to produce compound **F**. A mixture of compound **F**, potassium carbonate and piperazine in acetonitrile was stirred for 1 to 2 hours at room temperature. The solution was then dried and the crude product was purified through column chromatography (hexane: ethyl acetate, 7:3) to give *N*-desmethylacetildenafil (**30**).

3.2.4 NMR analysis

The synthesised compounds were dissolved in $CDCl_3$ for NMR analysis. ^{1}H spectra were recorded on a JEOL Lambda 400 FT-NMR spectrometer. ^{13}C spectra were recorded on a Bruker AVN400 FT-NMR spectrometer.

3.2.5 HPLC analysis

An Agilent High Performance Liquid Chromatography (HPLC) was employed for purity evaluation. The analytical column used for separation was Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 μm). The mobile phase consisted of acetonitrile-1m trifluoroacetic acid (50:50) and at a flow rate of 0.8 ml/min. The UV detector wavelength was operated at 254 nm. The total runtime for analysis was 15 minutes.

3.3 Results and discussion

3.3.1 NMR result

The ^{1}H NMR signals of all the synthesised compounds are presented in Appendix A and are consistent with their chemical structures. The ^{1}H NMR spectra for all of the sildenafil analogues are almost identical because the signals of the aromatic moiety are unaffected by the signals from the amine group.

3.3.2 Purity Test by HPLC

All the synthesised compounds were evaluated for their purity. Each compound was directly dissolved in acetonitrile-water (50:50) to 10 µg/ml and analysed using HPLC. The purity of the compounds purity was determined based on the peak area percentage, as this indicates the percentage of the peak components compared to the total detectable peaks in the sample chromatogram. The peak area percentages for the compounds are tabulated in Table 3.1. An example of HPLC chromatogram and UV spectrum for one of the compounds – thiodimethylsildenafil is shown in Figure 3.2.

Table 3.1 Peak area percentage for the synthesised compounds

No	Synthesised Compound	Peak area (%)	Yield (%)
1	Dimethylsildenafil (13)	98.5%	91%
2	*N*-desmethylacetildenafil (30)	99.9%	94%
3	*N*-desmethylsildenafil (31)	98.4%	98%
4	Propoxyphenylhydroxyhomosildenafil (42)	99.9%	75%
5	Propoxyphenylsildenafil (43)	96.9%	77%
6	Propoxyphenylthiodimethylsildenafil (44)	99.9%	90%
7	Propoxyphenylthiohomosildenafil (45)	99.9%	91%
8	Propoxythio-*N*-desmethylsildenafil (48)	99.9%	92%
9	Thio-*N*-desmethylsildenafil (54)	92.9%	92%
10	Thiodimethylsildenafil (55)	99.9%	72%

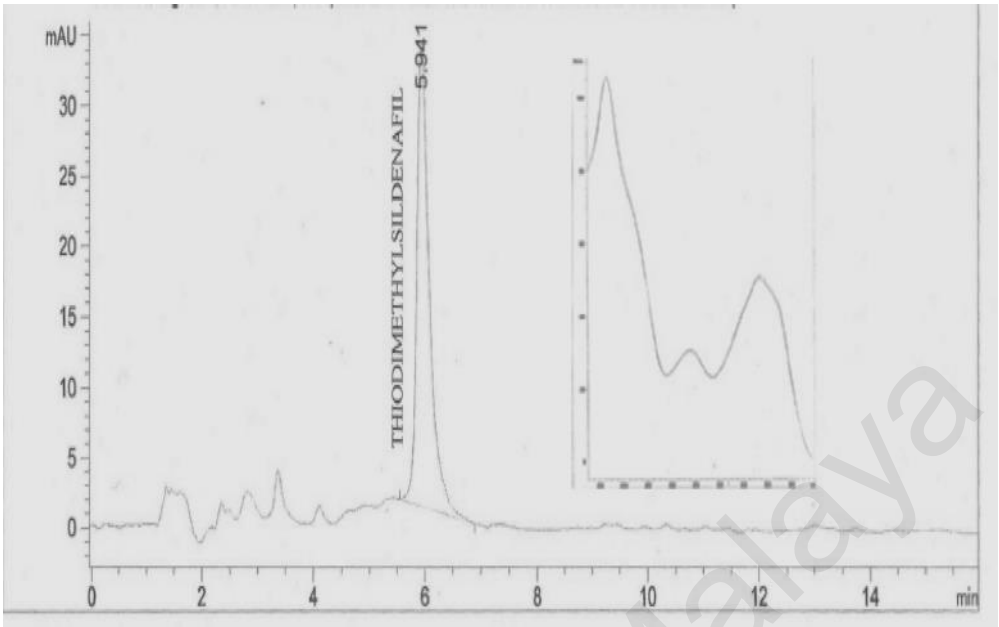

Figure 3.2 HPLC chromatogram and UV spectrum of thiodimethylsildenafil

3.4 Conclusion

The following ten analogues of sildenafil were successfully synthesised in-house: dimethylsildenafil (**13**), *N*-desmethylacetildenafil (**30**), *N*-desmethylsildenafil (**31**), propoxyphenylhydroxyhomosildenafil (**42**), propoxyphenylsildenafil (**43**), propoxyphenylthiodimethylsildenafil (**44**), propoxyphenylthiohomosildenafil (**45**), propoxythio-*N*-desmethylsildenafil (**48**), thio-*N*-desmethylsildenafil (**54**) and thiodimethylsildenafil (**55**). These analogues will be used for building the LCMS library database and structural confirmation by comparing the fragment ion patterns of the sample and the synthesised analogues.

CHAPTER 4:

SPECTRAL LIBRARY DATABASE AND LIQUID CHROMATOGRAPHY MASS SPECTROMETRY ION TRAP TIME-OF-FLIGHT METHOD DEVELOPMENT

4.1 Introduction

This chapter describes the utilisation of LCMS to build a spectral library and also to develop a simultaneous determination method for PDE-5 inhibitors and their analogues. A LCMS spectral library database consisting of 61 compounds of PDE-5 inhibitors and their analogues were created by standard analysis. This database includes retention times and accurate mass ion fragmentation of the compounds. Following that, a LCMS IT-TOF method was developed and validated for simultaneous determination of the 61 compounds.

LCMS has been previously shown to be the best tool for detection of PDE-5 inhibitors and their analogues, both known and unknown (Singh et al., 2009; Venhuis & de Kaste, 2012). The advantages of LCMS in analysing such adulterant have been discussed in detail in section 2.2.3. LCMS produces compound information on accurate masses and ion fragmentation patterns, which can be used to construct an in-house library.

In previous studies, the largest number of PDE-5 inhibitors and their analogues that have been simultaneously detected by LCMS is around 65 compounds (Lebel et al., 2014). However, a number of these compounds are impurities from the synthesis process, such as sildenafil dimer impurity and tadalafil impurity A. Furthermore, some common adulterants were not included, such as propoxyphenylhydroxyhomosildenafil and propoxyphenylthiosildenafil. In this study, a total of 61 compounds were analysed, of which fourteen were not included in the study by Lebel et al.

4.2 Spectral library database development

4.2.1 Experimental

4.2.1.1 Chemicals

HPLC grade acetonitrile and water solutions containing 0.1%v/v formic acid were purchased from Sigma-Aldrich and LCMS-grade methanol was purchased from J.T. Baker.

Fifty-one PDE-5 inhibitor standards were purchased from suppliers as listed below: Eli Lilly - tadalafil; Cachesyn - acetildenafil, acetylvardenafil, benzylsildenafil, chlorodenafil, chloropretadalafil, cinnamyldenafil, cyclopentynafil, depiperazinothiosildenafil, desmethylcarbodenafil, dimethylacetildenafil, dioxohongdenafil, dithiodesmethylcarbodenafil, dithiodimethylcarbodenafil, hydroxychlorodenafil, hydroxyhomosildenafil, hydroxypropylnortadalafil, hydroxythiovardenafil, hydroxyvardenafil, mirodenafil, *N*-butylnortadalafil, *N*-desethylvardenafil, *N*-octylnortadalafil, nitrodenafil, norneovardenafil, nortadalafil, oxohongdenafil, propoxyphenyldimethylisobutylsildenafil, propoxyphenyldimethylsildenafil, propoxyphenylthiosildenafil, propoxyphenyl-thiohydroxyhomosildenafil, pyrazole-*N*-desmethylsildenafil, sildenafil-*N*-oxide, vardenafil oxopiperazine; ClearSynth - vardenafil intermediate (2-(2-ethoxyphenyl)-5-methyl-7-propyl-3*H*-imidazo[5,1-*f][*1,2,4]triazin-4-one)); Pfizer – sildenafil; TLC Pharmachem - aminotadalafil, avanafil, gendenafil, homosildenafil, hydroxyacetildenafil, hydroxythiohomosildenafil, mutaprodenafil, noracetildenafil, norneosildenafil, piperiacetildenafil, pseudovardenafil, thiohomosildenafil, thiosildenafil, udenafil, vardenafil.

Ten sildenafil analogues were synthesised in-house as described previously in Chapter 3. Individual standard stock solutions of 250 μg/ml were prepared in methanol.

Further dilutions to 10 μg/ml were carried out for individual and mixtures of standards. All solutions were stored at -20°C (optimum storage temperature to ensure stability as recommended by suppliers and also used by Lee et al. (2013) and Lee et al. (2015))

4.2.1.2 Equipment

Analysis of individual standard stock solutions was carried out using an LCMS IT-TOF system (Shimadzu, Kyoto, Japan) equipped with an autosampler and quaternary pump. Separation was achieved on a Kinetex C_{18} (2.1 x 100 mm, 2.7 μm XB). Data acquisition and analysis were performed using Shimadzu PostRun analysis software.

4.2.1.3 LCMS IT-TOF method

The operating conditions were adopted from the current NPRA LCMS test method. The analytical column was maintained at 40°C, while the autosampler was maintained at 8°C to avoid sample/standard degradation. The binary mobile phase consisted of 0.1% formic acid in acetonitrile (mobile phase A) and 0.1% formic acid in water (mobile phase B). Separation was achieved using the following gradient elution: 0–0.5 min, 95–90% B; 0.5–1 min, 90–70% B; 1–5.5 min, 70–30% B; 5.5–7 min, 30–10% B; 7–8.5 min, 10–95% B; 8.5–13.5 min, 95% B. The mobile phase was pumped at a flow rate of 0.4 ml/min. The manufacturer default settings for MS parameters were adopted and are as follows: CDL temperature, 200°C; detector voltage, 1.6 kV; nebulising gas flow, 1.5 L/min; drying gas, nitrogen, 188 kPa; heat block temperature, 200°C; ion accumulation time, 30 ms; CID parameters, energy, 50%; collision gas, argon; ESI, positive ionisation mode; mass resolution > 10,000. Two scan events for targeted screening were performed to obtain the MS^2 for each compound. Event 1 was a full scan MS data acquired over a mass range of 10–700 m/z, whereas event 2 involved MS^2 data acquisition through further fragmentation of the precursor ion from the parent compound.

4.2.2 Results and discussion

A library database of 61 PDE-5 inhibitors and analogues, complete with the accurate mass spectra data and retention time for each compound was developed (see Appendix B for the chemical structures of the standards). Table 4.1 summarises the exact fragment ions for each compound. See Appendix A in the supplementary material for the chemical structures of the standards.

Table 4.1 LCMS data for the 61 PDE-5 inhibitor standards included in the spectral library

No	Compound	Chemical formula	Retention time (min)	Precursor ion $[M+H]^+$ (m/z)	Product ions (m/z)
1	Acetildenafil	$C_{25}H_{34}N_6O_3$	6.141	467.2732	297.13, 325.13, 339.17, 353.16, 396.20, 420.22, 439.24.
2	Acetylvardenafil	$C_{25}H_{34}N_6O_3$	5.373	467.2629	341.15, 297.13, 317.19, 355.17, 396.20
3	Aminotadalafil	$C_{21}H_{18}N_4O_4$	8.572	391.1359	135.04, 250.09, 269.10
4	Avanafil	$C_{23}H_{26}CIN_7O_3$	6.729	484.1773	349.13, 375.11
5	Benzylsildenafil	$C_{28}H_{34}N_6O_4S$	9.258	551.2352	377.12, 403.21, 459.17, 508.23
6	Chlorodenafil	$C_{19}H_{21}CIN_4O_3$	13.317	389.1357	285.13, 297.13, 311.11, 361.10
7	Chloropretadalafil	$C_{22}H_{19}CIN_2O_5$	13.683	427.0986	262.08, 278.02, 302.07, 334.10, 395.07
8	Cinnamyldenafil	$C_{32}H_{38}N_6O_2$	9.177	555.3011	339.14, 355.17, 437.22
9	Cyclopentynafil	$C_{26}H_{36}N_6O_4S$	7.324	529.2574	283.12, 311.15, 329.16, 377.13, 404.14, 458.18
10	Depiperazinothio-sildenafil	$C_{17}H_{20}N_4O_4S_2$	11.346	409.0977	257.06, 272.07, 285.08, 301.11, 315.09, 381.07
11	Desmethylcarbodenafil	$C_{23}H_{30}N_6O_3$	5.474	439.2470	311.11, 339.14
12	Dimethylacetildenafil	$C_{25}H_{34}N_6O_3$	6.329	467.2678	297.13, 325.13, 339.17, 353,17, 382.18, 410.21
13	Dimethylsildenafil	$C_{23}H_{32}N_6O_4S$	7.221	489.2230	377.12, 283.12,

					311.15, 313.16, 331.08, 432.17
14	Dioxohongdenafil	$C_{25}H_{30}N_6O_5$	9.381	495.2223	313.11, 285.12, 325.12, 166.09
15	Dithiodesmethyl-carbodenafil	$C_{23}H_{30}N_6OS_2$	9.933	471.1996	343.07, 371.10, 414.14
16	Dithiodimethyl-carbodenafil	$C_{24}H_{32}N_6OS_2$	10.324	485.2016	371.09, 343.06, 428.15, 468.18
17	Gendenafil	$C_{19}H_{22}N_4O_3$	11.805	355.1900	285.13, 298.10, 313.16, 327.14
18	Homosildenafil	$C_{23}H_{32}N_6O_4S$	6.966	489.2255	255.12, 283.12, 311.15, 331.09, 377.13, 461.20
19	Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$	5.865	483.2646	297.13, 311.12, 339.16, 353.15, 396.20, 439.34, 465.25
20	Hydroxychlorodenafil	$C_{19}H_{23}CIN_4O_3$	11.633	391.1419	285.12, 313.12, 345.10, 363.11
21	Hydroxyhomosildenafil	$C_{23}H_{32}N_6O_5S$	6.770	505.2214	377.12, 461.19, 487.21
22	Hydroxypropylnor-tadalafil	$C_{24}H_{23}N_3O_5$	9.463	434.1514	312.13, 250.08, 294.12
23	Hydroxythiohomo-sildenafil	$C_{23}H_{32}N_6O_4S_2$	9.538	521.1984	503.19, 461.19
24	Hydroxythiovardenafil	$C_{23}H_{32}N_6O_4S_2$	7.953	521.1861	315.08, 328.12, 345.12, 393.09, 477.16, 503.18
25	Hydroxyvardenafil	$C_{23}H_{32}N_6O_5S$	5.952	505.2134	283.11, 299.10, 312.15, 329.15, 377.12, 461.192
26	Mirodenafil	$C_{26}H_{37}N_5O_5S$	9.095	532.2552	268.14, 296.13, 344.10, 377.12, 404.16, 419.17, 488.23, 514.24
27	Mutaprodenafil	$C_{27}H_{35}N_9O_5S_2$	9.095	630.2072	377.12, 487.20, 600.22
28	*N*-Butylnortadalafil	$C_{25}H_{25}N_3O_4$	13.314	432.1832	310.15, 262.08, 282.15
29	*N*-Desethylvardenafil	$C_{21}H_{28}N_6O_4S$	5.952	461.1920	299.11, 284.12, 151.08, 312.15, 329.15, 376.10
30	*N*-Desmethylacetildenafil	$C_{23}H_{30}N_6O_3$	5.776	439.2400	297.13, 325.13, 339.18
31	*N*-Desmethylsildenafil	$C_{21}H_{28}N_6O_4S$	6.782	461.1918	283.11, 299.11, 311.14, 329.15,

					377.12
32	*N*-Octylnortadalafil	$C_{29}H_{33}N_3O_4$	17.083	488.2465	250.07, 262.07, 302.08, 366.21
33	Nitrodenafil	$C_{17}H_{19}N_5O_4$	13.661	358.1487	256.09, 284.12, 313.11, 330.12
34	Noracetildenafil	$C_{24}H_{32}N_6O_3$	5.977	453.2607	353.16, 297.13, 325.13, 339.16, 396.20
35	Norneosildenafil	$C_{22}H_{29}N_5O_4S$	15.201	460.2006	283.12, 299.11, 329.16, 347.08, 377.13
36	Norneovardenafil	$C_{18}H_{20}N_4O_4$	7.456	357.1455	151.08, 189.06, 300.08, 313.08, 329.11
37	Nortadalafil	$C_{21}H_{17}N_3O_4$	8.782	376.1200	169.07, 226.09, 254.09
38	Oxohongdenafil	$C_{25}H_{32}N_6O_4$	8.152	481.2510	355.17, 396.20, 410.21, 435.25, 453.26
39	Piperiacetildenafil	$C_{24}H_{31}N_5O_3$	6.514	438.2413	325.12, 297.13, 353.15, 380.20, 408.23
40	Propoxyphenyldimethyl-isobutylsildenafil	$C_{24}H_{34}N_6O_4S_2$	8.846	517.2590	269.14, 297.13, 339.18, 363.11, 405.16
41	Propoxyphenyldimethyl-sildenafil	$C_{24}H_{34}N_6O_4S$	8.029	503.2420	283.12, 325.16, 347.08, 391.14, 446.19
42	Propoxyphenylhydroxy-homosildenafil	$C_{24}H_{34}N_6O_5S$	7.550	519.2270	283.11, 331.08, 349.08, 391.13, 501.21
43	Propoxyphenylsildenafil	$C_{23}H_{32}N_6O_4S$	7.628	489.2248	255.12, 283.12, 325.16, 347.08, 447.18
44	Propoxyphenylthio-dimethylsildenafil	$C_{24}H_{34}N_6O_3S_2$	11.149	519.2191	271.10, 299.10, 315.09, 341.14, 363.06, 407.12
45	Propoxyphenylthiohomo-sildenafil	$C_{24}H_{34}N_6O_3S_2$	10.897	519.2167	299.11, 327.12, 369.17, 393.10, 435.15
46	Propoxyphenyl-thiohydroxyhomosildenafil	$C_{24}H_{34}N_6O_4S_2$	10.447	535.2167	517.21, 299.10, 325.11, 359.12, 475.21
47	Propoxyphenylthio-sildenafil	$C_{23}H_{32}N_6O_5S$	10.633	505.2036	313.10, 329.10, 299.09, 355.15, 379.08, 421.13

48	Propoxythio-*N*-desmethylsildenafil	$C_{22}H_{31}N_6O_3S_2$	10.492	491.1898	299.29, 271.10, 315.09, 341.14
49	Pseudovardenafil	$C_{22}H_{29}N_5O_4S$	12.249	460.1918	151.08, 256.09, 284.12, 299.11, 312.15, 329.15, 377.12
50	Pyrazole-*N*-desmethylsildenafil	$C_{21}H_{28}N_6O_4S$	5.637	461.1900	163.05, 269.10, 285.09, 297.13, 363.11
51	Sildenafil	$C_{22}H_{30}N_6O_4S$	6.814	475.2101	283.12, 311.15, 329.16, 377.13
52	Sildenafil *N*-oxide	$C_{22}H_{30}N_6O_5S$	7.153	491.1801	404.14, 312.15, 331.08, 377.13
53	Tadalafil	$C_{22}H_{19}N_3O_4$	9.701	390.1442	135.04, 240.11, 268.11, 302.08
54	Thio-*N*-desmethylsildenafil	$C_{21}H_{29}N_6O_3S_2$	9.545	477.1733	271.10, 299.09, 315.09, 329.14, 345.13, 363.06, 393.10
55	Thiodimethylsildenafil	$C_{23}H_{32}N_6O_3S_2$	10.180	505.2046	393.10, 299.09, 327.12, 448.15
56	Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$	9.928	505.2029	327.12, 299.10, 355.16, 373.17, 393.10, 421.13
57	Thiosildenafil	$C_{22}H_{30}N_6O_3S_2$	9.662	491.1862	283.07, 312.10, 313.11, 341.14, 407.12
58	Udenafil	$C_{25}H_{36}N_6O_4S$	7.575	517.2508	191.08, 255.12, 283.11, 299.11, 325.16, 347.07, 406.15, 418.15, 474.21
59	Vardenafil	$C_{23}H_{32}N_6O_4S$	6.085	489.2200	284.12, 299.11, 312.15, 339.15, 376.10
60	Vardenafil intermediate (2-(2-Ethoxyphenyl)-5-methyl-7-propyl-3H-imidazo[5,1-f][1,2,4]triazin-4-one))	$C_{17}H_{20}N_4O_2$	9.930	313.1522	151.08, 169.09, 256.08, 285.12
61	Vardenafil oxopiperazine	$C_{21}H_{26}N_6O_5S$	7.577	475.1630	312.15, 299.10, 329.15, 349.09, 377.12

Some of these compounds are isomers and have exactly the same precursor ion and elute at very similar retention times. Examples of such isomers, include propoxyphenylthiosildenafil (**47**), thiodimethylsildenafil (**55**) and thiohomosildenafil (**56)**, which all have precursor ions with m/z 505. These compounds can usually be differentiated by their product ions. However, a method with good resolution needs to be developed in order to simultaneously detect their presence without any false positive results.

4.3 LCMS IT-TOF method development for simultaneous determination of PDE-5 inhibitors and their analogues

4.3.1 Experimental

4.3.1.1 Chemicals

The same chemicals as in Section 4.2.1.1 were used.

4.3.1.2 Standard preparation

The individual stocks solution prepared in Section 4.2.1.1 were used to produce mixtures of standards. The mixtures were further diluted to 10 µg/ml and stored at -20°C.

4.3.1.3 LCMS IT-TOF method

The LCMS system and operating conditions described in Sections 4.2.1.2 and 4.2.1.3 were utilised except for the gradient elution and flow rate. After method optimisation, separation of all the 61 compounds was achieved using the following mobile phase gradient elution: 0–2 min, 90–85% B; 2–4 min, 85–75% B; 4–12.5 min, 75–50% B; 12.5–15.5 min, 50–30% B; 15.5–17 min, 30–90% B; 17–20 min, 90% B. The mobile phase was pumped at a flow rate of 0.35 ml/min.

4.3.1.4 Method validation

Since the aim of this study was to detect and identify PDE-5 inhibitors and analogues, validation was limited to the following parameters: specificity, limit of detection (LOD) and matrix effects. Three different forms of blank samples, pills (small, spherical, Chinese herbal medicine), capsules and coffee powder, were used in the validation process. These samples, which were obtained from NPRA, had all been tested beforehand to ensure that no PDE-5 inhibitors or their analogues were present using the method described in section 4.3.1.3. Ten representative PDE-5 inhibitor standards were selected to validate the method to cover the different classes and structures of the compounds. The standards chosen were avanafil, sildenafil and representative analogues lacking the sulfonamide moiety, acetildenafil (**1**) and desmethylcarbodenafil (**11**), thio analogue, thiodimethylsildenafil (**55**) and propoxy analogue, propoxyphenylsildenafil (**43**), vardenafil (**59**) and its analogue, pseudovardenafil (**49**), and tadalafil (**53**) and its analogue aminotadalafil (**3**).

The blank samples were prepared by 1 g of samples in 10 ml of methanol (blank solution).The standard stock solution used was 0.01 mg/ml. For example, in order to produce a spiked solution of 5 μg/ml, 5 ml of stock solution were diluted into the 10 ml blank solution, resulting in a solution of 5 μg/ml.

Specificity was assessed by observing for peak interferences from other substances in spiked blank samples. Matrix effects were studied by analysing the peak area and retention time differences between spiked blank samples and individual standards. The LOD was determined by the lowest detectable concentration in spiked blank samples at a signal-to-noise ratio of 3 or more (S/N>3). Equation 4.1 was used to convert the LOD value from μg/ml to μg/g.

$$\text{LOD}\left(\frac{\mu g}{g}\right) = \frac{\text{LOD}\left(\frac{\mu g}{ml}\right)}{\text{Concentration of blank sample}\left(\frac{g}{ml}\right)} \qquad \textbf{Equation 4.1}$$

4.3.2 Results and discussion

4.3.2.1 Simultaneous determination method by LCMS

An LCMS IT-TOF method was developed to ensure that the 61 PDE-5 inhibitor standards were separated with good resolution and high sensitivity (see Fig.4.1 until 4.4 for the extracted ion chromatograms of the standards). The total runtime required was 20 minutes after gradient elution profile optimisation. The method previously developed by NPRA (described in section 4.2.1.3) failed to give good separation for most of the isomer compounds. Gradient elution was then changed accordingly to get the best separation. The initial mobile phase was set up to 25% A (acetonitrile). However, this causes early elution of the compounds which in return leads to co-elution of some the compounds, hence poor resolution. Therefore, the initial mobile phase was set up to only 10% A. The runtime was varied, from 8–15 minutes. Test results with the changes in the gradient elution, still showed poor resolution and co-elution of some of the standards, especially for isomeric compounds with the same $[M+H]^+$, such as thiodimethylsildenafil (**55**) and thiohomosildenafil (**56**). Therefore, gradient elution with a total runtime of 20 minutes was selected as it gives the best peak separation. Peak separation with good resolution is important because PDE-5 inhibitors are normally found as adulterants in high abundance, therefore co-eluting peaks may not be clearly observed but rather as one irregular peak due to their overlapping with one another (Lee et al., 2013). Mobile phase A was increased up to 70% and gradually reduced to the initial mobile phase composition of 10% A in order to condition the column. Isocratic hold of the highest mobile phase A composition was not performed as all compounds

have been eluted earlier before reaching the 70% point. Mobile phase A composition at 10% was held constant for 3 minutes to permit column equilibration. Equilibration time was set by multiplying the internal volume of the column. Flow rate was also optimised based on the gradient elution time. Longer gradient elution time requires a faster flow rate to ensure a good peak separation and resolution. The optimised flow rate was 0.35 ml/min.

All instrumental aspects were taken into consideration during the method development. The mobile phase and column were chosen based on their ability to generate good chromatographic peak shape, separation and sensitivity (Lee et al., 2013). 0.1% formic acid acted as the mobile phase additive as the analysis were operated in positive ionisation mode, to produce better peak shape and to increase protonation (University of Illinois at Urbana Cahampaign, 2016; Vaclavik et al., 2014). Three reversed-phase columns were tested for their ability to separate all 61 PDE-5 inhibitors and their analogues. These columns are Poroshell C_{18} (3.0 x 100 mm, 2.7 µm particles) from Agilent; Kinetex C_{18} (2.1 x 100 mm, 1.7 µm PFP) and Kinetex C_{18} (2.1 x 100 mm, 2.7 µm XB) from Phenomenex. Each column was subjected to gradient optmisation. The Kinetex C_{18} (2.1 x 100 mm, 1.7 µm PFP) column showed broad peak shapes and poor resolution, thus was excluded from the list. Analysis with the Poroshell C_{18} column showed good resolution and the performance was at par with that of the Kinetex C_{18} (2.1 x 100 mm, 2.7 µm XB) column, which displayed good peak separation and increased efficiency. The Kinetex C_{18} (2.1 x 100 mm, 2.7 µm XB) column also has a smaller diameter compared to the Poroshell C_{18} column, which is likely to reduce the solvent volume required and increase sensitivity as higher analyte concentrations. Due to these considerations, the Kinetex C_{18} (2.1 x 100 mm, 2.7 µm XB) column was chose.

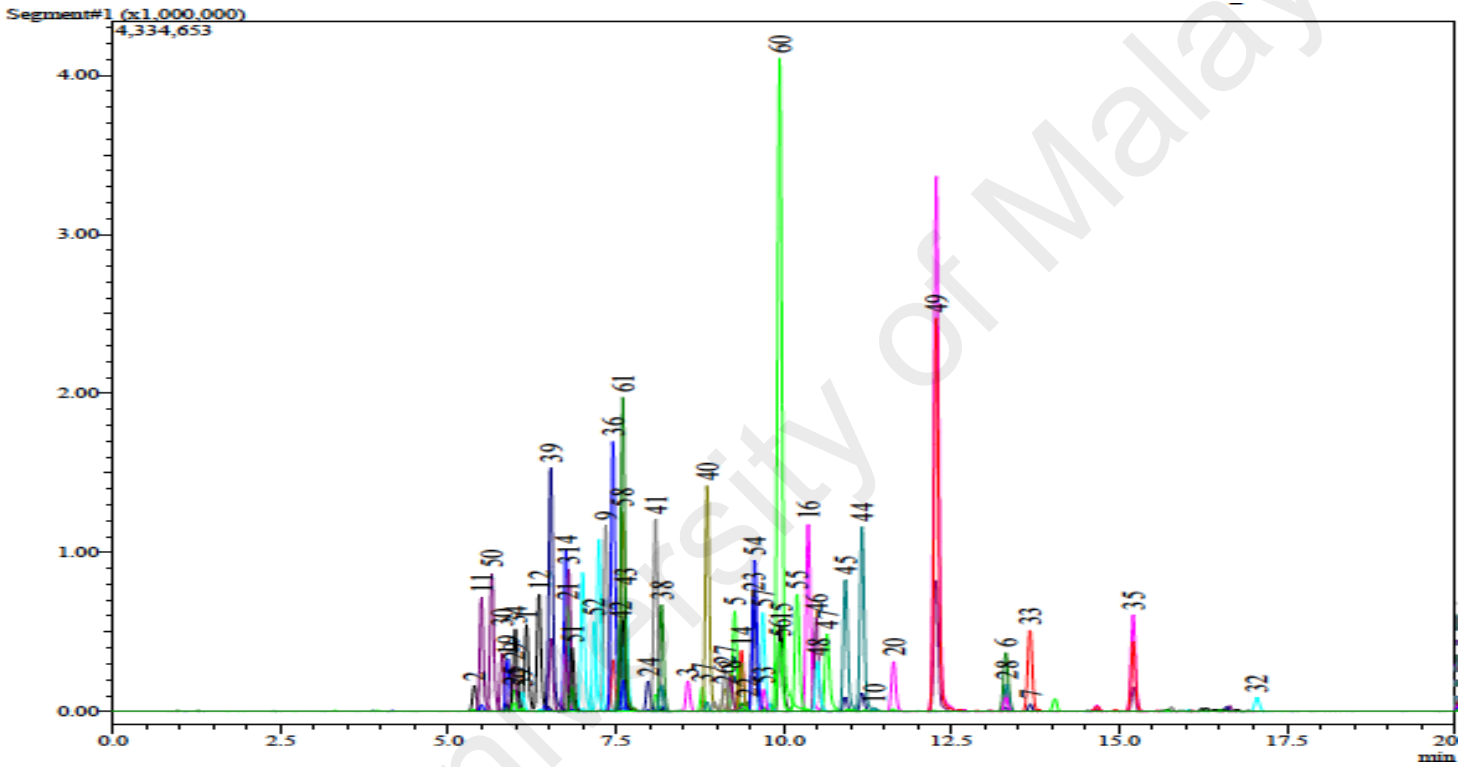

Figure 4.1 Total ion chromatogram of the mixture of 61 standards acquired on the LCMS IT-TOF

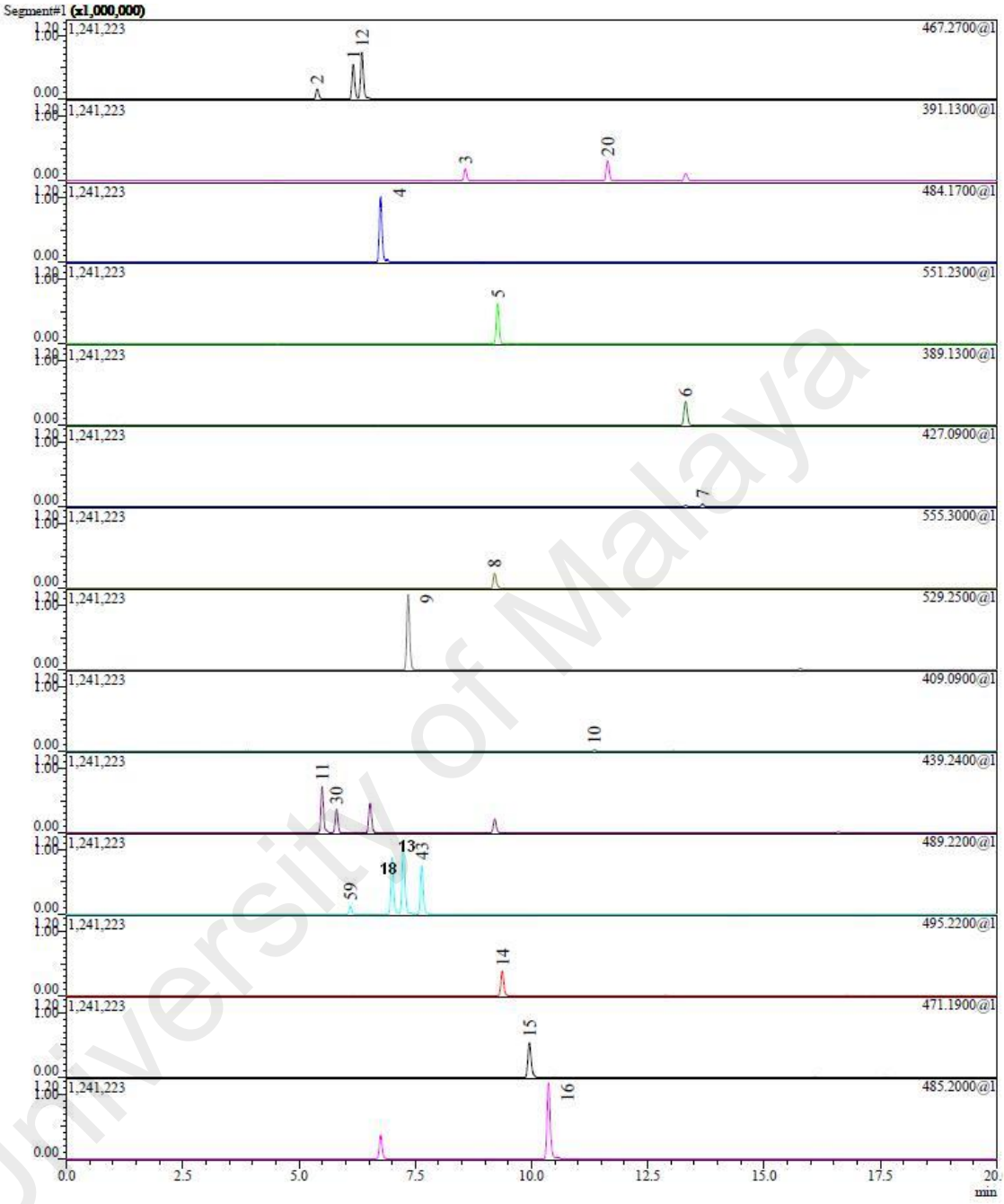

Figure 4.2 Extracted ion chromatograms obtained from the analysis of a mixture of PDE-5 inhibitor standards by LCMS IT-TOF (presented in alphabetical order and numbered as in Table 4.1)

Chromatograms obtained for 1, Acetildenafil; 2, Acetylvardenafil; 3, Aminotadalafil; 4, Avanafil; 5, Benzyl Sildenafil; 6, Chlorodenafil; 7, Chloropretadalafil; 8, Cinnamyldenafil; 9, Cyclopentynafil; 10, Depiperazinothiosildenafil; 11, Desmethylcarbodenafil; 12, Dimethylacetildenafil; 13, Dimethylsildenafil; 14, Dioxohongdenafil; 15, Dithiodesmethylcarbodenfil; 16, Dithiodimethylcarbodenafil; 18, Homosildenfil; 20, Hydroxychlorodenafil; 30, *N*- Desmethylacetildenafil; 43, Propoxyphenylsildenafil; 59, Vardenafil.

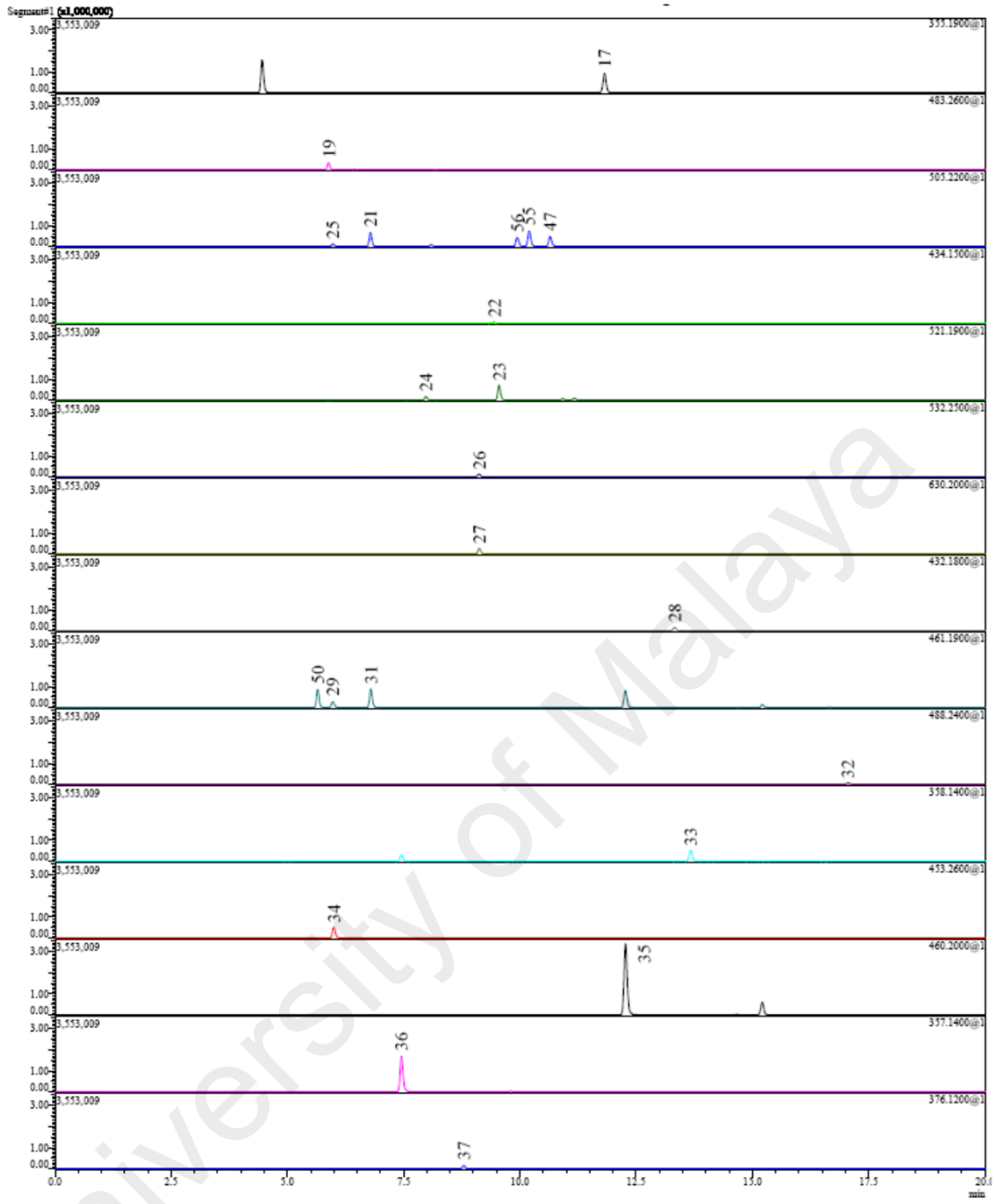

Figure 4.3 Extracted ion chromatograms obtained from the analysis of a mixture of PDE-5 inhibitor standards by LCMS IT-TOF (presented in alphabetical order and numbered as in Table 4.1)

Chromatograms obtained for: 17, Gendenafil; 19, Hydroxyacetildenafil; 21, Hydroxyhomosildenafil; 22, Hydroxypropylnortadalafil; 23, Hydroxythiohomesildenafil; 24, Hydroxythiovardenafil; 25, Hydroxyvardenafil; 26, Mirodenafil; 27, Mutaprodenafil; 28, *N*-Butylnortadalafil; 29, *N*-Desethylvardenafil; 31, *N*-Desmethylsildenafil; 32, *N*-Octylnortadalafil; 33, Nitrodenafil; 34, Noracetildenafil; 35, Norneosildenafil; 36, Norneovardenafil; 37, Nortadalafil; 47, Propoxyphenylthiosildenafil; 50, Pyrazole-*N*-Desmethylsildenafil; 55, Thiodimethylsildenafil; 56, Thiohomosildenafil.

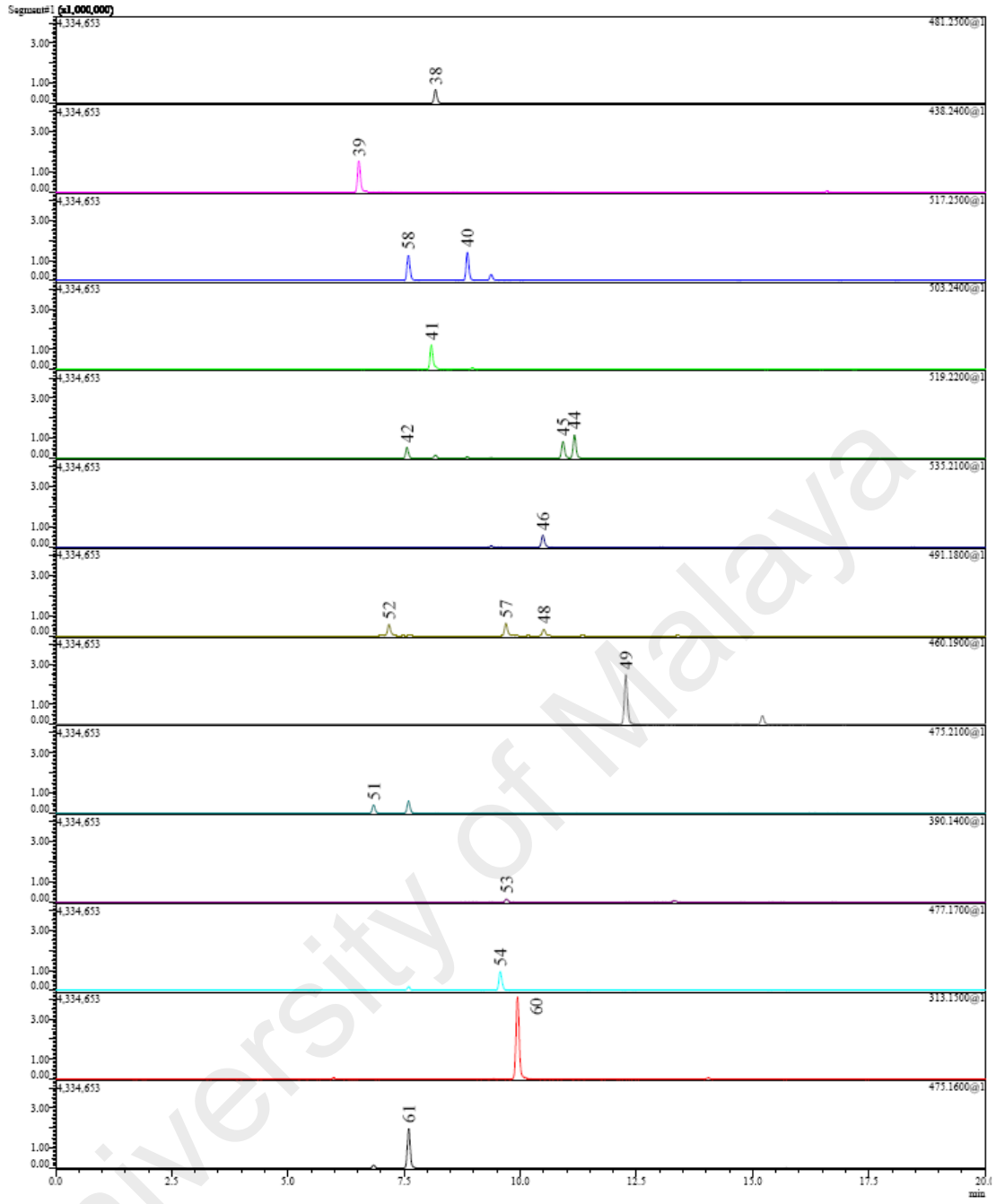

Figure 4.4 Extracted ion chromatograms obtained from the analysis of a mixture of PDE-5 inhibitor standards by LCMS IT-TOF (presented in alphabetical order and numbered as in Table 4.1)

Chromatograms obtained for: 38, Oxohongdenafil; 39, Piperiacetildenafil; 40, Propoxyphenyldimethylisobutylsildenafil; 41, Propoxyphenyldimethylsildenafil; 42, Propoxyphenylhydroxyhomosildnafil; 44, Propoxyphenylthiodimethylsildenafil; 45, Propoxyphenylthiohomosildenafil; 46, Propoxyphenylthiohydroxyhomosildenafil; 48, Propoxythio-*N*-desmethylsildenafil; 49, Pseudovardenafil; 51, Sildenafil; 52, Sildenafil *N*-oxide; 53, Tadalafil; 54, Thio-*N*-Desmethylsildenafil; 57,Thiosildenafil; 58, Udenafil; 60, Vardenafil intermediate; 61, Vardenafil oxopiperazine.

4.3.2.2 LCMS IT-TOF method validation

Validation was done by spiking a solution containing a mixture of the 10 representative standards into the blank samples; pills, capsules and coffee powder, respectively. No other peak interferences were observed coming from the blank samples, showing that the method is specific for its intended purpose. Comparison of peak area and retention time of standards in spiked blank sample solutions and standards in diluent solution showed no big differences. Therefore, it could be concluded that there were no interferences from the matrices. The LOD values for the 10 standards are presented in Table 4.2. The detection limit ranged from 0.05–0.4 μg/g of dry sample.

Table 4.2 Limit of detection (LOD) values for 10 representative PDE-5 inhibitor standards spiked in different blank matrices.

Matrix	Standards	Limit of Detection (LOD) (μg/g)
Capsule	Acetildenafil	0.3
Coffee		0.1
Pill		0.3
Capsule	Aminotadalafil	0.4
Coffee		0.4
Pill		0.4
Capsule	Avanafil	0.05
Coffee		0.05
Pill		0.1
Capsule	Desmetjylcarbodenafil	0.2
Coffee		0.3
Pill		0.2
Capsule	Propoxyphenylsildenafil	0.3
Coffee		0.3
Pill		0.2
Capsule	Pseudovardenafil	0.1
Coffee		0.2
Pill		0.2
Capsule	Sildenafil	0.2
Coffee		0.28
Pill		0.28
Capsule	Tadalafil	0.1
Coffee		0.3
Pill		0.3
Capsule	Thiodimethylsildenafil	0.3
Coffee		0.25
Pill		0.4
Capsule	Vardenafil	10
Coffee		30
Pill		10

4.3.3 Conclusion

An LCMS method was successfully developed to simultaneously detect 61 PDE-5 inhibitors and their analogues with good separation and resolution. The method was validated for specificity, matrix effects and limit of detection. This method will be used for screening for adulteration with PDE-5 inhibitors and their analogues by comparing accurate mass and ion fragmentation patterns.

CHAPTER 5:

MALAYSIA MARKET SAMPLE ANALYSIS

5.1 Introduction

In this chapter, an analysis of sixty-two herbal and food products marketed in Malaysia with indications to be used for increasing men's health was made. The samples were screened for adulteration with PDE-5 inhibitors and their analogues.

The effectiveness of synthetic PDE-5 inhibitors in treating erectile dysfunction has led to frequent occurrences of adulteration in HHSs. The danger imposed by such adulteration and its prevalence in different countries has been discussed in detail in section 2.2.3. One study conducted a HPLC test on five herbal powder samples from Malaysia market, in which four were adulterated with sildenafil, while the other one was adulterated with tadalafil (Siri, Lai, & Haris, 2013). Another study disclosed a LCMS/MS analysis of five different brands of coffee powder from the Malaysia market which are suspected to be adulterated with sildenafil (Rahim Yaacob, Sofia Onn, & Balkiah Ismail, 2013). However, the adulteration of PDE-5 inhibitors and their analogues in Malaysia have never been reported extensively in any study. Most of the other data on HHSs adulteration in previous years were obtained from official government website, reports and presentation, which basically only provide the statistical data of the adulteration without further details about the sample analysis as a whole. Due to that, we made an effort through this study to explore, analyse and report on adulterated HHSs marketed in Malaysia.

5.2 Experimental

5.2.1 Samples and sample preparation

Sixty-two samples were obtained from the NPRA between April 2014 and April 2016. Samples included unregistered herbal and food products, seized by the pharmacy

enforcement team (n = 39), products sent to the NPRA for registration (pre-registration testing) (n = 9) and registered products investigated under the post-registration market surveillance programme (n = 14). These samples do not represent the true total number of samples sent to the NPRA within this period of time. Some samples had been previously tested with another method such as HPLC and there was insufficient quantity available for further LCMS analysis. In the case of duplicate samples with the same product name, only one sample was chosen at random for analysis.

The samples were in the form of capsules, tablets, powders, pellet, pills and liquids. Homogenisation of the samples was performed beforehand. Tablets, pellets and pills were crushed and finely ground. About 0.2 g of powder/ground sample or 10 ml of liquid sample was mixed with 25 ml of methanol and then sonicated for 15 minutes. In the case of capsule-form samples, the capsule shells were included in the preparation since previous studies have detected the presence of adulterant in the capsule shell rather than the content (Lanzarotta, Crowe, Witkowski, & Gamble, 2012). The methanol solutions were then filtered through a 0.45 μm PTFE filter and finally diluted in methanol to 0.01 mg/ml.

5.2.2 LCMS analysis

Sample analysis was carried out using an LCMS IT-TOF system (Shimadzu, Kyoto, Japan) equipped with an autosampler and quaternary pump. Separation was achieved on a Kinetex C_{18} (2.1 x 100 mm, 2.7 μm XB). Data acquisition and analysis were performed using Shimadzu PostRun analysis software. The LCMS method developed and validated in Section 4.4.1 was applied.

5.3 Results and discussion

A total of 62 samples were analysed by using the validated LCMS IT-TOF method and compared against the in-house spectral library to give a similarity index (match

score) based on statistical calculations when comparing the intensity and height between the fragment ions of the sample and those of standards in the library (Table 5.1). The majority (82%; n = 32/39) of the unregistered products seized by the enforcement team were found to be adulterated, of which half were coffee products. Only two of products investigated under the post-registration market surveillance programme and none of the products from pre-registration testing contained adulterants. These findings are indicative of an increasingly common problem due to food products not needing to undergo a pre-registration process in order to be marketed and may be freely sold in any location, thus delaying or avoiding detection by the regulatory authorities.

Table 5.1 LCMS results of samples compared against the in-house spectral library

No	Sample code	PDE-5 inhibitor/analogue detected (library match score)	Dosage form
1	CPF001	Sildenafil (95%)	Capsule
2	CPF002	Desmethylcarbodenafil (96%)	Powder
3	CPF003	Propoxyphenylhydroxyhomosildenafil (96%)	Powder
4	CPF004	Aminotadalafil (93%), Dimethylsildenafil (93%), Thiodimethylsildenafil (98%)	Powder
5	CPF005	Sildenafil (97%), Sildenafil-*N*-oxide (94%), Dimethylsildenafil (96%)	Powder
6	CPF006	Desmethylcarbodenafil (96%)	Powder
7	CPF007	Sildenafil (94%), Sildenafil-*N*-oxide (96%), Pyrazole-*N*-desmethylsildenafil (61%)	Pellet
8	CPF008	Sildenafil (97%)	Powder
9	CPF009	Tadalafil (88%)	Powder
10	CPF0010	Sildenafil (95%)	Powder
11	CPF0011	Tadalafil (94%), Sildenafil (82%), Propoxyphenylsildenafil (84%)	Powder
12	CPF0012	Noracetildenafil (93%), Propoxyphenyl-hydroxyhomosildenafil (93%), Acetil acid	Capsule
13	CPF0013	Sildenafil (96%)	Powder
14	CPF0014	Desmethylcarbodenafil (95%)	Powder
15	CPF0015	Undetected	Powder
16	CPF0016	Undetected	Pellet
17	CPF0017	Undetected	Pellet
18	CPF0018	Undetected	Pellet
19	CPF0019	Dimethylsildenafil (95%)	Powder
20	CPF0020	Tadalafil (87%)	Powder
21	CPF0021	Sildenafil (96%), Sildenafil-*N*-oxide (96%)	Powder
22	CPF0022	Undetected	Liquid
23	CPF0023	Sildenafil (86%), Dimethylsildenafil (95%), Thiosildenafil (92%), Thiodimethylsildenafil (97%)	Powder
24	CPF0024	Desmethylcarbodenafil (95%)	Powder
25	CPF0025	Undetected	Powder
26	CPF0026	Propoxyphenylhydroxyhomosildenafil (95%), Propoxyphenylthiohydroxyhomosildenafil (93%)	Powder
27	CPF0027	Sildenafil (97%)	Powder
28	CPF0028	Sildenafil (97%)	Pill
29	CPF0029	Sildenafil (96%)	Capsule

30	CPF0030	Undetected	Capsule
31	CPF0031	Sildenafil (97%)	Powder
32	CPF0032	Desmethylcarbodenafil (95%)	Powder
33	CPF0033	Sildenafil (96%)	Capsule
34	CPF0034	Sildenafil (97%)	Powder
35	CPF0035	Sildenafil (91%), Propoxyphenylsildenafil (84%), Tadalafil (87%), Thiosildenafil (95%), Thiodimethylsildenafil (80%)	Powder
36	CPF0036	Sildenafil (79%), Tadalafil (85%)	Powder
37	CPF0037	Sildenafil (96%)	Capsule
38	CPF0038	Tadalafil (86%)	Powder
39	CPF0039	Dimethylsildenafil (94%), Thiodimethylsildenafil (97%)	Powder
40	PEN0001	Undetected	Capsule
41	PEN0002	Undetected	Capsule
42	PEN0003	Undetected	Capsule
43	PEN0004	Undetected	Capsule
44	PEN0005	Undetected	Tablet
45	PEN0006	Undetected	Capsule
46	PEN0007	Undetected	Capsule
47	PEN0008	Undetected	Capsule
48	PEN0009	Undetected	Capsule
49	SRV0001	Dithiodesmethylcarbodenafil (97%), Xanthoantrafil	Capsule
50	SRV0002	Undetected	Capsule
51	SRV0003	Undetected	Tablet
52	SRV0004	Propoxyphenylhydroxyhomosildenafil (66%)	Capsule
53	SRV0005	Undetected	Capsule
54	SRV0006	Undetected	Capsule
55	SRV0007	Undetected	Capsule
56	SRV0008	Undetected	Liquid
57	SRV0009	Undetected	Capsule
58	SRV0010	Undetected	Capsule
59	SRV0011	Undetected	Capsule
60	SRV0012	Undetected	Capsule
61	SRV0013	Undetected	Capsule
62	SRV0014	Undetected	Capsule

Table 5.2 demonstrates that the most frequent classes of adulterants detected were sildenafil and its analogues. This could be due to the fact that raw materials for the synthesis of sildenafil and its analogues are much cheaper and easily obtained compared to those of tadalafil (Patel et al., 2014; Venhuis & de Kaste, 2012). Vardenafil and its analogues were not detected in any of the samples. These compounds have been found in other studies to be the least common class of adulterants (Patel et al., 2014; Venhuis & de Kaste, 2012). One of the explanations that have been proposed is the fact that vardenafil has a shorter duration of action compared to sildenafil (4 h) and tadalafil (36 h), which is contrary to the 'purpose' of these illegal products of promising an extended time of satisfactory sexual performance.

Table 5.2 List of adulterants detected and their frequency of occurrence in products from different sources

Compound	Type of sample / total number of samples		
	CPF/39	PEN/9	SRV/14
Acetil acid	1	-	-
Aminotadalafil	1	-	-
Desmethylcarbodenafil	5	-	-
Dimethylsildenafil	5	-	-
Dithiodesmethylcarbodenafil	-	-	1
Noracetildenafil	1	-	-
Propoxyphenylhydroxyhomosildenafil	3	-	1
Propoxyphenylsildenafil	2	-	-
Propoxyphenylthiohydroxyhomosildenafil	1	-	-
Pyrazole-*N*-desmethylsildenafil	1	-	-
Sildenafil	18	-	-
Sildenafil *N*-oxide	3	-	-
Tadalafil	6	-	-
Thiodimethylsildenafil	4	-	-
Thiosildenafil	2	-	-
Xanthoantrafil	1	-	-

CPF: Illegal products seized by the enforcement team;
PEN: Pre-registration products;
SRV: Post market surveillance products

The distribution of adulterated samples according to the number of distinct PDE-5 inhibitors and analogues that they contained is shown in Fig. 5.1. Although only one inhibitor was detected in the majority of the adulterated samples (64%; n = 22/34), the fact that there were a number of samples that contained two or more adulterants is a cause for concern, especially when one was found to contain five different inhibitors. A study conducted in France in 2014 disclosed that out of 150 dietary supplements tested, 61% were adulterated with PDE-5 inhibitors and their analogues and 36% of the adulterated supplements were found to contain more than one adulterant (Gilard et al., 2015). Similarly, of herbal supplements sampled from the Dutch market from 2003–2012 (n = 71), 32% were found to be adulterated with PDE-5 inhibitors and their analogues and 13% contained more than one adulterant (Reeuwijk et al., 2013). The reason for the presence of multiple adulterants in one product might either be due to the manufacturers trying to boost the products' performance or simply a lack of Good Manufacturing Practice (GMP) during production. Such multiple adulterations could

lead to serious adverse effects to the consumers without them being aware of the danger.

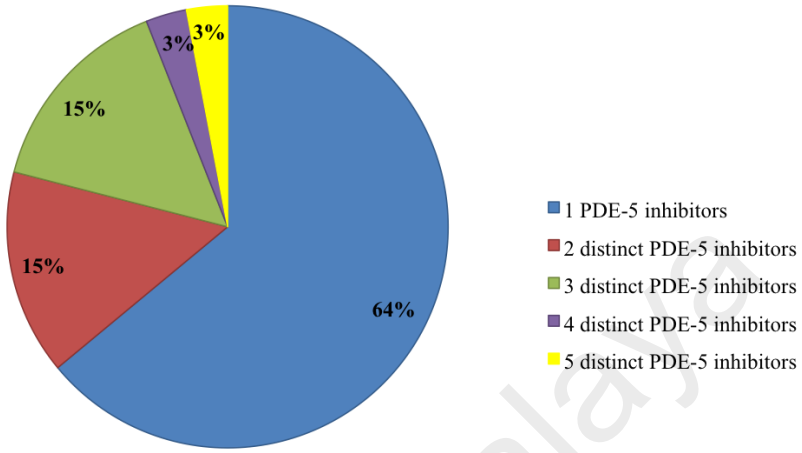

Figure 5.1 Distribution of adulterated samples according to the number of distinct PDE-5 inhibitors and analogues that they contained**.**

LCMS IT-TOF analysis of sample CPF0012, detected the presence of analogue noracetildenafil (**34**), propoxyphenylhydroxyhomosildenafil (**42**) and unknown compound A, which gave a prominent peak that eluted at 9.6 minutes for the protonated ion $[M+H]^+$ at *m/z* 357.1559. Fragmentation of the parent ion generated several distinguishable product ions at *m/z* 285, 311 and 329 (Fig. 5.2). The product ion data did not give a high match score with any of the compounds in the in-house library. Shimadzu Formula Predictor software was therefore used to predict the molecular formula of the compound, which was found to be $C_{18}H_{20}N_4O_4$. This software uses isotopic patterns and MS^n spectral filtering to refine the molecular formula prediction. By referring to previous studies, the predicted molecular formula and observed fragmentation pattern were found to be in accordance with sildenafil analogue, acetil acid, previously detected in Singapore (Health Sciences Authority, 2008; Lebel et al., 2014).

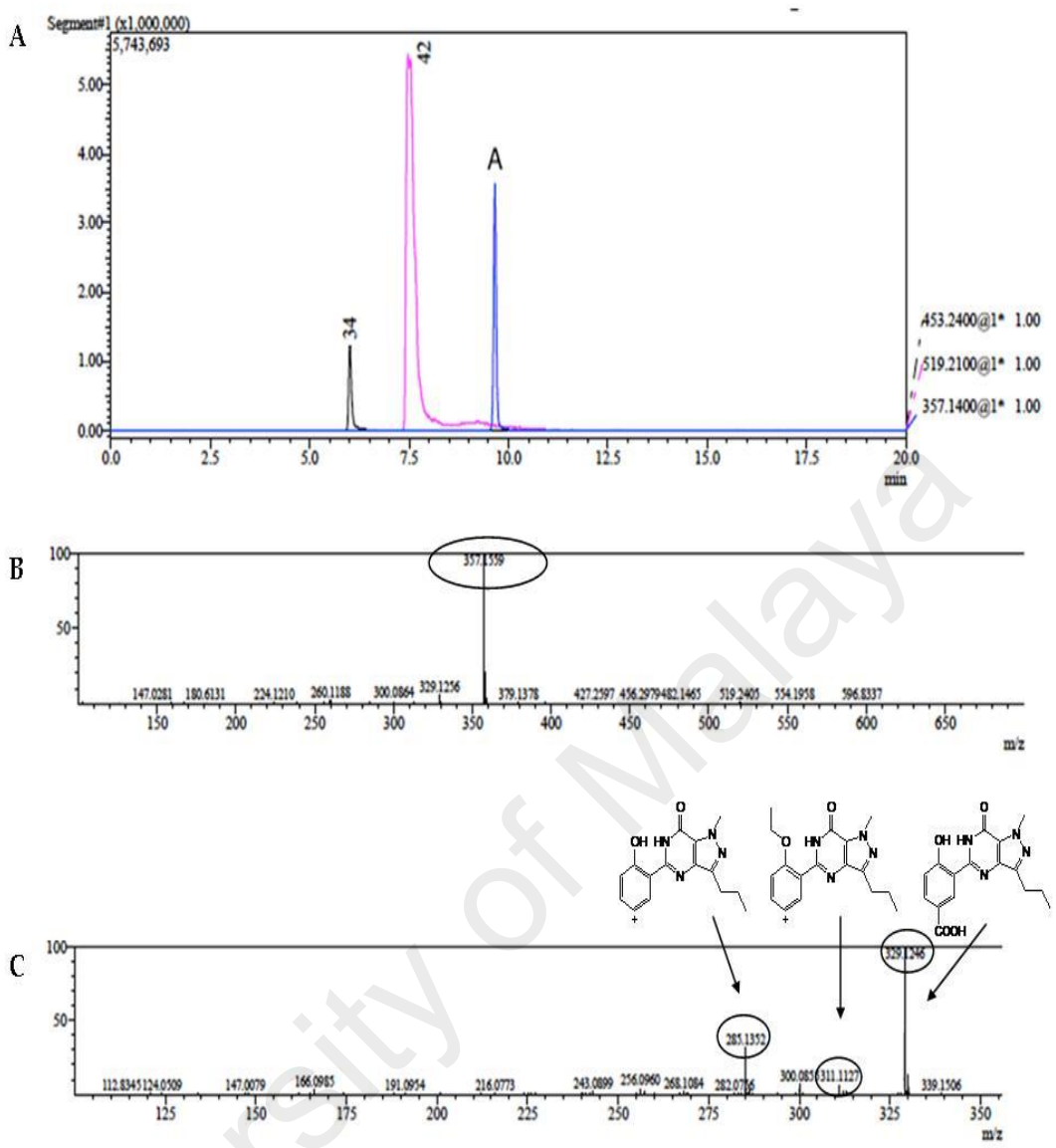

Figure 5.2 A. Extracted ion chromatogram obtained for sample CPF0012, showing peaks for noracetildenafil (34), propoxyphenylhydroxyhomosildenafil (42) and compound A; B. MS spectrum of the $[M+H]^+$ ion from compound A with *m/z* 357.1559; C. MS spectrum of the corresponding product ions arising from the $[M+H]^+$ ion.

Similarly, analysis of sample SRV0001 detected dithiodesmethylcarbodenafil (**15**) and unknown compound B, which gave a prominent peak that eluted at 9.7 minutes for the protonated ion $[M+H]^+$ at *m/z* 390.1666 and fragment peaks at *m/z* 151 and 252 (Fig. 5.3). Searching against the in-house library gave 83% similarity index (match score) to vardenafil intermediate (**60**). However, the vardenafil intermediate should give a parent

ion at *m*/*z* 313.1522, suggesting that compound B has a different identity. The formula predictor software predicted the molecular formula for the compound to be $C_{19}H_{23}N_3O_6$. This formula and the observed fragmentation pattern were in accordance with xanthoanthrafil, which is a PDE-5 inhibitor that is not an analogue of the sildenafil, tadalafil or vardenafil. This compound has previously been detected in dietary supplements in Japan and Singapore (Kumasaka et al., 2008; Zou, Hou, Oh, Ge, et al., 2008).

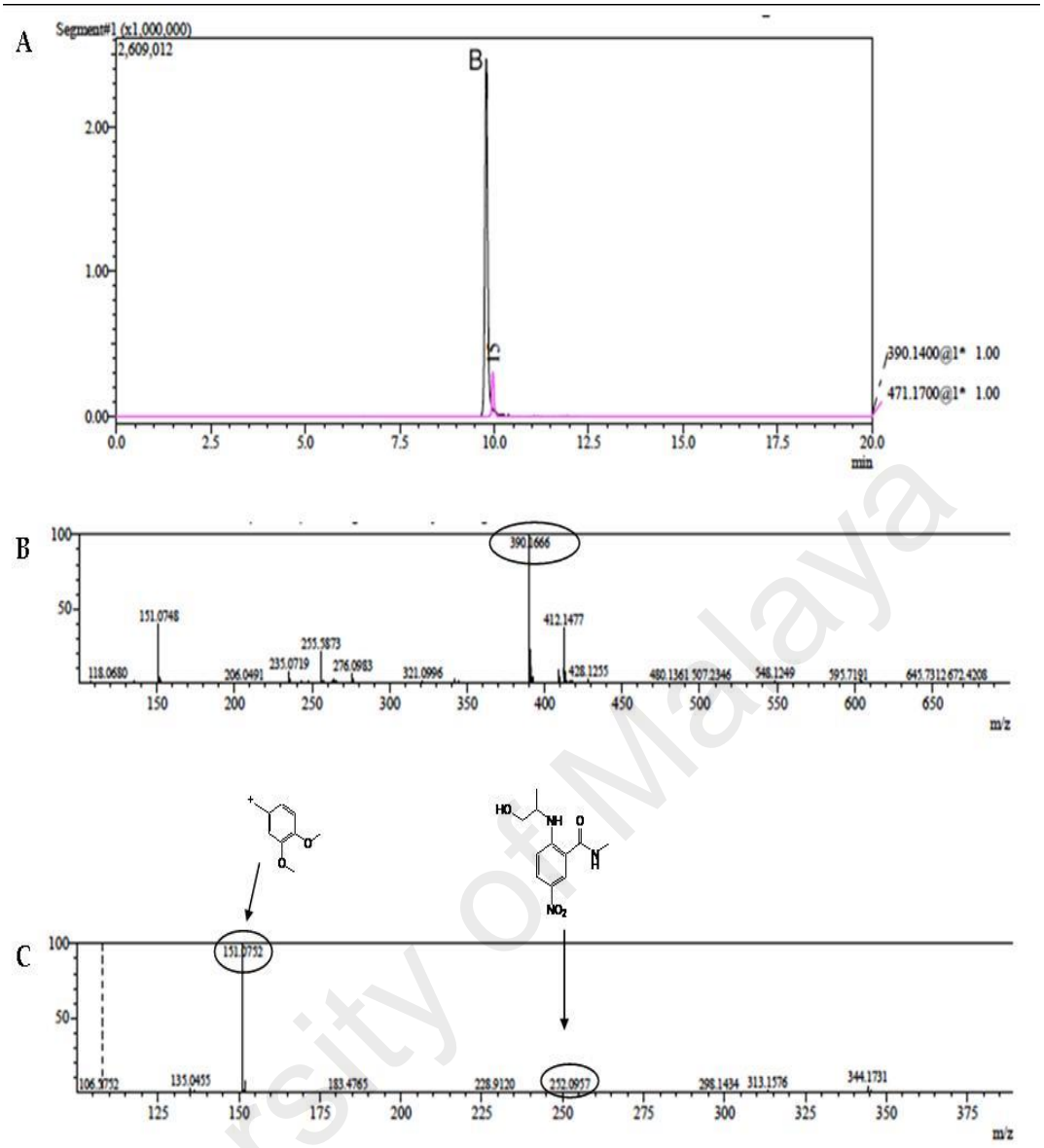

Figure 5.3 A. Extracted ion chromatogram obtained for sample SRV0001, showing peaks for dithiodesmethylcarbodenafil (15) and compound B; B. MS spectrum of the [M+H]+ ion from compound A with m/z 390.1666; C. MS spectrum of the corresponding product ions arising from the $[M+H]^+$ ion

Although these identifications of unknown adulterants A and B seem quite plausible, matching of their observed LCMS data against reference standards would be required to give confirmation.

5.4 Conclusion

In this study, sixty-two herbal and food products marketed in Malaysia with indications to be used for increasing men's health were analysed to detect the presence of PDE-5 inhibitors and their analogues. More than half the number of the products analysed were found to be tainted with at least one of these adulterants. The highest adulterated products are those of unregistered products. Health professionals, regulators and consumers should be made aware of the danger of these adulterated unregistered products sold freely in the market.

CHAPTER 6:

CONCLUSION AND FUTURE WORK

6.1 Conclusions

HHSs are considered and expected to be a safe alternative for users. This study focused on the detection of PDE-5 inhibitors and their analogues in HHSs specifically formulated or sold for the purpose of increasing men's sexual health. .

Firstly, ten different sildenafil analogues were successfully synthesised in-house. The main purpose was to develop the capability to produce additional PDE-5 inhibitor standards for the ever-increasing number of new analogues being used as adulterants. The price of analogues has been increasing lately, making it a burden to purchase the ever-increasing number of new analogues. Due to that, in-house synthesis may help in curbing this situation.

An in-house LCMS library database was then built using 51 purchased PDE-5 inhibitors and their analogues together with the 10 synthesised analogues. The library stored information of the retention time and accurate mass fragmentation of the compounds. Subsequently, an LCMS method was developed and optimised for simultaneous detection of the 61 compounds. The identification method was validated for matrix effect, specificity and limit of detection. The validated LCMS method offers improved separation between a large numbers of PDE-5 inhibitor compounds compared with the previous NPRA LCMS method and will be further used for ISO 17025 accreditation.

Lastly, a total of sixty-two samples marketed in Malaysia as sexual enhancement HHSs were collected from NPRA from April 2014 till April 2016. Samples included unregistered herbal and food products, seized by the pharmacy enforcement team (n = 39), products sent to the NPRA for registration (pre-registration testing) (n = 9) and

registered products investigated under the post-registration market surveillance programme (n = 14). These samples were analysed for adulteration of PDE-5 inhibitors and their analogues using LCMS IT-TOF method developed in this study. The results showed that thirty-four out of the sixty-two samples were adulterated. Thirty-two (94%) of the adulterated samples are those of the unregistered products. This indicates that the supply and usage of unregistered HHSs requires a very strict control as it may put consumers' safety at stake. Testing was carried out in accordance with Malaysia regulations under the Poison Act 1952 which prohibits the presence of PDE-5 inhibitors and their analogues in herbal supplements at any level or concentration. Therefore quantitative analysis was not performed.

In conclusion, the work in this study has provided a wider screening method using LCMS IT-TOF for adulteration of PDE-5 inhibitors and their analogues. Testing of samples was performed using high-end laboratory equipment, LCMS IT-TOF, with a validated method and run against purified standards, which indicates that the results of the analysis are strong and could hold in a court of justice. The method developed and applied are important to ensure that HHSs are of the highest quality in order to safeguard the health and safety of consumers.

6.2 Limitations and future work

Although this research has fulfilled its aims and objectives, there were still some inevitable limitations. Due to time constraints, the synthesis part only involved sildenafil analogues. In future studies, synthesis involving tadalafil, vardenafil and their analogues, as well as other types of sildenafil analogues can be done. This will eventually increase the capability of producing in-house synthesised standards.

The sample preparation method used in this study was performed by direct dissolution in methanol. Although this simple method is suitable for the highly sensitive

LCMS analysis, proper studies have not been done beforehand to prove it. It is necessary to do a comparison study between different kinds of sample preparation method, such as direct dissolution, solid phase extraction and liquid-liquid extraction.

The samples analysed for this study were obtained solely from NPRA. The enforcement team sent most of the samples to the lab without their original packaging, as the samples were still under investigation. Therefore, the origin and ingredients of the products are unknown. This information should be addressed in future studies to provide better understanding of the samples and also to determine the geographic sources of the adulterated samples.

The analysis of suspected adulterants, which were not included in the list of 61 standards, could have been strengthened by running the samples against the suspected standards. NMR studies would also helped to confirm their respective molecular structures. However, these were not performed due to budget and time constrains and should be initiated in further studies for unknown compounds.

The pharmacokinetics properties, PDE-5 inhibitory activities and toxicity effects of most of the analogues are also unknown. Therefore, it is necessary to carry out studies to determine these profiles, in order to have a better understanding on the effect of these analogues to consumers. Furthermore, quantitative analysis could be included in future to determine whether or not the amount of PDE-5 inhibitors and analogues in the sample would exert a pharmacological effect in consumers.

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