ERGOGENIC EFFECTS OF INTAKE OF SALBUTAMOL, CAFFEINE AND THEOBROMINE ON NON-ASTHMATIC SUBJECTS

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ABSTRACT

Inhaled short acting β_2–agonists are commonly used in the treatment of asthma, exercise induced bronchoconstriction (EIB) and airway hyperresponsiveness (AHR). The World Anti-doping Agency (WADA) permits asthmatic athletes to use inhaled salbutamol with an accumulated dosage not exceeding 1600 µg over a 24-hour period. A key driver for the inclusion of β_2-agonists on the WADA restricted list is associated with its potential impact on athletic performance according to previous research. However, the findings from previous studies are equivocal and focus almost exclusively on endurance performance. Furthermore, there are no available data examining the ergogenic effect of inhaling a single bolus of the upper daily limit of 1600 µg or the impact on urine concentration of the short-acting β_2-agonist and its relationship with the WADA code. Meanwhile, caffeine and theobromine have been regarded as bronchodilators and whilst the potential ergogenic effects of caffeine have led to its WADA monitored status no such information exists for theobromine.

Study 1 investigated the ergogenic effect of 800 µg and 1600 µg of salbutamol in a randomised, single-blind and cross-over design on 5-km running time-trial performance in non-asthmatic endurance athletes (n = 7). The study revealed no significant improvement in performance after the inhalation of either doses of salbutamol (t = 1683.29 ± 179.74 sec for 800 µg and t = 1683.57 ± 190.69 sec for 1600 µg) compared with placebo condition (t = 1714.71 ± 186.22 sec). Study 2 employed a simulated football protocol following the inhalation of 800 µg and 1600 µg of salbutamol in a randomised, single-blind placebo controlled trial in male (n = 7) and female (n = 6) football players. Results demonstrated there is an improvement of inhaled salbutamol on performance. Whilst the average urine salbutamol concentrations did not breach the WADA urinary threshold (1000 ng·mL^{-1}), five individuals, two male and three female, recorded urine drug concentrations that exceeded the threshold. Study 3 examined the urine concentration of salbutamol following passive body mass loss of 2% or 5% following the inhalation of 800 µg and 1600 µg of salbutamol across ethnicity and gender (n = 32). The study demonstrated that following the inhalation of 1600µg it is possible to present urine salbutamol concentrations above the current WADA upper limit that imposes a urinary drug threshold of 1000 ng·mL^{-1}. Study 4 explored the ergogenic effect of caffeine and theobromine on non-asthmatic athletes during a 3-km time trial (n = 10). The findings suggest that both caffeine and theobromine resulted in an improved time-trial performance (t = 1168.7 ± 78.12 sec and t = 1176.5 ± 75.15 sec, respectively) compared with placebo condition (t = 1260.6 ± 110.65 sec), while caffeine resulted in a larger and more rapid enhancement compared to theobromine. Overall, salbutamol, the intake of which is restricted by WADA, did not have an ergogenic effect on athletes, while on the other hand, caffeine and theobromine, the usage of which are not controlled at all in sports competitions, had a performance enhancing effect on athletes, according to our results. Furthermore, although the inhalation of salbutamol did not improve the overall performance, athletes inhaling these substances might record urine concentration exceeding the WADA threshold, resulting in an adverse analytical finding (AAF).

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DECLARATION

I declare that the work contained in this thesis is entirely my own. Individuals acknowledged above were involved with data collection.

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Abbreviations

AAF	Adverse Analytical Finding
AAF	Antitussive and Antiashthmatic Film
AHR	Airway Hyperresponsiveness
AMPK	AMP-activated Protein Kinase
ANOVA	Analysis of Variance
ASM	Airway Smooth Muscle
ATPS	Ambient Temperature and Pressure, Saturated
ATS	American Thoracic Society
BASES	British Association of Sport and Exercise Sciences
BM	Body Mass
BPT	Bronchial Provocation Tests
BW	Body Weight
CAMP	Cyclic Adenosine Monophosphate
CHO	Carbohydrates
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
EIA	Exercise Induced Asthma
EIAH	Exercise-induced Arterial Hypoxemia
EIB	Exercise Induced Bronchoconstriction
ERS	European Respiratory Society
EVH	Eucapnic Voluntary Hyperpnoea

FeCO$_2$	Fraction of Expired Carbon Dioxide
FeO$_2$	Fractions of Expired Oxygen
FEF$_{25\text{-}75}$	Forced Expiratory Flow between 25 and 75% of Forced Vital Capacity
FEV$_1$	Forced Expiratory Volume in 1 second
FIFA	Fédération Internationale de Football Association
FFA	Free Fatty Acid
FVC	Forced Vital Capacity
GLUT4	Glucose Transporter 4
HR	Heart Rate
HSL	Hormone Sensitive Lipase
IOC	International Olympic Committee
IOC-MC	International Olympic Committee – Medical Committee
MVV	Maximal Voluntary Ventilation Rate
OH	Hydroxyl
PEF	Peak Expiratory Flow (rate)
PRN	Pro re nata
RER	Respiratory Exchange Ratio
RH	Relative Humidity
RNA	Ribonucleic Acid
RPE	Rating of Perceived Exertion
STPD	Standard Temperature and Pressure, Dry
SPSS	Software Package used for Statistical Analysis

TUE	Therapeutic Use Exemptions
UEFA	European Football Union
VO$_2$	Oxygen Uptake
VCO$_2$	Carbon Dioxide Production
V$_E$	Minute Ventilation
WADA	World Anti-doping Agency
WHO	World Health Organization

CHAPTER I

GENERAL INTRODUCTION

1.1 Introduction

Asthma is a chronic inflammatory disorder of the airways and is associated with bronchial (airway) hyperresponsiveness. According to World Health Organization (WHO) estimates, there are 235 million people suffering from asthma worldwide. Furthermore, asthma is the most common chronic disease among children (WHO, 2016). In the UK 5.4 million people (8.4% of UK population) are receiving treatment for asthma, including 1.1 million children (Asthma UK, 2015).

Exercise induced bronchoconstriction (EIB), sometimes termed Exercise Induced Asthma (EIA), has been shown to develop during long duration exercise resulting in a reduction in endurance capacity leading to an impaired athletic performance and prolonged recovery time following exercise. Associated with different sport types, diagnostic methods used, and environments, reports on the prevalence of EIB in athlete populations have ranged from 9% to 55%. Many studies have shown that athletes who participate in summer sports have a lower incidence of EIB than those who participate in winter sports (Dickinson *et al.*, 2005). The number of athletes using inhalers in Olympics Games has been increasing from 11% in 1984 to 17% in 1998 in the US Olympic team alone (Dickinson *et al.*, 2005).

Exercise induced bronchoconstriction describes acute, transient airway narrowing that occurs during and most often after exercise. It can be objectively defined as a >10% decline in forced expiratory volume in 1 second (FEV_1) after appropriate exercise provocation (Dheda *et al.*, 2009). However, the decline in FEV_1 is reversible either through inhalation of short

acting β_2-agonists which give immediate reversibility or natural recovery which can take up to four hours to reverse bronchoconstriction. Clinical symptoms of EIA include coughing, wheezing, shortness of breath, excessive mucus production, chest tightness, and chest pain (Donna *et al.*, 2010). Exercise is the most common trigger of bronchospasm in those who are known to be asthmatic, and 50% to 90% of all individuals with asthma have airways that are hyperreactive to exercise (Rundell *et al.*, 2002). However, EIB also occurs in up to 10% of subjects who are not known to be atopic or asthmatic (Dheda *et al.*, 2009; Parsons *et al.*, 2014).

Standard treatment for asthma and EIB includes short acting β_2-agonists *i.e.* salbutamol, terbutaline, procaterol and fenoterol. In 2001, The International Olympic Committee - Medical Committee (IOC-MC) established the requirement for athletes using short acting β_2-agonists to present evidence of current asthma, EIB or airway hyperresponsiveness (AHR) through the therapeutic use exemptions (TUE) process. This regulation has recently been amended no longer requiring a full TUE, only confirmation of asthma and/or EIB from a certified medical professional.

According to Lafontan (1988), the use of oral β_2-agonists can increase muscle mass by stimulating protein anabolism as well as increasing the metabolism of lipids and carbohydrates. In addition, some studies from 1980s have reported effects of oral β_2-agonists on the central nerve system consistent with antidepressant activity (Belmaker, 1982). A number of studies have reported remarkable changes in body composition following oral β_2-administration. Over comparatively short periods of time (between four and twelve weeks), specific β_2-agonists (*i.e.* clenbuterol and salmeterol) have been shown to enhance skeletal

muscle size and strength in some animal species (Baker, 1983). Moreover, these treatments resulted in body fat loss irrespective of mode of administration (*i.e.* oral or implanted osmotic mini-pumps; Baker, 1983). These results may indicate the underlying reason why athletes searching for performance enhancement commonly use β_2-agonists. However, in contrast to oral β_2-agonists the ergogenic effect of inhaled (topical) β_2-agonists is equivocal (Meeuwisse, 1992; Freeman, 1989) indicating the need for further study.

The IOC-MC investigated the use of inhaled β_2-agonists at the 2000 Olympic Games and were concerned that some non-asthmatic athletes were using these medications inappropriately. Accordingly, the IOC-MC introduced its β_2-agonists policy before the 2002 Olympic Winter Games in Salt Lake City for health, not doping reasons. At that time a Therapeutic Use Exemption (TUE) certificate was required for the use of inhaled short acting β_2-agonists. To carry out this policy, a medic was required to provide evidences through either a bronchodilator response or one of several Bronchial Provocation Tests (BPT) before an athlete was granted approval to use β_2-agonists (Anderson *et al.*, 2003). As a result, only 445 athletes at the 2004 Summer Olympic Games in Athens provided evidence for the use of inhaled short acting β_2-agonists, which was 162 athletes less than four years earlier in Sydney where TUE's were not required (Anderson *et al.*, 2006).

In line with these findings, the International Olympic Committee (IOC) and World Anti-Doping Agency (WADA) prohibited systemic use of β_2-agonists, putting them on their doping lists from 2004 stating: "All β_2-agonists (including both optical isomers where relevant) are prohibited except salbutamol (maximum 1600 micrograms over 24 hours) and salbutamol by inhalation which require a declaration of use in accordance with the

International Standard for TUE. The presence of salbutamol in urine in excess of use of the substance and will be considered as an adverse analytical finding (AAF) unless the athlete proves, through a controlled pharmacokinetic study, that the abnormal result was the consequence of the use of a therapeutic dose (maximum 1600 micrograms over 24 hours) of inhales salbutamol" (WADA, 2012).

Recently WADA removed the requirement for TUE for inhaled short acting β_2-agonists however, they remain on the WADA Monitoring Program. The World Anti-doping Agency (WADA) stipulates that athletes who declare the use of salbutamol in order to treat asthma and/or EIB should not exceed an accumulated dose of >1600 µg over a 24-hour period (~16 inhalations of a standard salbutamol inhaler; WADA, 2013). This recommendation appears out with the scientific evidence where the majority of studies have only investigated doses of up to 800 µg of salbutamol. While the majority of these studies have demonstrated no ergogenic effect on performance there remains questions regarding the use of doses up to the recommended daily limit of 1600 µg. Furthermore, the majority of previous studies have focused on continuous, aerobic exercise challenges. To date, there is no available data for the impact of inhaled short acting β_2-agonists on repeat sprint team sport performance, such as association football (soccer).

Concern has been raised recently that the inhalation of 1600 µg in a single dose may result in urine concentrations close to the WADA upper limit of 1000 ng·mL^{-1} and, therefore, result in an adverse analytical finding (AAF). A small number of recent studies have examined the impact of inhaling the WADA daily upper limit on urine concentrations. These studies have focused on multiple dosing regimens such as 4 x 400 µg of salbutamol as opposed to a single

high dose (Elers *et al.*, 2011). Whilst athletes are usually prescribed 200-400 μg of inhaled salbutamol they are often instructed to use their inhaler '*pro re nata*' (*i.e.* on an as needed basis), that could be interpreted as a clearance to inhale unlimited amounts of salbutamol to combat respiratory symptoms. Individuals encouraged to administer salbutamol *pro re nata* may dose over and above the maximal recommended daily dose of 1600 μg either intentionally or inadvertently, however in both instances the individuals intent to dope for performance enhancement purposes may be nil. Such circumstances may lead to the current threshold being unintentionally breached and thus bring about an Antitussive and Antiashthmatic Film (AAF). A number of cases have been reported in the literature where athletes have tested positive following inhalation of high doses of short acting β_2-agonists (Kindermann *et al.*, 2006). Recently, a team sport athlete escaped a doping violation after he inhaled in excess of 1600 μg over the course of a match and then tested positive in the post-match anti-doping test. The player's defense was based on a prescribed 'as needed basis' with no guidance on an upper limit to its use (personal communication with UK Anti-Doing). Accordingly, in practice, 16 inhalations in a short period of time prior to competition may occur in poorly controlled, less well informed athletes, as well as potentially unscrupulous athletes, testing positive despite medicating within the recommended limit (WADA, 2013).

In addition to the inhalation of high, single bolus doses of short acting β_2-agonists the impact of dehydration through sweating associated with high intensity exercise in hot environments may have a profound effect on urine concentration. To date, little is known of the impact of dehydration on urine concentrations of short acting β_2-agonists. In addition, WADA impose global regulations and as yet no data exists on the impact of ethnicity on the pharmacokinetics of inhaled short acting β_2-agonist and their subsequent appearance in the

urine. To date, no studies have examined the impact of dehydration and ethnicity on the excretion of short acting β_2-agonists.

Caffeine (1,3,7-Trimethylpurine-2, 6-dione; $C_8H_{10}N_4O_2$) and theobromine (3,7-dimethyl-1,H-purine-2, 6-dione; $C_7H_8N_4O_2$) are closely related alkaloids which can be found in a variety of food sources including: chocolate; tea leaves; cocoa; and kola nut (Wikipedia, 2016). Theobromine, caffeine and their related compounds are considered pharmacologically beneficial as they demonstrate significant positive effects.

Caffeine, taken prior to exercise, has been shown to be an effective prophylaxis for EIB (Inman, 1996). Vanhaitsma (2010) comparative study on effects of Caffeine and β_2-agonists for asthmatic athletes, showed that in athletes with asthma and EIB, moderate (6 mL/kg body mass) to high doses (9 mL/kg body mass) of caffeine provide a significant protective effect against EIB, and that a high dose of caffeine is equally as effective as salbutamol in attenuating the bronchoconstrictor response to exercise. Whilst the action of theobromine on the central nervous system (CNS) is generally considered weak or non-existent, a small number of studies have reported that theobromine primarily acts a diuretic and a bronchial smooth muscle relaxant. Theobromine is an active ingredient of bronchodilator drugs that are used in the treatment of acute and chronic asthma, and persistent cough (Irwin, 1997). The IOC does not consider theobromine an illicit substance in humans, though their administration to racing animals is prohibited. A large evidence base exists for the performance enhancing effects from caffeine. In contrast, little is known of the ergogenic effect of theobromine.

Accordingly, the global purpose of this thesis was to examine the impact of inhaling a short acting β_2-agonist (salbutamol) at doses up to and including the maximum daily dose (1600 µg) in a single bolus on endurance and team game performance. In addition, this thesis aims to contribute to the understanding of the impact of dehydration and ethnicity on the drug elimination in the urine following the inhalation of short acting β_2-agonists at doses up to and including the maximal dose (1600 µg) as stipulated on the 2013 WADA Prohibited List. Finally, the impact of theobromine and caffeine, known bronchodilators, were assessed for their ergogenic impact on endurance performance.

CHAPTER II

LITERATURE REVIEW

2.1 Exercise Induced Asthma (EIA)

Exercise-induced asthma (EIA), sometimes termed 'Exercise Induced Bronchoconstriction (EIB)', is a syndrome resulting in coughing or wheezing, and chest tightness or pain associated with continuous or strenuous exercise in individuals with and without known asthma (Randolph, 2007). EIA is presently defined as a transient narrowing of the airways following exercise and is present in approximately 80-90% of people with asthma (Anderson, 1997). EIA is connected with airway inflammation leading to smooth muscle contraction and mucus production developing maximally about five to ten minutes following the cessation of exercise (Rundell *et al.*, 2000).

2.2 Prevalence of EIA

The prevalence of EIA in athletes is higher than the 8% asthma prevalence in the general population of the UK (Asthma UK, 2001; Helenius *et al*., 1998; Wilber *et al.*, 2000). The prevalence of EIA in athletic populations has progressively increased since data collection on the subject began in the early 1980's. Data from the United States Olympic team reported an 11% incidence in 1984 that grew to 14% in 1996 (Weiler *et al.*, 1998). Athletes who compete in summer sports have a lower prevalence of EIA than those who compete in winter sports *i.e.* 17% of team U.S.A. athletes at the 1998 Winter Olympics were diagnosed with EIA (Weiler and Ryan, 2000; Rundell *et al.*, 2000), which suggests the environment in which an individual trains and competes may be an important precursor in the development of EIA (Mannix *et al.*, 1996).

2.3 Diagnosis of EIA

The presence of EIA is difficult to diagnose through symptoms alone as clinical symptoms are often nonspecific (Jonathan, 2005). The main symptoms are cough, wheezing, tight chest and difficulties breathing; although other symptoms include a disparity between health and performance, and difficulties in sleeping (Storms, 1998).

Methods for diagnosis of EIA that have been used include: questionnaire and symptom history; and exercise challenge, together with a range of indirect airway challenges including: eucapnic voluntary hyperpnoea (EVH); metacholine; saline; mannitol; and histamine (Anderson *et al.*, 2001). In athletes with normal findings on resting spirometry the Eucapnic Voluntary Hyperpnoea (EVH) Challenge is recommended as the 'gold standard' assessment of EIA (Anderson *et al.*, 2001).

Previous studies using FEV_1 to identify EIA have suggested a magnitude of change ranging from a 7-20% fall in FEV_1 (Anderson *et al.*, 1971; Helenius *et al.*, 1998). The International Olympic Committee - Medical Commission (IOC-MC) has ruled that an exercise or EVH challenge is positive for EIA when the FEV_1 falls ≥10% from the baseline measurement. However, work carried out by Helenius *et al.* (1998) suggested that a fall of 10% in FEV_1 following an exercise test is not sensitive enough to diagnose EIA in elite athletes. Whilst lacking ecological validity, an EVH test conducted in the laboratory has a better level of standardisation and may be more perceptive than an exercise challenge in the diagnosis of EIA (Rundell *et al.*, 2004).

2.4 Asthmatic Therapy

There are pharmacologic and non-pharmacologic therapies available for asthmatics that decrease the severity of asthma and control its progression. In general, both pharmacologic and non-pharmacologic therapies are necessary to optimize the management of asthma and avoid adverse effects. These are presented in Table 1 and Table 2 (Randolph, 2009).

Pharmacologic	
Inhaled short-acting β_2-agonists	Short-acting β_2-agonists relax smooth muscle, decrease vascular permeability, increase airflow, and moderately inhibit mediator release, but only last for 3 - 6 hours (Williams and Shapiro, 1995). Typical forms include salbutamol (albuterol) and terbutaline.
Inhaled long-acting β_2-agonists	Long-acting β_2-agonists relax smooth muscle, decrease vascular permeability, increase airflow, and moderately inhibit mediator release and work up to 12 hours following inhalation (Williams and Shapiro, 1995). Typical forms include salmeterol and formoterol.
Inhaled corticosteroids	Inhaled corticosteroids reduce the number of airway inflammatory cells including: mast cells; eosinophils; and lymphocytes, and decreases the number of mononuclear cells, CD4+ type 2 T-helper cells, in order to improve airway epithelium (Ward *et al.*, 2002; Bocchino *et al.*, 1997).

Oral anti-histamines	Anti-histamines have been proved only in some circumstances to prevent inflammation of airways and thereby reducing responses to stimuli such as exercise and cold air, leading to a reduction in symptoms (Bianco, 1989)
Oral anti-leukotrienes	Anti-leukotrienes are a form of anti-inflammatory drug, the use of which reduces bronchoconstriction and inflammation of the airways, thereby enhancing the control of asthma (Ducharme, 2002).
Inhaled cromolyn sodium	Cromolyn sodium prevents the release of substances that cause inflammation or swelling of the airways. Therefore, it is used to prevent shortness of breath, wheezing, and troubled breathing induced by asthma. It is also used to prevent breathing difficulties during exercise (Medline Plus, 2014).

Table 1. Pharmacologic interventions for the treatment of asthma

Non-Pharmacologic	
Refractory period (Warm-up)	A warm-up induces a refractory period, which limits the magnitude of EIA in subsequent vigorous exercise and therefore resulting in fewer symptoms, decreased medication use, and improved exercise performance (Stickland *et al.*, 2012)
Change training environment	It has been well recognised that the role of environmental is a trigger of asthma. Changing training environment can decrease the

	exposure to allergens, as a result reducing asthma-associated symptoms (NEEF, 2005).
Breathing exercises	Researchers found that the asthmatics breathe faster than non-asthmatics, which exposes the lung to cooler and drier air and triggers asthma. Breathing exercises that encourage shallow breathing at a controlled rate have been proved to reduce asthma symptoms (Bottrell, 2011)
Low salt diet	Low sodium diet decreases the bronchoconstrictor response to exercise in asthmatics and improves lung function in adults with asthma (Mickleborough and Fogarty, 2006).
Poly-unsaturated fatty acids	Poly-unsaturated fatty acids reduce airway narrowing and as a result prevent asthmatic symptoms. A typical form is fish oil (Mickleborough and Rundell, 2005).
Anti-oxidants (Vitamin C and E)	An efficient endogenous antioxidant defence mechanism prevents excessive production of oxidants. An unbalanced exposure to oxidants and endogenous antioxidants will lead to oxidative stress which can trigger a number of diseases including lung pathologies such as asthma. Anti-oxidants can prevent asthma by maintaining the right balance (Van Toan and Hanh, 2013).

Table 2. Non-pharmacologic interventions for the treatment of asthma

2.4.1 Non-Pharmacological Treatment in Asthma

There is increasing interest in breathing retraining techniques in asthma, particularly among

patients and the lay press. The Buteyko technique, for example, which uses hypoventilation in an attempt to raise the partial pressure of carbon dioxide in the blood, has been advocated as a method to allow reductions in, or even withdrawal of, asthma medication. Unfortunately rigorous trials of these methods have not yet been published and they should therefore be viewed with caution. It has recently been recognized, however, that many patients treated for asthma in primary care also have symptoms suggestive of dysfunctional breathing patterns (Thomas, 2001). Results of a physiotherapy based breathing retraining programme in such patients have reported significant improvements in health status in the short-term (Thomas, 2003). It is likely that retraining techniques may improve symptoms and health status where there is dysfunctional breathing, either in the context of mild asthma or where asthma has been misdiagnosed. Physical training methods have been shown to improve cardiovascular fitness but not lung function in patients with asthma but effects on symptoms and quality of life have not been assessed (Ram, 2000).

The exposure of patients with atopic asthma to the allergens that they are sensitized to has been shown to increase asthma symptoms and airway hyper-responsiveness leading to bronchoconstriction (Boulet, 1993). Changes of training environment is in order to control the exposure of house dust mite and pet allergens, however, have not conclusively been shown to improve asthma outcomes and larger trials have been advocated (Gotzsche, 2001). Studies of allergen control measures in infancy have shown reductions in respiratory symptoms (Custovic, 2001; Chan-Yeung, 2000), but it remains to be seen if such measures will prevent the development of atopy and asthma in later life.

Allergen specific immunotherapy, or desensitization, involves the administration of specific

allergen extracts via subcutaneous injections of increasing concentration with the aim of inducing immunological tolerance. The process may work by generating interleukin-10 producing regulatory T-cells. Immunotherapy appears to be particularly useful in allergic rhinitis but has also been shown to improve symptoms and airway responsiveness in patients with allergic asthma (Abramson, 2000). Overall the benefits appear to be modest, the technique is labour intensive, and major concerns about its safety remain since life threatening anaphylactic reactions can occur. Thus, while some patients may gain dramatic benefits immunotherapy for asthma is not recommended in the UK (Report of a BSACI working party, 1993).

According to the information listed above, the non-pharmacological treatments for asthma or EIA might not be the ideal treatments for symptom relief or fundamentally therapy, therefore further studies are still needed to prove their efficacy and reliability.

2.4.2 Pharmacological Treatment in Asthma

There are a number of treatment methods that can treat asthma according to the severity (see Table 1). Mild intermittent asthma is normally treated with inhaled short acting β_2-agonists, such as salbutamol and terbutaline, they are effective bronchodilators and should be prescribed to all patients with symptomatic asthma (British Thoracic Society, 2003; Global Initiative for Asthma, 1995). They are useful in preventing symptoms of exercise-induced asthma when given before the start of exercise (Anderson, 1976), and are important in the treatment of acute severe asthma. Their mechanism of action is thought to occur primarily by the relaxation of airway smooth muscle cells, but they also increase mucociliary clearance.

They do not have any effective anti-inflammatory activity. Although sympathomimetic agents, short acting β_2-agonists have few side effects when inhaled, but tremor, palpitation, and tachycardia can occur with high doses. They should be used for symptom relief on an as required basis, since studies have shown that their regular use provides no additional benefit and may even be harmful (Dennis, 2000; Walters, 2000). Furthermore, individual patients' requirement for short acting $\beta2$-agonists provides a useful guide to the need for a step-up in treatment; current guidelines suggest that if they are used on a daily basis for symptom control then regular anti-inflammatory agents are indicated (Global Initiative for Asthma, 2003). The use of more than one canister of short acting β_2-agonists per month has been particularly associated with poorly controlled disease and should therefore alert the prescriber to the need for increased regular anti-inflammatory treatment (Suissa, 1994). Tolerance to the effects of short acting β_2-agonists can occur, particularly to the protection against bronchoconstriction induced during indirect challenges (O'Connor, 1992).

Corticosteroids are currently the most effective anti-inflammatory agents for the treatment of asthma and inhaled corticosteroids are currently recommended for all patients with mild persistent asthma who require short acting β_2-agonists more than once per day (British Thoracic Society, 2003) or those with intermittent asthma who experience severe deteriorations (Global Initiative for Asthma, 1995). They exert their anti-inflammatory effects through a diverse range of mechanisms including the activation of the glucocorticoid receptor leading to the regulation of transcription of target genes, and the direct inhibition of a range of inflammatory cells, particularly eosinophils. Studies have consistently shown that treatment with regular inhalations of corticosteroids results in significant improvements in airway inflammation in asthma, an effect demonstrated on bronchial biopsies (Djukanovic *et al.*, 1992) and also on non-invasive markers of airway inflammation such as the differential

eosinophil count in induced sputum or nitric oxide concentrations in exhaled breath (Jatakanon *et al.*, 1999). Furthermore, there is evidence that corticosteroid treatment is not helpful in the absence of eosinophilic airway inflammation (Pavord *et al.*, 1999). In conjunction with these improvements in airway inflammation, inhaled corticosteroids improve symptoms (Djukanovic *et al.*, 1992), health status (Mahajan *et al.*, 1997), airway hyper-responsiveness and lung function (Haahtela *et al.*, 1991), and reduce asthma exacerbations (O'Byrne *et al.*, 2001).

The crommones sodium cromoglycate and nedocromil sodium, both given by inhalation, have also been used as controller therapies in mild persistent asthma (Blobal Initiative for Asthma, 1995). Their mechanism of action is not fully understood, although they are believed to suppress IgE-mediated inflammatory responses and may inhibit inflammatory cells (Diaz *et al.*, 1984). Sodium cromoglycate has been shown to reduce symptoms and deterioration frequency (Edwards *et al.*, 1994) and nedocromil sodium to improve symptoms, lung function, and airway responsiveness (Bel *et al.*, 1990). Overall, however, they appear to be rather less effective than low dose inhaled corticosteroids (Szefler *et al.*, 1998) and their long-term effects on airway inflammation are unknown. Therefore the use of these agents in adults has been aborted, instead is to use low dosed of inhaled steroids for most of mild persistent asthma patients.

For moderate persistent asthma, there are a number of options that patients can use:

i. **Long acting β$_2$-agonists (salmeterol and formoterol),** they are currently generally recommended as the first choice for patients who have symptoms that persist despite

regular inhaled corticosteroids. Salmeterol is a partial agonist of the β_2-receptor while formoterol is a full agonist. Both appear to have similar clinical effects, but formoterol has a more rapid onset of action (van Noord *et al*., 1996). Side effects of tachycardia, tremor, and muscle cramps are rarely a problem unless given in high doses. Tolerance to the effects of long acting β_2-agonists with loss of bronchodilator activity after the subsequent administration of both short and long acting β_2-agonists has been reported (Newnham *et al*., 1995; Grove *et al*., 1995). As with short acting β_2-agonists, these agents work primarily via the relaxation of airway smooth muscle, with additional effects on mast cells and vascular permeability, but without significant anti-inflammatory activity (Nelson *et al*., 1995). This lack of anti-inflammatory activity precludes their use as first line agents in asthma (Lazarus *et al*., 2001) and current guideline recommend that they are only prescribed alongside regular inhaled corticosteroids (British Thoracic Society, 2003; Global Initiative for Asthma, 1995). When uses in this way, long acting β_2-agonists have been shown to improve daytime and night time symptoms and the need for reduce β_2-agonists (Pearlman *et al*., 1992; Kesten *et al*., 1991). In a randomized controlled trial of 852 patients treated with low dose inhaled corticosteroids (the FACET study) the addition of formoterol to inhaled low or high dose budesonide improved symptoms and lung function. In addition, the number of both mild and severe asthma exacerbations was reduced, where mild deteriorations are defined as a fall in peak expiratory flow (PEF) of > 20% from baseline on two or more days, increase use of rescue short acting β_2-agonists or nocturnal wakening and severe deteriorations defined as a fall in PEF of > 30% from baseline on two or more days or deterioration in symptoms requiring rescue oral corticosteroids (Pauwel *et al*., 1997).

ii. **Increasing the dose of inhaled corticosteroids.** The traditional approach to patients with persistent symptoms despite low doses of inhaled corticosteroids was to increase the corticosteroid dose, but the evidence for this is somewhat inconsistent. While some studies have demonstrated clear dose related improvements in symptoms and lung function (Pauwels *et al*., 1997; Nathan *et al*., 2000; Dahl *et al*., 1993), others have not demonstrated clinically important benefits with moderate or high doses (Adams *et al*., 2000). Overall the beneficial effects of increasing the dose of inhaled corticosteroids appear to be modest and may be largely outweighed by the increased risk of side effects. Some studies have suggested that higher doses of inhaled steroids are less effective at controlling symptoms and peak flow variability compared with the addition of long acting β_2-agonists (Pauwels *et al*., 1997; Greening *et al*., 1994). There is increasing evidence that asthma deteriorations are associated with eosinophilic airway inflammation (Pizzichini *et al*., 1999; Turner, 1998), and the benefits of the high doses of inhaled corticosteroids on deterioration frequency are therefore likely to reflect dose related anti-inflammatory effects. Turner and colleagues (1998) have shown that a doubling of the dose of beclomethasone in subjects with symptomatic asthma and a persistent sputum eosinophilia despite treatment with inhaled corticosteroids improved symptoms and significantly reduced the sputum eosinophil count, whereas the addition of salmeterol led to improvements in symptoms but no change in the sputum eosinophil count. Similarly, in a study of increasing doses of budesonide in patients with steroid naïve asthma, Jatakanon *et al.* (1999) demonstrated a dose dependent reduction in the percentage of eosinophils in induced sputum. While low doses of inhaled corticosteroids are therefore probably appropriate for the majority of patients, higher doses of these drugs may be indicated in some patients who experience frequent severe deteriorations of asthma or who have persistent airway inflammation.

iii. **Leukotriene antagonists.** Monteukast and zafirlukast are both effective cysteinyl leukotriene receptor antagonists capable of markedly inhibiting exercise-induced bronchoconstriction (Finnerty, 1992; Manning, 1990) and the early and late response to inhaled allergen (Taylor, 1991; Diamant, 1999). When added to as required β_2-agonists, clinical trials have shown improvement in lung function (Spector, 1994; Reiss, 1997), reduction in the need for rescue bronchodilators (Spector, 1994; Leff, 1998), and some evidence of a reduction in eosinophilic airway inflammation (Pizzichini, 1999). In the UK, leukotriene antagonists are currently licensed for use in patients who remain symptomatic despite treatment with inhaled corticosteroids. Clinical trials have shown evidence of efficacy in patients taking high doses of inhaled steroids (Christian, 2000), and the introduction of montelukast has been shown to allow a reduction in the dose of inhaled corticosteroid without loss of asthma control (Lofdahl, 1999). Some studies have also shown that the addition of long acting β_2-agonists results in greater improvements in asthma control than the addition of leukotriene antagonists (Nelson, 2000; Fish, 2001).

iv. **Theophylline.** It has been used for many years in relatively high doses as a bronchodilator, but due to adverse effects it has often been reserved for use in patients with more severe asthama. Gastrointestinal upset is particularly common (Pollard *et al.*, 1997) but tachycardia and arrhythmia can also occur and measurements of serum concentrations are generally advised with high dose treatment (Blobal Initiative for Asthma, 1995). Recent interest has been in the use of theophylline at lower doses where the risk of side effects is minimized. The combination of low dose inhaled corticosteroids and theophylline has been shown to result in comparable asthma control as higher doses of inhaled corticosteroids and may provide slightly greater improvements in lung function (Evans *et al.*, 1997; Ukena *et al.*, 1997; Lim *et al.*, 2000). A metanalysis has suggested that long acting β_2-agonistsare more effective than

theophylline in patients taking low doses of inhaled corticosteroids and result in fewer side effects (Wilson, 2000). Unlike long acting β_2-agonists, however, theophylline has been shown to have possible anti-inflammatory activity and may therefore have a role in some patients (Sullivan, 1994).

2.5 β_2-Adrenergic Receptor Agonists

2.5.1 Development of β_2-Adrenergic Receptor Agonists

β_2-agonists are the most common and effective therapy for the anticipation of symptoms of EIA in asthmatic patients (Dempsey, 1977). β_2-agonists relax smooth muscle, decrease vascular permeability, increase airflow, and moderately inhibit mediator release (Williams and Shapiro, 1995). For most patients, inhaled β_2-agonists are the first-line treatment of asthma and EIA (Collomp *et al.*, 2010). An extracted alkaloid identified nowadays as ephedrine was found from ma huang (Ephedra equisetina), a traditional Chinese medicine, which was used for more than 2000 years for the short-term treatment of aspiratory symptoms. The nonselective α-Adrenergic Receptor agonist and β-Adrenergic Receptor agonist epinephrine was brought into clinical practice and conducted by the subcutaneous route for the treatment of acute asthma at the beginning of 20th century (Bullowa and Kaplan, 1903). Epinephrine was far from the ideal drug even though it was a major advancement in medical field, the side effects caused hypertension and tachycardia due to its effect on α_1- and β_1-Adrenoreceptors in the cardiovascular system in bronchodilation use. In addition, it has a short duration of action and has to be administered parenterally due to its metabolic instability (Waldeck, 2002). The nonselective β-agonist became the standard-of-care bronchodilator, though the adverse effects were complicated (Waldeck, 2002), and caused

tachycardia and predisposes patients to cardiac dysrhythmias. Isoproterenol has another defect in its short duration of action due to its ready transportation into cells by the uptake process for catecholamines (Gryglewski and Vane, 1970), except in the gut, it is converted by catechol-O-methyltransferase (COMT) to 3-O-methylisporenaline (Blackwell *et al.*, 1974). A noncatechol resorcinol derivative of isoproterenol named metaproterenol was subsequently developed in the early 1960s. Although it was an effective bronchodilator when inhaled, it still produced cardiac side effects because it did not distinguish between β_1- and β_2-Adrenoreceptors. The modern era of selective β_2-agonists started after the discover of salbutamol (albuterol in USA) by Sir David Jack and colleagues working at Allen and Hanburys in the UK (Jack, 1991).

β_2-agonists consist of a benzene ring with a chain of two carbon atoms and either an amine head group or a substituted amine head group, and the majority of the β_2-agonists currently used in asthma are structurally related to adrenaline (Figure 1). If a hydroxyl (OH) group is present at positions 3 or 4 on the benzene ring, the structure is catechol nucleus and hence the agent is termed a catecholamine. If these OH groups are substituted or repositioned, the drug is generally less potent than the synthetic catecholamine isoproterenol, which has both strong β_1-agonist and β_2-agonist properties and is the more powerful bronchodilator (McFadden, 1981).

Adrenaline (Epinephrine)
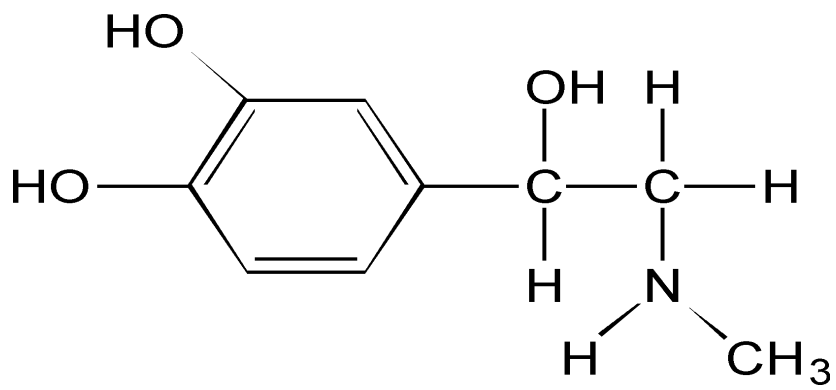
Short-acting β₂-agonisits

Salbutamol
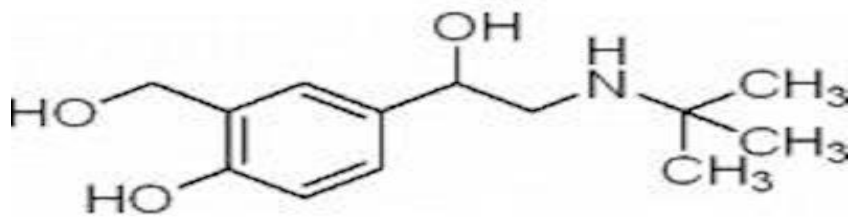
Terbutaline
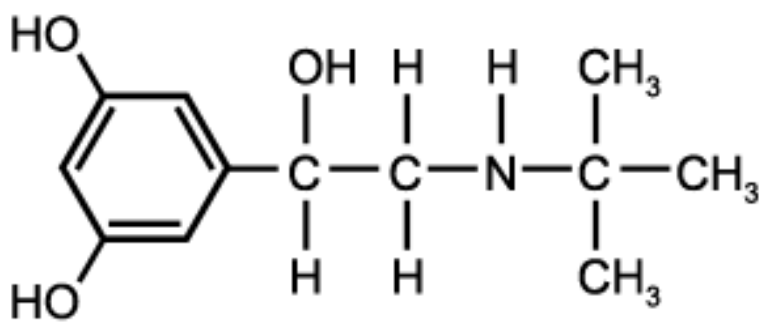
Salmeterol
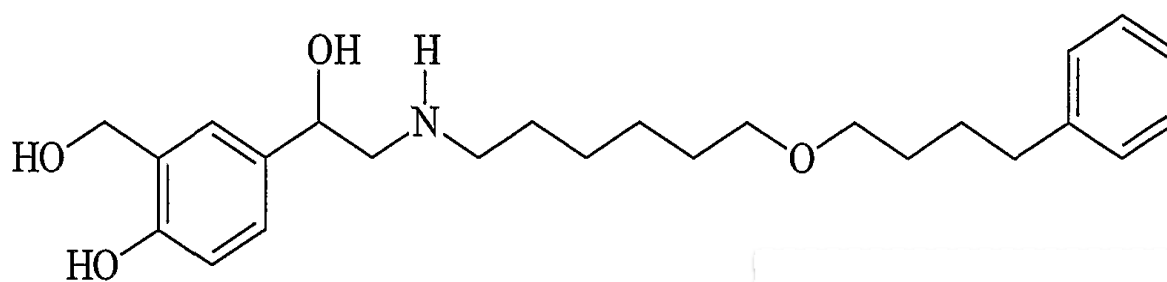
Formoterol
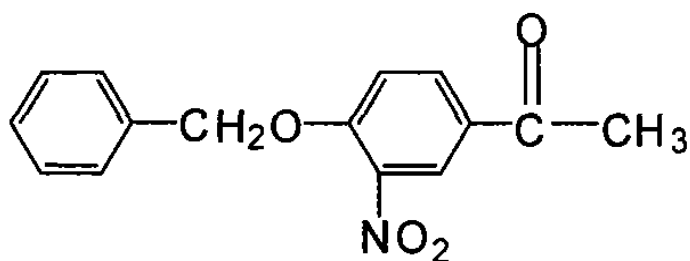
Figure 1. Chemical structure of adrenaline (epinephrine) and currently used short- and long-acting beta-2 agonists.

The short duration of action (4 to 6 hours) of β₂-agonists was the major limitation of its use between 1960s and 1970s, thus, the next step of β₂-agonists was the development of the long-acting drugs salmeterol and formoterol, the duration of action of which is about 12 hours, and it made their use for maintenance treatment (*e.g.*, for reducing nighttime symptoms) more appealing (Lotvall, 2002). A pure R-isomer of salbutamol, levalbuterol (Berger, 2003), and the R,R-enantiomer of formoterol, arformoterol (Cazzola *et al.*, 2010b), have been developed. It is claimed that they do not have the S-enantiomer and therefore safer, at least for (S)-salbutamol, which is now known to have unwanted effects in the lung (Page and Morley, 1999). At present, several once-a-day ultra-long-acting β₂-agonists are in different stages of clinical development (Cazzola and Matera, 2009; Cazzola *et al.*, 2011).

2.5.2 Short-Acting β₂-Adrenergic Receptor Agonists

Short-acting β₂-agonists are mainly used to alleviate asthma symptoms rapidly and often in emergent situations. They can normally be divided into two broad groups according to duration of action after inhalation of conventional doses: (1) The catecholamines isoprenaline and rimiterol, which have a very short action of one to two hours; and (2) Those conventionally described as short-acting, such as fenoterol, salbutamol, and terbutaline, which are active for three to six hours, though the action of fenoterol may be slightly longer (Beardshaw *et al.*, 1974). In general, higher dosage of any inhaled β₂-agonist has a considerably longer action, for instance, 1600 μg salbutamol works longer than 200 μg salbutamol (Corris *et al.*, 1983). It is not recommended to use short-acting β₂-agonists as regular treatment for asthma as they are only reliever medication (Karjalainen, 2008) and might lead to increased asthma exacerbation (Sears *et al.*, 1990) and worsen airway inflammation (Manolitsas *et al.*, 1995).

Salbutamol has negligible α-agonist activity at recommended clinical doses and demonstrates a substantial greater selectivity between β₂- and β₁-Adrenoreceptors than any other product previously available (Sears and Lotvall, 2005). In *vitro* tests that used guinea pig isolated atria for β₁-Adrenoreceptors and tracheal preparations for β₂-Adrenoreceptors, respectively, documented that isoproterenol has equal affinity for β₂- and β₁-Adrenoreceptors, orciprenaline is slightly more selective for β₁-Adrenoreceptors, whereas salbutamol is more selective for β₂-Adrenoreceptors (O'Donnell, 1972). Therefore salbutamol has approximately equivalent potency at relaxing human isolated bronchi in *vitro* and bronchodilator potency in subjects with asthma compared with epinephrine (Baldwin *et al.*, 1994). However, the effects of salbutamol and epinephrine on histamine-induced contraction in *vitro* are significantly

different from their effects on histamine reactivity in *vivo*. Salbutamol has no effect on the maximal response to histamine, whereas epinephrine reduces it by 54% in *vitro*. In contrast, salbutamol is more potent in *vivo* (Baldwin *et al.*, 1994). Other in *vitro* studies have also demonstrated that salbutamol acts as a partial agonist at β_2-Adrenoreceptors compared with isoproterenol (O'Donnell and Wanstall, 1978). In human isolated bronchi, salbutamol is a partial relaxant of airway smooth muscle (ASM), whereas isoproterenol has greater efficacy (Goldie *et al.*, 1986). Nonetheless, compared with isoproterenol, salbutamol is at least as potent a bronchodilator, has a much longer duration of action, and is much less likely to influence blood pressure or heart rate. Nevertheless, isoproterenol produces tachycardia that runs parallel to bronchodilation. In contrast, salbutamol causes the same maximum bronchodilation but with minimal cardiovascular responses. After inhalation of salbutamol, maximum brochodilation can be seen within 15 minutes of inhalation (Price and Clissold, 1989). Salbutamol binds only weakly to the receptor and quickly diffuses back into the microcirculation accounting for its short duration of action (4-6 hours; Sears and Lotvall, 2005). However, because of its rapid onset of action, which is a clear clinical advantage for the reversal of bronchoconstriction, salbutamol is usually considered the drug of choice as relief medication for symptoms of bronchospasm. This thesis will discuss the results of the experiments on non-asthmatic athlete especially taking salbutamol in later content.

2.5.3 Long-Acting β_2-Adrenergic Receptor Agonists

Long-acting β_2-agonists such as salmeterol and formoterol provide twelve-hour bronchodilation (Lotvall, 2002). It has been demonstrated that long-acting β_2-agonists can be used for treatment of asthma as an add-on to inhaled corticosteroids, which have the effect of improving lung functions, relieving asthma symptoms and reducing asthma exacerbations

(Lemanske *et al.*, 2001, cited in Karjalainen, 2008).

The different chemical structures of formoterol and salmeterol grant remarkably different pharmacological characteristics, even though they are both potent and effective β_2-Adrenergic Receptor agonists. It has been suggested that salmeterol specifically binds to the β_2-AR via the salbutamol "head group", whereas a secondary exosite has been proposed in which the lipid tail binds to grant the long duration of action (Green *et al.*, 1996). The exosite is an auxiliary binding site, a domain of highly hydrophobic amino acids within the fourth domain of the β_2-AR (Johnson, 1998). When the lipid tail is in association with the exosite, the salmeterol is prevented from dissociating from the β_2-AR, but the head can freely engage and disengage the active site by the Charniere (hinge) principle, the fulcrum being the oxygen stom in the side chain. The position of this oxygen atom is critical for the long duration of action (Johnson, 2001). The salmeterol molecule is >10,000 times more lipophilic than salbutamol. It partitions rapidly into the cell membrane and then diffuses laterally to approach the active site of the β_2-AR through the membrane. The process is relatively slow (>30 minutes) and accounts for the slow onset of action of salmeterol compared with salbutamol (Johnson, 2001).

By contrast, formoterol is not thought to be able to access the exosite and an alternative hypothesis for its long duration of action has been propsed whereby the lipophilic, basic nature of this drug allows formoterol to partition effectively into the lipid bilayers of ASM after inhalation. This partitioning then permits an effective concentration of agonist to be present over time in the form of a depot within the ASM, from where formaterol progressively leaches out to interact with the active site of the β_2-AR, providing a prolonged

duration of action. This is known as the diffusion microkinetic theory (Anderson *et al.*, 1994). The size of the depot is determined by the concentration or dose of formoterol applied. In airway preparation, the onset of action of formoterol is somewhat delayed compared with salbutamol, and the duration of relaxant activity, although longer, is concentration-dependent (Johnson, 2001). In any case, formoterol is somewhat less lipophilic than salmeterol and is believed to diffuse more rapidly through the lung tissues, reaching the site of action faster than salmeterol (Anderson, 1993).

2.6 Action of β_2-adrenergic receptor agonists

For more than four decades, β_2-agonists have been used to relieve asthma symptoms. Potent bronchodilators exist in the lungs to provide rapid relief from bronchoconstriction for asthmatic athletes. Bronchodilators are known to relax smooth muscle of the airway, which will then increase airway calibre (Sporer, 2006). β_2-agonists act through the β_2-receptor which can be found in high concentrations in both the bronchial epithelium and bronchial smooth muscle (Lotvall, 2001). β_2-receptors are a member of the seven transmembrane domains G protein-coupled family of receptors. β_2-receptors in airway cells are stimulated by the sympathomimetic agents to generate a number of effects, including smooth muscle relaxation and bronchodilation mediated by the activation of adenyl cyclase, which will produce cyclic 3'5' adenosine monophosphate (cAMP). This leads to an inhibition of myosin-actin biding in the bronchial smooth muscle (Sporer, 2006). The end result is smooth muscle relaxation, which has been shown in both asthmatics and non-asthmatics.

2.6.1 β2-Adrenergic Receptor Agonists on Non-asthmatic Athletes

Oral β_2-agonists are generally used for asthma patients' brochodilating effect, it is also known that oral β_2-agonists increase muscle mass as well, by stimulating protein anabolism and they also increase the metabolism of lipids and carbohydrates (Lafontan, 1988; Price, 1989; Martineau, 1992). In addition, some early studies identified effects of oral β_2-agonists on the central nervous system consistent with antidepressant activity (Belmaker, 1982).

A number of studies have pointed out that a fall in arterial oxygen saturation may take place in highly trained athletes during high intensity exercise (Dempsey, 1999). There are many possible reasons which cause this exercise-induced arterial hypoxemia (EIAH), temperature- and acid-induced shifts in O_2 dissociation at any given arterial PO_2 contribute to EIAH, as do both an excessive alveolar-to-arterial PO_2 difference and inadequate compensatory hyperventilation (Harms, 2000). However, the effect on performance level after a drop in arterial oxygen saturation is questioned (Koskolou, 1994). Notionally, the use of β_2-agonists may result in an improvement in the oxygen transfer from the alveoli to the arterial blood in elite endurance athletes with extreme demands to the oxygen transport system, because it reduces the risk of pulmonary edema by up-regulating the sodium-driven clearance of alveolar fluid (Sartori, 2002). In contrast, Stewart *et al.* (2003) demonstrated no enhancement of arterial oxygen saturation during exercise when an acute, inhaled, therapeutic dose of salbutamol was administered to highly trained non-asthmatic athletes with EIAH.

It is improbable that salbutamol and other β_2-agonists bronchodilators are ergogenic, because in healthy, non-asthmatic subjects bronchoconstriction is not a performance-limiting factor.

Thus, β₂-agonists would need to reach the systemic circulation in order to display its assumed ergogenic action. Comparing with orally administered therapeutic doses, clinically relevant doses in the form of aerosols result in much lower systemic plasma concentrations (Walker, 1972). The adaption of the human body to exercise is strongly associated with β₂-agonists stimulation. Increased muscle contractility, transport capacity as well as increased availability of substrates for energy metabolism are all, more or less, mediated by stimulation of β-adrenergic receptors. Some studies have demonstrated increased plasma lactate concentrations after systemic administration of β-adrenergic agonists during exercise, which together with changes in the respiratory exchange ratio (RER), could be seen as indirect measures of carbohydrate oxidation and glycolytic flux (Collomp, 2000; Van Baak, 2000; Hallen, 1996).

2.7 Inhaled β2-agonists

In the case of acute break through episodes of asthma, corticosteroids and other similar treatments are ineffective and bronchodilator therapy in the form of inhaled short-acting β₂-agonists is recommended (Rundell and Jenkinson, 2002). Inhaled short-acting β₂-agonists relax smooth muscle, increase airflow, decrease vascular permeability and moderately inhibit mediator release (Williams and Shapiro, 1995). In addition to their use following an acute episode, it is recommended that the short acting β₂-agonist should be inhaled 30 minutes before exercise. Treatment with two 'puffs' (200 μg; a standard dose 'puff' is 100 μg) of a short-acting β₂-receptor agonists shortly prior to exercise will give peak bronchodilation in 15 to 60 minutes and protection from EIA for at least 3 hours in most patients (Bierman, 1984). This type of treatment has been shown to improve pulmonary function in 90% of individuals with EIA (Anderson *et al.*, 1979). The degree of attenuation to EIA observed following short-

acting β_2-agonist administration has ranged from 50-100% in clinical trials using both adults and children (Anderson *et al.*, 1976; Boulet *et al.*, 1989).

Short acting β_2-agonists are not recommended as the only source of treatment for EIA if they are inhaled more than three times a week (BTS, 2004). Furthermore, Anderson and Brannan (2004) suggest the following limitations in the use of β_2-agonists: (1) daily use of inhaled β_2-agonists can result in the development of tolerance and reduction in the duration of their protective effect; (2) the severity of EIA may increase when exercise is performed between 8-12 hours following the last inhaled dose and; (3) prolonged recovery of lung function after an asthma attack. These responses are believed to be due to desensitisation of the β_2-receptors on mast cells leading to greater mediator release. Since inhaled β_2-agonists are used by a large number of asthmatics in the UK, these findings may have implications for initial therapy given to individuals diagnosed with mild EIA (See Table 3).

Step	Medication
1. Mild Intermittent Asthma	Inhaled short-acting β_2-agonists
2. Introduction of regular preventer therapy	Corticosteroids
3. Add on therapy	Increase current medication, Inhaled long acting β_2-agonists, theophylines, leukotrienes receptor antagonists, anti-histamines
4. Poor control on moderate dose of corticosteroid and add on therapy	Add forth drug from list above
5. Continuous or frequent use of oral corticosteroids	Oral corticosteroids

Table 3. The British Thoracic Society 5 Step asthma medication guidelines (British Thoracic Society. (2004). 'British Guidelines on the Management of Asthma: A national clinical guideline')

2.8 Anti-Doping: The World Anti-Doping Authority (WADA) and the International Olympic Committee – Medical Commission (IOC-MC)

The Prohibited List published by the World Anti-doping Authority (WADA, 2014) states that all β_2-agonists, including all optical isomers (*e.g.* d- and l-) where relevant, are prohibited with the exception of three formats: inhaled salbutamol (maximum 1600 micrograms over 24 hours); inhaled formoterol (maximum delivered dose 54 micrograms over 24 hours); and salmeterol only when taken by inhalation in accordance with the manufacturers' recommended therapeutic regimen.

Prior to the 2002 Salt Lake City Winter Olympic Games, short-acting β_2-agonists were permitted for use by an asthmatic athlete with a letter of support from a medical officer to explain symptoms and diagnosis. For the 2002 Winter Olympics the IOC-MC mandated the requirement for a formal Therapeutic Use Exemption (TUE) for the use of inhaled salbutamol, formoterol, salmeterol and terbutaline for asthmatic athletes. This rule was changed again in 2010, where TUE is no longer required for salbutamol and salmeterol with formoterol and terbutaline remaining on the restricted list. Athletes who have asthma, EIA, EIB or AHR can use inhaled salbutamol and salmetorol as long as they declare they are using the drug therapeutically to treat their condition. The regulations for the use of salbutamol and salmeterol are relatively relaxed when compared with terbutaline and formotorol.

2.9 Ergogenic (performance enhancing) Effects of Short Acting β_2-agonists

It has been suggested that oral salbutamol appears to have performance-enhancing effects mediated by its role in increasing strength (Martineau *et al.*, 1992). Oral salbutamol is therefore not permitted to be used by athletes. Oral salbutamol, however, is distinguishable from the inhaled form and can be easily identified by anti-doping involving a urinary concentration upper threshold of 1000 ng/mL salbutamol (free plus gluconuride) and the ratio of the S1 and R-enantiomers (Fitch *et al.*, 2008; Berges *et al.*, 2000).

In contrast, the majority of studies examining the efficacy of inhaled short acting β_2-agonists have failed to observe an ergogenic effect (Wolfarth *et al.*, 2010). The majority of previous studies has focused on endurance performance in Caucasian males and has reported no effect of inhaled short acting β_2-agonist on performance (Larson *et al.*, 2005). A very small number

of studies have examined the ergogenic effect of inhaled short acting β_2-agonist outside of endurance performance. One study reported an increased peak power output in a 15s supra-maximal cycling test (Signorile *et al.*, 1992) however; others have failed to replicate this result (Sporer *et al.*, 2008) following the administration of 200 μg, 400 μg and 800 μg of salbutamol.

Kindermann and Meyer (2006) reviewed twenty previous studies that addressed the performance-enhancing effects of inhaled β_2-agonist on non-asthmatic athletes, most of which have failed to show an improvement in athletes' performance. Meeuwisse *et al.* (1992), Fleck *et al.* (1993), Lemmer *et al.* (1995) and Norris *et al.* (1996) conducted four separate and different endurance cycling studies on male athletes, with dosage of salbutamol ranging from 200 μg to 400 μg. All four studies reported no significant improvement in the athletes' performance as well as no significant enhancement in VO_{2max}. Similarly, McKenzie *et al.* (1983) and Morton *et al.* (1992) conducted studies of middle and long distance running with a dosage of 200 μg salbutamol and concluded no significant improvement compared with placebo condition. Of note, these two studies included female athletes as well as male athletes, therefore expanding the study results across gender. A small number of other studies in a similar field were conducted with cross-country skiers, triathletes, or a combination of these exercises (Goubault *et al.*, 2001; Sandsund *et al.*, 1998; Stewart *et al.*, 2002) and similar conclusions were drawn showing no significant performance enhancing effects of inhaled salbutamol.

A small number of studies have also demonstrated the ergogenic effects of inhaled β_2-agonist on non-asthmatic athletes. Bedi *et al*. (1988) have performed a study on 14 male and one

female cyclist and triathletes with inhalation of 180 μg salbutamol and report a significant increase in ride time during a maximal effort workload to exhaustion following one-hour heavy continuous exercise. Van Baak *et al.* (2004) also conducted a placebo controlled study with 16 cyclists and triathletes, all male, and concluded a significant improvement in cycling time trial after the inhalation of 800 μg salbutamol. Signorile *et al.* (1992) conducted a study on recreational athletes reporting a significant increase in peak power during a 15-second Wingate test following inhalation of 180 μg salbutamol, they report.

In contrast, a number of studies have revealed the opposite findings, where the performance after inhalation of β_2-agonist was reduced compared with placebo conditions (Heir and Stemshaug, 1995; Carlsen *et al.*, 1997). Carlsen *et al.* (1997) reported a significant decrease in running time to exhaustion after the administration of 800 μg of inhaled salbutamol. Heir and Stemshaug (1995) reported a similar conclusion and found a reduced endurance time at 110% VO_{2max} following the inhalation of 0.05 mg·kg^{-1} of salbutamol. In another study Carlsen *et al*. (1997) conducted the same test over the same participant, following the inhalation of 50 μg of salmeterol, demonstrating similar results compared with the use of salbutamol.

A recent systematic review on previous studies of the effects of inhaled β_2-agonist on physical performance was conducted by Pluim *et al.* (2011), which reviewed 26 placebo-controlled studies involving 403 participants. The results revealed no significant enhancement on VO_{2max}, peak power and exercise time to exhaustion. Another important finding was that the types of β_2-agonist used, whether salbutamol, salmeterol, formoterol, or terbutaline, made no significant difference. However, care is warranted in the interpretation

of this finding given the low and moderate doses of inhaled β_2-agonists used in previous studies. Limited data exists examining the use of higher doses of salbutamol up to the WADA recommended daily upper limit of 1600 µg, particularly when inhaled in a single bolus. Furthermore, limited data exists examining the impact of inhaled short-acting β_2-agonists on team game performance. Given the popularity of team games such as association football, it is clear that research is required to investigate any potential ergogenic impact.

2.10 Urinary concentration of inhaled salbutamol

Sporer, Sheel and McKenzie (2008) undertook a time-trial performance with healthy non-asthmatic athletes. The results from their study show the urine concentration of salbutamol increases as the dose of inhaled salbutamol increases, yet the highest urine concentration did not exceed the WADA daily upper limit of 800 µg even with the largest inhaled dose of 800 µg. Pichon *et al*. (2006) examined urine concentration after inhalation of multiple small doses of salbutamol and reported the urine concentrations far below the WADA daily upper limit. Elers *et al*. (2010) studied athletes with doctor-diagnosed asthma and found peak urine concentration below the WADA upper threshold after inhalation of 800 µg of salbutamol. Elers *et al*. (2011) investigated the differences in urine concentrations between asthmatic and healthy subjects. No significant difference was found and urine concentrations for both groups did not exceed the WADA daily upper limit when corrected for the urine specific gravity. To date, a limited number of studies have examined the impact of inhaling the WADA recommended daily limit of 1600 µg in a single administration. Accordingly, it is not known what impact this dose of salbutamol would have on urine concentration. Dehydration in exercise can significantly reduce body mass, which might affect the urine concentration to exceed the WADA limit. However, little is known in this potential impact of dehydration and

we believe it is important to understand this relationship between dehydration and urine concentration.

2.11 The pharmacological and ergogenic effect of Caffeine

Caffeine (1,3,7-Trimethylpurine-2, 6-dione; $C_8H_{10}N_4O_2$) is a bitter, white crystalline purine, a methylxanthine alkaloid, and is closely related chemically to the adenine and guanine contained in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Caffeine can be found in a variety of food sources including: coffee, tea, soft drinks, chocolate, and kola nuts.

2.11.1 Caffeine Action

An important mode of action for caffeine has been identified as the inhibition of adenosine receptors within the physiological concentration range of caffeine (normally less than 70 µmol/L). Caffeine has a similar structure to adenosine and can bind to cell membrane receptors for adenosine, hence blocking their action. Adenosine receptors are found in the brain, heart, smooth muscle, adipocytes and skeletal muscle etc., most tissues. The ubiquitous nature and varied types of adenosine receptor facilitates caffeine simultaneously affecting a variety of tissues, resulting in a wide range of often interacting responses (Palmer *et al.*, 1995; Shryock *et al.*, 1997; Ralevic *et al.*, 1998; Fredholm, 1995).

The mechanisms listed below may explain why caffeine could possibly enhance athletes' performance during the competition:

1. Antagonism of adenosine (Holtzman *et al.,* 1991): caffeine blocks adenosine receptors because of its close conformity to adenosine, thus emulously restraining its action (Rieiro *et al.,* 2010). Caffeine can reduce cerebral blood flow (Cameron *et al.,* 1990), and adenosine-mediated vasodilatation can be reduced by antagonize adenosine receptor in blood vessels and myocardial blood flow is reduced accordingly (Namdar *et al.,* 2009).

2. Increased fatty acid oxidation: enhancement of lipolysis results in sparring of glycogen (Spriet *et al.,* 1992). Caffeine switches the substrate preference from glycogen to fat by increasing hormone sensitive lipase (HSL) activity and inhibition of glycogen phosphorylase activity (Rush *et al.,* 2001).

3. Caffeine acts as a nonselective competitive inhibitor of the phosphodiesterase enzymes (Umemura *et al.,* 2006): Phosphodiesterases hydrolyze the phosphodiesterase bond in molecules such as cyclic adenosine monophosphate (cAMP), inhibiting the breakdown of cAMP. CAMP activates lipolysis by activating HSL and is an important molecule in the epinephrine cascade (Chesley *et al.,* 1995). It further activates protein kinase A, which in turn can phosphorylate a number of enzymes involved in glucose and lipid metabolism (Graham, 2001).

4. Increased post-exercise muscle glycogen accumulation: enhanced recovery by increased rate of glycogen resynthesis following exercise (Taylor *et al.* 2011). Battam *et al.* (2004) reported that caffeine ingestion has no effect on glycogen accumulation during recovery in recreationally active individuals. Pedersen *et al.* (2008) recently reported that caffeine (8 mg/kg body weight) co-ingested with carbohydrates (CHO) increases rates of post-exercise muscle glycogen accumulation compared with consumption of CHO alone in well-trained athletes after exercise-induced glycogen depletion. Caffeine added to post-

exercise CHO feeding seems to have the potential to improve glycogen resynthesis, although this issue needs further study.

5. Mobilization of intracellular calcium: It has been shown that caffeine can enhance calcium release from the sarcoplasmic reticulum (Supinski *et al.*, 1984) and can also inhibit its reuptake (Endo, 1977). Via this mechanism, caffeine can enhance contractile force during submaximal contractions in habitual and non-habitual caffeine consumers (Tarnopolsky *et al.*, 2000). Intracellular calcium favors the activation of endothelial nitric oxide synthase, which increases nitric oxide (Echeverri *et al.*, 2010). Some of the ergogenic effects of caffeine might therefore as well be mediated partly by affecting the neuromuscular system and increasing contractile force (Tarnopolsky, 2008).

2.11.2 Caffeine and athletic performance

The possible ergogenic effect of caffeine has been recognised by the researchers since the late 18th century (Jacobson and Kulling, 1989). Schirlitz (1930) (cited in Jacobson and Kulling, 1989) was the first to present laboratory evidence that the use of caffeine could increase work output. This finding was confirmed by Ivy *et al.* (1979) who reported that total work output was increased after the ingestion of 250 mg caffeine. Similarly, Costill *et al.* (1977) concluded that the cycling time to exhaustion at 80% VO_{2max} was significantly increased after the administration of 330 mg caffeine. Given the potential for caffeine to act as powerful ergogenic aid a large number of studies over the last four decades have examined the role of caffeine in enhancing athletic performance across a range of sports.

Since these early findings about caffeine could be a powerful ergogenic aid, the research in this field has expanded rapidly. Researchers tested the ergogenic effect of caffeine on different types of sports, using different doses as well as controlling for other conditions. Jenkins *et al*. (2008) found that the cycling performance was significantly improved in 13 cyclists following the administration of low doses of caffeine (2-3 mg/kg) compared with placebo conditions. A more recent study by Desbrow *et al*. (2012) investigated this effect by placing two different doses of caffeine, 3 mg/kg and 6 mg/kg, using a randomised, placebo controlled, double-blind design. The results demonstrated a significant improvement in cycling performance with dose of 3 mg/kg of caffeine, but no additional improvement was observed with of the higher caffeine intake (6 mg/kg).

Caffeine with a concentration > 12 μg/mL in the urine was considered doping, and was on the WADA banned list from 1962 to 1972 and then 1984 till 2003 when it was placed on the monitored list in order to monitor the possible potential of misuse in sport. In opposition to this move by WADA, caffeine has been demonstrated to be ergogenic at doses lower than those that result in a urine concentration of 12 μg/mL, and higher doses appear to reveal no additional performance enhancing effect (Graham, 2011). A large number of athletes tested positive for caffeine during the second banned period. The sanctions ranged from warnings up to 2-year suspensions, although suspensions usually were only 2 to 6 months. According to WADA, one of the reasons caffeine was removed from the Prohibited List was that many experts believe it to be omnipresent in beverages and food and that having a threshold might lead to athletes being sanctioned for social or dietary consumption of caffeine (Prohibited list, 2012). Furthermore, urinary caffeine concentrations can very considerably because it is metabolized at very different rate in individuals (Fenster *et al*., 1998), and does not always correlate to the dose ingested.

A number of studies have reported significantly improved cycling time-trial performance, which is most likely related to a greater reliance on fat metabolism (Mc Naughton, *et al.*, 2008); or improved maximal cycling power, which is related to decreased neuromuscular fatigue (Del Coso, *et al.*, 2008). According to Greer *et al.* (2000), theophylline (a metabolite of caffeine) is more effective in doing so. However, the effect of caffeine on fat oxidation may only be significant during lower exercise intensities and may be blocked at higher intensities (Gonzalez, 2012). Spriet *et al.* (1992) discovered that ingestion of a high dose of caffeine before exercise reduced muscle glycogenolysis in the initial 15 minutes of exercise by increasing free fatty acid (FFA) levels, which inhibits glycolysis and spares glycogen for later use. Caffeine's effect on the inhibition of glycogen phosphorylase has also been shown in *vitro* (Rush *et al.*, 2001) as well as its effect on increasing HSL activity (Donsmark *et al.*, 2003). Following caffeine administration prior to and after the onset of cycling, Ivy *et al.* (1979) found that plasma free fatty acid levels were increased 30% compared to placebo. This action might be mediated by inhibition of the enzyme phosphodiesterase, thereby yielding higher levels of cAMP, which has been identified as an important molecule for glycogen metabolism and lipolysis (Nehlig & Debry, 1994). Pedersen *et al.* (2008) found higher rates of muscle glycogen accumulation after the co-ingestion of caffeine with CHO during recovery in highly trained subjects. This might be partly mediated by the activation of AMP-activated protein kinase (AMPK) (Egawa *et al.*, 2009) as it is involved in the translocation of glucose transporter 4 (GLUT4) to the plasma membrane. This mechanism enables the cell to take up glucose from the plasma and store it as glycogen.

While the ergogenic impact of caffeine in moderate-to-highly trained endurance athletes is quite clear and well documented, its effects on anaerobic, high-intensity tasks are less well investigated. Mora-Rodriguez *et al.* (2012) found that caffeine ingestion of 3 mg/kg could

counter reductions in maximum dynamic strength and muscle power output in the morning (2.5-7.0%) thereby increasing muscle performance to the levels found in the afternoon. Especially with regard to anaerobic performance caffeine's adenosine receptor blocking effect in the CNS may be important (Daves & Green, 2009). Whilst caffeine seems to benefit trained athletes who show specific physiological adaptations whereas performance gains in untrained subjects might be lost or masked by a high variability in performance.

Graham *et al.* (1998) have found out that coffee contains phenolic compounds such as chlorogenic acids, which elicits metabolic effects independent of caffeine. These compounds may counteract the physiological responses of caffeine. The issue therefore is whether absorbing the same amount of caffeine through a food source is as effective as absorbing isolated caffeine in the form of a tablet. As discussed above that the performance enhancing effect of caffeine is very clear. However, only a few studies have shown a positive effect of coffee on performance (McLellan & Bell, 2004; Wiles, *et al.*, 1992; Hodgson, *et al.*, 2013; Costill, *et al.*, 1978), and others did not (Graham, *et al.*, 1998; Butts, 1985; Lamina & Musa, 2009).

One of the earlier studies by Costill *et al.* (1978) found improvements in time-trail performance of cyclists only in the coffee trail group (330 mg caffeine an hour prior to exercise) but not in the decaffeinated coffee trail. Graham *et al.* (1998) studied exercise endurance in different groups of runners, to ingest caffeine (4.45 mg/kg body weight (BW)) or placebo capsule with water or either decaffeinated coffee, decaffeinated coffee with added caffeine or regular coffee. They found only caffeine significantly improved running time to exhaustion at 75% VO_{2max} but neither did regular coffee nor decaffeinated coffee plus

caffeine. Based on these results, Graham *et al*. (1998) concluded that some elements in coffee possibly influence in the ergogenic response of caffeine alone.

This is over against Hodgson *et al.* (2013) who studied at time trail performance in trained subjects after administration of caffeine (5 mg caffeine/kg BW), coffee (5 mg caffeine/kg BW), decaffeinated coffee and placebo an hour prior to exercise. They have reported similar significant improvement of ~5% in time trail performance in both the caffeine and the coffee supplemented group with no effects in the decaf or placebo group. The authors speculated that coffee consumed an hour prior to exercise, at a high caffeine dose improved performance to the same degree as caffeine.

One of the reasons for the variance of the two studies mentioned above could be the different performance tests used. While Graham *et al*. (1998) used a time to exhaustion test which reportedly can exhibit a coefficient of variation as high as ~27% (Jeukendrup, 1996), Hodgson *et al*. (2013) used a time trail which have been shown to be more renewable, and they have concluded that due to lower statistical power, Graham *et al*. (1998) were not able to detect a difference between caffeine and coffee absorption on performance. In a word, both coffee and caffeine seem to exhibit a performance enhancing effect and further research will be needed to help us understand this issue.

Not only does caffeine impact endurance, it has been reported to benefit cognitive function and fine motor skills (Foskett *et al*., 2009). Accordingly, caffeine is commonly used by athletes as an ergogenic aid due to its associated reduction in fatigue, and enhancement of

concentration and alertness (Paluska, 2003). Trained athletes benefit from a moderate dose of 5 mg/kg (Woolf *et al.*, 2008), although lower doses of caffeine (1.0-2.0 mg/kg) may improve performance (Cox *et al.*, 2002). Another reason explains why caffeine is widely used in competitive activities might be its small but significant paregoric effect (Derry *et al.*, 2012), possibly mediated by augmenting plasma endorphin concentrations (Grossman & Sutton, 1985). It is also defined that caffeine reduces the rate of perceived exertion during exercise (Doherty & Smith, 2005), proposing that athletes are able to undertake higher intensities but do not perceive this effort to be different from placebo conditions.

Of note, there are a small number of studies reporting no ergogenic effect on work performance when participants received a high dose of caffeine, including endurance (Cameron *et al.*, 1990; Tarnopolsky, 2008; Davis & Green, 2009; Mora-Rodriguez *et al.*, 2012; Butts, 1985; Grossman & Sutton, 1985; Goniewicz *et al.*, 2013; Ryder, 1994; Martinsen & Sundgot-Borgen, 2012). Perkins and Williams (1975) performed an exercise to exhaustion under four different conditions: placebo; caffeine doses in 4 mg/kg; 7 mg/kg; and 10 mg/kg. The results demonstrated no significant difference in time to exhaustion between different doses compared with placebo. Butts and Crowell (1985) made a similar conclusion in time to exhaustion at 75% VO_{2max} with a dosage of 300 mg caffeine.

2.12 The pharmacological and ergogenic effect of theobromine

Theobromine (3,7-dimethyl-1, H-purine-2, 6-dione; $C_7H_8N_4O_2$) is a closely alkaloid to caffeine (1,3,7-Trimethylpurine-2, 6-dione; $C_8H_{10}N_4O_2$), which can be found in a variety of food sources including: chocolate; cocoa; and kola nut.

2.12.1 Theobromine and athletic performance

Due to its similarity with caffeine, theobromine may also provide performance enhancement as well as protection against bronchoconstriction, although the studies around this specific field are rare. Theobromine is widely consumed in the diet and is currently used in metabolism-enhancing sporting supplements for its stimulant effects (Pearce, *et al.*, 2012). Furthermore, theobromine is not prohibited or restricted in any form by the WADA.

Despite the absence of subjective or behavioral changes, Mumford *et al.* (1994) did note an increased: alertness; headache; and irritability in some individuals, suggesting the possibility of individual differences in sensitivity. Using a higher dose, Mitchell *et al.* (2011) found that 700 mg theobromine lowered blood pressure, decreased self-reported calmness and increased subjects' ratings of how interesting they found performance of study tasks. Mattew *et al.* (2013) demonstrated that theobromine responses differed according to dose with limited subjective effects at 250 mg and negative mood effects at higher doses. They also observed a dose-dependent increased in heart rate. Therefore the study concluded that theobromine at normal intake ranges may contribute to the positive effects to exercise performance, but at higher intakes the performance effects become negative.

2.12.2 Clinical Use of Theobromine

Clinically, theobromine is used as a vasodilator, a diuretic, and cardiac contractile stimulant (William, 1943). Accordingly, theobromine has been used to treat high blood pressure (Theobromine Chemistry, 2015). A recent paper published suggested that the decrease in blood pressure might be associated with flavanols (Kelly, 2005). Importantly, previous

empirical research has claimed a potential bronchodilator effect of theobromine in *vitro*, although the effect is not as significant compared with caffeine (Simons, Becker and Simons, 1985). Whilst theobromine may be used to control asthma the effects of theobromine on athletic performance are under-researched. Although there is no evidence that consumption of theobromine will increase athletic performance, Pearce *et al*. (2012) strongly advice the need for further research.

Theobromine is useful in asthma and in other respiratory tract problems such as cough for which no definitive drug has been developed. Codeine is very effective but its metabolism to compounds acting on opioid receptors limits its use (Prieto-Lastra, *et al*., 2006). A safety and natural alternative could be theobromine since it is able to prevent cough provoked by citric acid in guinea-pigs and by capsaicin (an irritant component of chili peppers) in humans. A report from van Zyl, *et al*. (2008) showed that the diffusion of theobromine in lung substructures is higher than that of other drugs used in the therapy of respiratory diseases. The authors indicate that not only lipophilicity but also the position of alkyl groups in the purine ring affect the ability of caffeine and theobromine to cross biological membranes. The differential capability of tissue penetration and accumulation may explain why theobromine may achieve higher effects than caffeine. The efficacy of theobromine may become higher if it readily crosses membranes and reaches high interstitial concentrations even though theobromine may have less affinity for receptors than caffeine.

2.12.3 Caffeine *vs*. Theobromine

According to Smit (2011), that theobromine appears to be toxic in some mammals, including pets. However, the action mechanisms of theobromine in humans may be different from those observed in other mammals with a number of clinical trials demonstrating that theobromine is not toxic for humans (Penleton *et al.*, 2012, 2013; Baggott *et al.*, 2013).

An increasing amount of evidence in the last decade has showed that theobromine has psychoactive actions in humans that are qualitatively different from those of caffeine (Mitchell, *et al*., 2011; Baggott *et al*., 2013). The effect of theobromine on blood pressure (van den Bogaard, *et al*., 2010) is also qualitatively different than that of caffeine (Mitchell, *et al*., 2011) but the reasons for these differences are not established. One possible reason for the conflict in the effects of caffeine and theobromine could be their different half-life. The half-life of caffeine is the time required for the body to eliminate one-half of the caffeine. It varies widely among individuals according to factors such as pregnancy status, body weight, medication intake, and liver enzyme function level (needed for caffeine metabolism). In healthy adults, the half-life is approximately 3 to 7 hours (Ananya, 2014). Nicotine decreases the half-life by 30–50% (Fredholm *et al*., 1999). Additionally, it is known that oral contraceptive use can double caffeine half-life (Abernethy and Todd, 1985; Patwardhan *et al*., 1980), however, the effects of newer oral contraceptives on caffeine half-life have not been studied. Pregnancy can raise it to as much as 15 hours during the last trimester (Fredholm *et al*., 1999). In newborn babies the half-life can be 80 hours or more, dropping very rapidly with age, possibly to less than the adult value by age 6 months (Fredholm *et al*., 1999). The antidepressant fluvoxamine (Luvox) reduces the clearance of caffeine by more than 90%, and increases its elimination half-life more than tenfold; from 4.9 hours to

56 hours (Drug Interaction: Caffeine Oral and Fluvoxamine Oral.). Caffeine can accumulate in individuals with severe liver disease, increasing its half-life (Verbeeck, 2008). A study conducted by Statland & Demas (1980) has showed that people with compromised liver function had a significantly longer half-life (a 49-year-old woman having alcoholic hepatic disease had a serum half-life of 168 hours).

Half-life of theobromine is higher than caffeine even in rodents, which have a faster hepatic metabolism. Therefore, half of the theobromine administered to rats is excreted unchanged (Bonati *et al.*, 1984). In humans, the mean half-life of theobromine in plasma from healthy volunteers is approximately 10 hours and the percentage of unmodified compound present in urine collected for 48 hours after a single dose of 10 mg/kg is relatively high (16-18% depending on the technique for isolation and quantitation) (Tarka *et al.*, 1983). The importance of this fact is evidenced when methylxanthines are used as bronchodilators in the management of asthma patients in whose serum the half-life is also higher for theobromine than for caffeine (Becker *et al.*, 1984). When one of the main xenobiotic metabolizing enzymes, cytochrome P450 1A2 (YP1A2), is expressed in heterologous cells the rate of transformation is much lower for theobromine (5%) than for caffeine (81%; Gu *et al.*, 1992) therefore confirming that caffeine is more labile in terms of degradation than theobromine.

In recent years, theobromine is starting to be widely studies to look for common and differential mechanisms with caffeine. The effect of theobromine in respiratory diseases is not due to inhibition of mediators of inflammation in asthma, histamine or slow reacting substance of anaphylaxis (Hillyard *et al.*, 1984). A novel differential target of methylxanthines is poly(ADP-ribose)polymerase-1, a nuclear enzyme that is poorly inhibited

by caffeine but significantly inhibited by theobromine (Geraets *et al.*, 2006). In this sense, Ahmad *et al.* (2015) have recently shown that inhibition of poly(ADP-ribose)polymerase-1 significantly reduces inflammation of lungs caused by gamma-carrageenan. Recent evidence demonstrates neovascularization in an animal model of asthma (Wagner *et al.*, 2015). Interestingly, theobromine may reduce neovascularization accompanying tumor growth and metastasis (Gil *et al.*, 1993) and, therefore it may reduce both acute symptoms and angiogenesis in asthma.

2.12.4 Theobromine and Performance

In terms of the psychoactive effect of theobromine, early studies failed to observe mood effects following theobromine ingestion (Brunk *et al.*, 1973; Dorfman & Jarvik, 1970) however, more recently Mumford *et al.* (1994) reported that 5 out of 7 subjects were able to recognize a high dose of theobromine (560 mg) from a placebo or caffeine dose. The combination of caffeine (19 mg) and theobromine (250 mg) in capsules increased the self-reported mood construct "energetic arousal", and improved epistemic function as measured with a simple reaction time test (Smit *et al.*, 2004) compared to placebo capsules.

Some animal studies have reported that theobromine has inappreciable effects on cerebral blood flow and glucose use (Brome & Stefanovich, 1986). Kuribara & Tadokoro (1992) have pointed out that animals do show increases in motor activity with high doses of theobromine. According to the report from Mumford *et al.* (1994), caffeine increased subjective alertness in subjects, whilst theobromine did not, and Mitchell *et al.* (2011) for the first time found that theobromine did not act as an incentive alone or combined with caffeine, however, it may

have a tension-raising effect. In conclusion, caffeine has strong alerting effects with inappreciable contribution from theobromine; caffeine produced ergogenic effects at early time points, while theobromine decreased calmness at a later time point; caffeine may have more CNS-mediated effects on alertness and theobromine may be acting mostly via peripheral physiology.

The performance improvement contributions of theobromine are less clear and its psychoactive effects appear subtle compared to caffeine. Mumford (1994) postulated that theobromine is one tenth as potent as caffeine. Pharmacological assays also confirm that theobromine is less active than caffeine. Furthermore, individual variation in sensitivity to caffeine and theobromine is likely to play a role in the ergogenic efficacy of these compounds. Similar to caffeine, the ingestion of lower doses of theobromine may result in performance improvement, however at higher doses may causes a negative effect.

2.13 Summary

There are a large number of studies examining the ergogenic effect of inhaled short-acting β_2-agonists, however, their findings are contradictory. No strict comparison can be made between the studies because of different types of exercise, methods, and doses employed in each study. The vast majority of evidence suggests no ergogenic effect of inhaled salbutamol, however, a single high dose of up to the WADA recommended upper daily limit of 1600 μg has not been investigated. Furthermore, the majority of previous studies have examined endurance performance with no available data in team game performance.

Compared with the ergogenic effect of salbutamol, the urine concentration of salbutamol has received much less attention. Among the existing findings, the urine concentration of salbutamol did not exceed the WADA upper limit after the inhalation of salbutamol up to 800 μg. To date, the impact of inhaling the WADA recommended daily upper limit of salbutamol in a single bolus on urine concentration remains unknown. Furthermore, dehydration has been previously implicated in positive anti-doping results despite the paucity of data examining the impact of body mass loss through dehydration on urine concentration of salbutamol.

Caffeine and theobromine have been used in the control of asthma. The ergogenic effect of caffeine has again been examined by many previous studies despite a strong-link to enhanced performance, it is not banned and remains on the WADA monitored list. In contrast, little is known of the ergogenic impact of theobromine, although theobromine has been proved to be somewhat similar to caffeine in a number of aspects.

Main Hypotheses

1. Inhalation of 800 μg or 1600 μg of salbutamol will have no ergogenic effect on 5 km time trial performance.

2. Inhalation of 800 μg or 1600 μg of salbutamol will have no effect on simulated association football (soccer) performance.

3. Dehydration resulting in either a 2% or 5% reduction in body mass will not result in a positive doping violation following the inhalation of 800 μg or 1600 μg of salbutamol.

4. Caffeine and theobromine will have no effect on 3 km running performance in non-asthmatic athletes.

CHAPTER III

GENERAL METHODOLOGY

EIB is closely related to asthma and is defined as a transient narrowing of the airways, limiting expiration that usually occurs during and most often after exercise, and is reversible spontaneously or through the inhalation of β_2-agonists (Anderson, 1997). This thesis has previously reported the prevalence of EIB is 55% in athletes' population, which is much higher than the prevalence of asthma in the general British population (8.4% in UK adults). Accordingly, the use of short-acting β_2-agonists is prevalent during training and competition. This thesis sought to examine the ergogenic effects of short-acting β_2-agonists in non-asthmatic athletes to establish possible ergogenic effects.

The eucapnic voluntary hyperpnoea (EVH) challenge is a surrogate for exercise (Anderson *et al.*, 2001), and has been demonstrated to possess a high specificity and sensitivity in the diagnosis of EIB in elite athletes (Dickinson *et al.*, 2006). Therefore, the IOC-MC recommends that athletes are tested for EIB using an EVH challenge in order to provide evidence of EIB and permit the use of inhaled short-acting β_2-agonists during training and competing (International Olympic Committee- Medical Commision, 2010).

This chapter describes general procedures and equipment that are common to more than one study in the thesis, and general information with regards to laboratory standards and equipment used.

3.1 Spirometry – Maximal Flow Volume Loop

Spirometry is a medical test that measures the volume of air an individual inhales or exhales as a function of time. It is an effort dependent manoeuvre that requires co-operation, coordination and understanding by the subject. For these reasons the American Thoracic Society (ATS) and European Respiratory Society (ERS) have collaborated to publish spirometry guidelines (Miller *et al*., 2005), which were employed when spirometry measurements were performed in this thesis. The ATS/ERS guidelines ensure there is a global standard for the manoeuvre and the equipment used to test flow volume that is reliable and specific.

Spirometer

In the following collection of studies all maximal flow volume loops were collected using a MicroLab ML3500 Spirometer (Cardinal Health Ltd, UK), which met the ATS/ERS guidelines for diagnostic spirometers. The volume accuracy of the spirometer was checked using a three-liter syringe.

Measurement of Maximal Flow Volume Loop

The maximal flow volume manoeuvre (see Figure 2) was conducted as described in the following section. Following explanation of the test to the participant they were asked about recent illness, medication use, smoking and training they had completed in the 24 hours immediately prior to the test. The participant's data was entered into the spirometer and a forced vital capacity manoeuvre was selected. Throughout the whole manoeuvre the

participant was asked to remain in a seated position. Verbal instruction (see Table 4) and a correct demonstration of the manoeuvre were given following ATS/ERS guidlines. The participant was asked to attach a nose clip and inhale maximally. They then placed the mouth-piece in their mouth and exhaled maximally until they felt they had reached residual volume. Once they had reached residual volume they were instructed to inspire maximally to total lung capacity. According to ATS/ERS recommendations (see Table 5), an adequate test requires a minimum of three acceptable forced vital capacity (FVC) manoeuvres. Acceptable repeatability is achieved when the difference between the largest and the next largest FVC is ≤ 0.150 L and the difference between the largest and next largest FEV_1 is ≤ 0.150 L (Hankinson & Bang, 1991). For those criteria are not met in three manoeuvres, additional trials should be attempted, up to, but usually no more than, eight manoeuvres, eight is generally a practical upper limit for most subjects (Ferris *et al.*, 1978; Kanner *et al.*, 1983). After several forced expiratory manoeuvres, fatigue can begin to take its toll on subjects and additional manoeuvres would be of little added value. Therefore, this manoeuvre was completed a minimum of 3 times and no more than 8 times. The maximal flow-volume loop with the best FEV_1 was recorded as long as the second highest FEV_1 was within 0.2 L. Each individual maximal flow volume loop effort was accepted if they met the criteria listed in Table 6.

Figure 2. Spirometry Measurement

1. Sit up straight and try to be relaxed

2. Place nose clip on

3. Hold the mouth piece to the side of you head

4. Inhale until your lungs are full

5. Place the mouth piece in your mouth and exhale as fast as possible

6. Keep breathing out until you feel your lungs are empty

7. Following complete exhalation keep the mouth piece in your mouth and inhale maximally until your lungs are completely full.

Table 4. Verbal instruction given to participant

ATS/ERS recommendations are as follow:	
FVC minimum duration	6 s, (3 s for children) or plateau in the volume-time curve. Subject cannot or should not continue to exhale.
FVC end of test criteria	• subject cannot or should not continue further exhalation, or • the volume time curve shows an obvious plateau, or • the forced exhalation is of reasonable duration
FVC maximum number of maneuvers	8, both in adults and children
FVC maneuver acceptability: none of the following applies	• unsatisfactory start of expiration • back extrapolated volume >5% of FVC or 150 mL, whichever is greater • coughing that interferes with the measurement of FEV1 and/or FVC • early termination of expiration • Valsalva maneuver • A leak • An obstructed mouthpiece, no glottis closure • Effort that is not maximal throughout
FVC and FEV_1 repeatability the largest and second largest FVC and or FEV_1 must not differ by more than 150 mL	

Table 5. ATS/ERS recommendations (Quanjer *et al.*, 1993; American Thoracic Society, 1994; Enright *et al.*, 1991; Ferguson *et al.*, 2000; Miller *et al.*, 2005)

| Within-manoeuvre criteria |
| Individual maximal flow-volume loops were accepted if: |
| Participant is free from |
| Cough during the first second of exhalation |
| Early termination or cut – off |
| Effort that is not maximal throughout |
| Leak |
| Obstructed mouth piece |
| Participant demonstrates satisfactory exhalation |
| Duration of >6 seconds or a plateau in volume time curve |

Table 6. Criteria for acceptance of maximal flow-volume loops (Adpated from Miller *et al.* (2005). 'Standardisation of Sprirometry'. European Respiratory Journal, vol. 26, pp. 319-338)

3.2 Bronchoprovocation Challenge

Bronchoprovocation challenges are used to establish a diagnosis of Exercise Induced Bronchoconstriction (EIB). Bronchoprovocation challenges such as methacholine and histamine were not used to test for EIB in this thesis as they are not specific to EIB (Mahler, 1993; Haby *et al.*, 1995; Clough *et al.*, 1991; Rundell *et al.*, 2002; Holzer *et al.*, 2002). Two challenges that are thought to be specific to diagnose EIA are: (1) exercise; and (2) eucapnic voluntary hyperpnoea (EVH). The poor reproducibility of exercise challenges has resulted in EVH becoming the 'Gold Standard' in the diagnosis of EIA (Pearsons *et al*, 2013). We have also considered the Mannitol challenge, which is an alternative choice for the EVH challenge. However, the Mannitol challenge is known to be more sensitive for detection of summer EIB athletes, while less sensitive in detection of winter EIB athletes (Sue-Chu *et al.*, 2010).

In preparation for all bronchoprovocation challenges participants were instructed to stop pulmonary medications (see Table 7). The participants were told not to exercise within 4 hours of the challenge as this may exert a protective effect against EIA (Edmunds *et al.*,

1978; Anderson, 1993). On the day of the test the participants completed a questionnaire stating any medication they were using and whether they were suffering from any illness or injury. If a participant was suffering from an illness or injury that may limit the results of the test, they were instructed to return when they were symptom free and fit to complete the test. The participants were also told not to drink coffee, tea, cola drinks or eat chocolate on the day of the test (Henderson *et al*., 1993). Following the bronchoprovocation challenge the participant was not allowed to leave until their FEV_1 was within 10% of their baseline FEV_1. If a participant had not returned to within 10% of FEV_1 within 15 minutes after stopping the challenge, bronchodilator therapy was offered in the form of an inhaled short acting β_2-agonist (*e.g.* 200 mcg salbutamol).

Medication	Minimum time interval from last dose to challenge	Reference
Inhaled Short Acting β_2-Agonist	8 hours	Ahrens *et al*., 1984; Greenspon *et al.,* 1984
Inhaled Long Acting β_2-Agonist	48 hours	Derom *et al*., 1992; Cockcroft and Swystun, 1997
Cromolyn Sodium	8 hours	ATS 2000
Leukotriene modifiers	24 hours	ATS 2000
Inhaled corticosteroids	24 hours	Anderson *et al.,* 2001

Table 7. Time scales for stopping Pulmonary Medication (adapted from: American Thoracic Society. (2000) 'Guidelines for Methacholine and Exercise Challenge Testing – 1999'. *American Journal of Respiratory Critical Care Medicine***, vol. 161, pp. 309-329)**

3.3 Eucapnic Voluntary Hyperpnoea (EVH) Challenges

An EVH test is conducted in the laboratory (see Figure 3). It is a surrogate for exercise to identify EIB in athletes (Anderson *et al*., 2001). Before the athlete starts the EVH challenge they completed 3 maximal voluntary flow-volume loops with the best FEV_1 being recorded as their baseline measurement. The athlete is then asked to ventilate at a target minute ventilation of 85% of their maximal voluntary ventilation rate (MVV). This was calculated by multiplying their baseline FEV_1 by 30. The air which is inspired during the EVH challenge consists of 21% O_2, 5% CO_2 and 74% N_2 and was delivered via a gas cylinder (see figure 3). There is a 5% CO_2 concentration present to prevent syncope during the test. The hyperventilation lasts for 6 minutes during which verbal feedback and encouragement is given to the athlete. During the EVH challenge minute ventilation (V_E) was monitored by calculating the volume of air passing through the dry gas meter every minute. This allowed the athlete to know whether to increase, maintain or decrease V_E. After stopping the EVH challenge maximal voluntary flow-volume loops are taken at 3, 5, 7, 10 and 15 minutes.

Figure 3. EVH Challenge

3.4 Urinalysis

All urinalysis was performed at HFL Sport Science (Fordham, UK), the world's largest independent provider of drug surveillance, doping control and research activities to human and equine sports; it is also a former part of the Quotient Bioresearch Group which has over 40 years' continuous experience in the science of sports doping control (equine, canine and human). This includes experience testing within the framework of the WADA. The HFL Laboratory in Fordham, UK, is one of the pioneering laboratories provide unrivalled and internationally trusted expertise in all aspects of doping control for sports; it has accreditation to ISO 17025, for laboratory testing and calibration services. HFL's qualified team of scientists delivers both operational screening services and innovative research into prohibited substance detection and, over the past 40 years, has published more than 300 scientific papers, pioneering innovative bioanalytical techniques (About HFL Sport Science, 2015). This study's aim was to examine urinary levels in line with WADA Anti-Doping rules, and it was important to use a lab that had experience in WADA procedures. HFL's accreditations and rich experience in laboratory testing demonstrate that they are able to provide valid data and reliable results, we therefore had chosen HFL Sport Science to conduct all of the urine tests and analyses in this study.

Sample preparation involved the addition of 200 mg of salbutamol-D3 (NMI) as an internal standard to 1 mL of urine. Following the addition of 2 mL of 0.1 M phosphate buffer pH 6.8 and 100 μL of E. Coli enzyme (β-glucuronidase) solution the mixture was incubated overnight at 37°C. Strata XC 60 mg solid phase extraction cartridges (Phenomenex, Macclesfield, UK) were conditioned with 3 mL of methanol followed by 3 mL of reagent grade water. Following centrifugation at 3500 rpm for 5 minutes the samples were applied to

the cartridges. The cartridges were then washed with 3 mL of 0.1 M acetate buffer pH 9.0 followed by 3 mL of reagent grade water, 3 mL of 0.1 M HCl, 3 mL of methanol and 3 mL of diethyl ether. The cartridges were then dried for 5 minutes under vacuum and samples were eluted into glass vials with two, 1 mL of basic drug elution solvent (160 mL ethyl acetate, 34 mL propan-2-ol and 6 mL 34% ammonia solution). Samples were then evaporated to dryness at ambient temperature using a centrifugal vacuum concentrator (Genevac Ltd, Ipswich, UK) and reconstituted in 10 μL of isopropanol followed by 200 μL of basic reconstitution solution (495 mL of 0.1 acetic acid mixed with 5 mL Benzyldimethylphenyl Ammonium). Samples were centrifuged at 3000 rpm for 10 minutes prior to LCMS submission. Samples were injected onto a Thermo Scientific Accela HPLC system coupled to a Thermo Scientific LTQ Orbitrap Discovery Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Chromatographic separation was performed on a Waters Atlantis T3 column (2.1 x 100 mm, particle size 3 um; Waters Ltd, Elstree, UK) at 35°C. The mobile phase was a gradient system of 0.1% acetic acid aqueous solution containing uracil (300 ng·mL^{-1}) and 0.1% acetic acid in acetonitrile containing uracil (300 ng·mL^{-1}) set at a flow rate of 0.4 mL·min^{-1}.

3.5 Statistical Data Collection
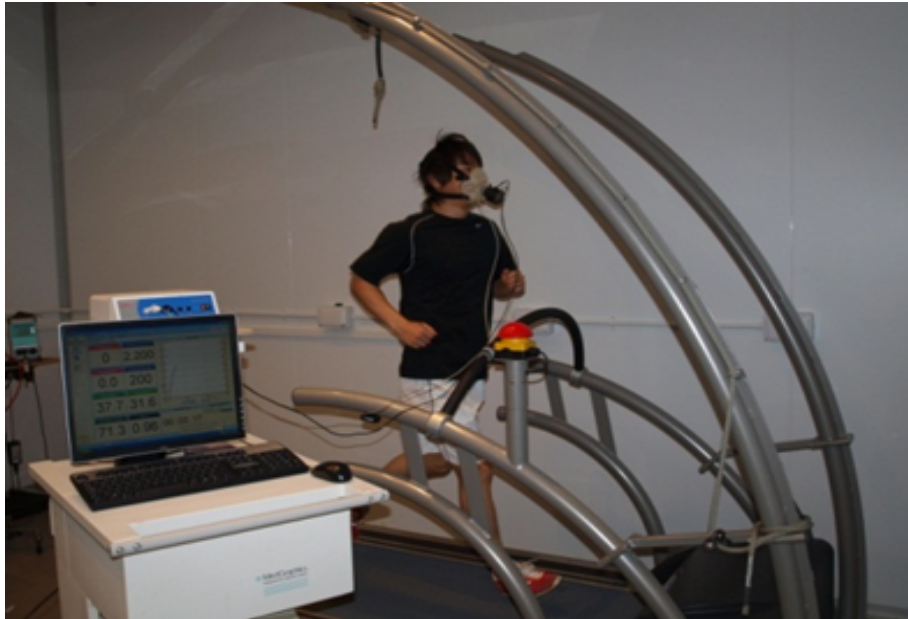
Figure 4. **Statistical Data Collection**

VO_{2max} was assessed using a graded, incremental treadmill (Woodway Ergo ELG 55 treadmill; Woodway GmbH, Weil am Rhein, Germany) protocols designed to elicit VO_{2max} in 8 to 12 minutes (See Figure 4). Expired ventilation (V_E) and fractions of expired oxygen (FeO_2) and expired carbon dioxide ($FeCO_2$) were measured during the test with gas exchange and ventilatory variables being analysed breath-by-breath using a calibrated computer-based exercise system (Jaeger Oxycon Pro, Germany). The analysers were calibrated before each test using a two-point measure: a calibration gas (CO_2 4%, O_2 16%, N_2 balance) and a reference gas room air after ATPS (ambient temperature and pressure, saturated) to STPD (standard temperature and pressure, dry). Heart rate (HR) and rating of perceived exertion (RPE) was continuously recorded (Polar; Lake Success, NY, USA).

Following the measurement of each subject's stature (Holtain Stadiometer; Holtain Ltd., Crymych, Wales) and body mass (Seca 888 Class III floor scale; Seca, Birmingham, UK) subjects performed 5 minutes light running at a self-selected pace. The protocol for the exercise test was preinstalled to allow automatic and accurate increments in speed ($km \cdot h^{-1}$) and gradient (%).

CHAPTER IV

STUDY 1 & STUDY 2

THE ERGOGENIC AND PHARMACOKINETIC IMPACT OF SHORT ACTING β_2-AGONISTS ON 5 KM TIME-TRIAL RUNNING PERFORMANCE & ASSOCIATION FOOTBALL PERFORMANCE

4.1 Introduction

Salbutamol is a short-acting β_2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease which was first brought to market in 1968 (Fitch *et al*., 2008). β_2-agonists are the most common and effective therapy for the anticipation of symptoms of exercise-induced bronchoconstriction (EIB) in asthmatic patients (Dempsey *et al*., 1977). β_2-agonists relax smooth muscle, decrease vascular permeability, increase airflow, and moderately inhibit mediator release (Williams and Shapiro, 1995). For those affected by EIB, treatment with two 'puffs' (2 x 100 μg) of a short-acting β_2-receptor agonists shortly following exercise will give peak bronchodilation in 15 to 60 minutes and protection from EIB for at least 3 hours in most patients (Bierman *et al*., 1984).

Due to fears of their potential ergogenic effect in non-asthmatic athletes, most forms of β_2-agonists are prohibited except salbutamol, salmeterol, formoterol and turbutaline by inhalation, which until recently required a declaration of use in accordance with the international standard for therapeutic use exemptions (TUE) (Dickinson *et al*., 2005). The World Anti-doping Agency (WADA) stipulates that athletes who declare the use of salbutamol in order to treat asthma or EIB should not exceed an accumulated dosage of 1600 μg over a 24-hour period. Evaluation of the potential abuse of salbutamol is undertaken during routine anti-doping testing with a urine concentration of greater than 1000 ng·L^{-1} considered a doping offence.

Oral salbutamol appears to have performance-enhancing effects mediated by its role in increasing strength (Martineau *et al.*, 1992) and is therefore not permitted for use by athletes. Oral salbutamol however, is distinguishable from the inhaled, topical form and is currently identified by anti-doping authorities associated with a urinary concentration upper threshold of 1000 μg/mL salbutamol (free plus gluconuride) and the ratio of the S1 and R-enantiomers (Fitch *et al.*, 2008; Berges *et al.*, 2000). In contrast, the majority of studies examining the efficacy of inhaled short acting β_2-agonists have failed to observe an ergogenic effect of acute inhalation of salbutamol in non-asthmatic individuals (Wolfarth *et al.*, 2010).

Oral β_2-agonists are acknowledged to induce significant changes in body composition. Relatively short periods (4-12 weeks) of specific β_2-agonists, especially clenbuterol and salmeterol, have been shown to increase skeletal muscle strength and size in several animal species. In addition, the administration of these β_2-agonists orally or via implanted osmotic minipumps, has been associated with a decline in body fat (Sporer *et al.*, 2008; Elers *et al.*, 2011; Anderson *et al.*, 2001; Drust *et al.*, 2000). Of note, this anabolic effect on skeletal muscle has, however, never been observed in either healthy animals or humans following the administration of oral salbutamol, possibly because of the short elimination half-life (Drust *et al.*, 2007; Draper *et al.*, 1997). In contrast, following an initial study from Martineau *et al.* (1993) reporting an improved endurance performance following the administration of oral salbutamol a number of further studies demonstrated improved endurance during intense submaximal exercise (Kindermann, 2007), increased strength following resistance exercise (Elers *et al.*, 2012), and increased strength and endurance (Haverkamp *et al.*, 2007) and power (Decorte *et al.*, 2008), although side effects could be unpleasant (The World Anti-Doping Agency 2010) including fine tremor, anxiety, headache, muscle cramps, dry mouth, and palpitation.

Previous studies have examined the effects of topical (inhaled) β_2-agonists on performance after acute administration (Dickinson *et al*., 2006a; Dickinson *et al*., 2006b; Anderson *et al*., 2003) and after short-term administration (Rundell *et al*., 2004; Parsons *et al*., 2007; Pluim *et al*., 2011; The World Anti-Doping Agency 2012; Elers *et al*., 2011; Anderson *et al*., 2011; Kindermann, 2007). Based on the results of these studies, the performance after acute therapeutic inhalation has no ergogenic impact. This has, in part, been explained by the lack of systemic bioavailability with such low-dose topical administration. There are, however, a number of limitations to these previous studies, for example, most studies investigating the effect of inhaled salbutamol on running performance have investigated doses of up to 800 μg while few higher dose trials including the permitted upper limit of 1600 μg are available in the literature. Furthermore, the majority of previous studies have focused on VO_{2max} or running time to exhaustion rather than a more ecologically valid assessment *i.e.* sports specific time-trial.

The World Anti-Doping Agency (WADA) stipulates that athletes who declare the use of salbutamol in order to treat asthma or exercise induced bronchoconstriction (EIB) should not exceed an accumulated dose of 1600 μg over a 24-hour period. To date, however, there are no available data examining the ergogenic effect of 1600 μg or the urine concentration of the short-acting β_2-agonist following the administration of a single bolus of 1600 μg and its relationship with the WADA code. Most studies investigating the effect of inhaled salbutamol on maximal flow volume values, such as FEV_1, have investigated doses of up to 800 μg. Furthermore, previous studies have failed to control for the presence of exercise induced bronchoconstriction (EIB) in apparently healthy subjects during exercise performance studies (Elers *et al*., 2010).

Whilst oral salbutamol appears to have an ergogenic effect, and despite its presence on the WADA restricted list, previous studies examining acute doses of inhaled salbutamol have failed to demonstrate an improved athletic performance (Dickinson *et al.*, 2005; Dickinson *et al.*, 2006b; Anderson *et al.*, 2003; Rundell *et al.*, 2004; Parsons *et al.*, 2007; Pluim *et al.*, 2011; Signorile *et al.*, 1992; Sporer *et al.*, 2008; Elers *et al.*, 2011; Anderson *et al.*, 2001; Miller *et al.*, 2005). In a recent meta-analysis (Pluim *et al.*, 2011), reported no performance enhancement as a result of inhaled salbutamol administration. The majority of previous studies have focused on continuous, aerobic exercise challenges. There are a limited number of studies that have examined the impact of a hot environment on combined salbutamol administration (up to 1600 μg) and endurance running performance, or on the resultant salbutamol elimination in urine; there is no available data for the impact of inhaled short acting β_2- agonists on association football (soccer) performance. In addition to the inhalation of high, single bolus doses of salbutamol, the impact of dehydration through sweating associated with high intensity exercise in hot environments may have a profound effect on urine concentration that will affect urinalysis.

Exercise affects the pharmacokinetics of inhaled drugs in several ways. Pulmonary absorption is higher during exercise through exercise-induced increases in pulmonary blood flow and alveolar permeability (Schmekel *et al.*, 1992). Exercise may also compromise blood flow to the splanchnic region and reduce gastro-intestinal uptake of the part of the drug that is swallowed during inhalation (up to 80% for inhaled salbutamol; Khazaeinia *et al.*, 2000; Lipworth, 1996). Furthermore, exercise-induced sweat loss concentrates the urine, thus leading to a higher urine concentration of β_2-agonists (Lipworth, 1996; Dickinson *et al.*, 2014; Hostrup *et al.*, 2014). Sporer *et al.* (2008) observed that the urine concentrations of salbutamol were close to exceeding the urinary threshold in exercising subjects after

inhalation of 800 µg. It is likely that the urinary threshold and decision limit for salbutamol would have been exceeded in that study, if subjects had inhaled the maximal allowed dose of 1600 µg salbutamol. Real-life doping control is mostly conducted immediately after a competitive event or a training session. Athletes using β_2-agonists, usually inhale them a prophylaxis prior to competition or training (McKenzie & Fitch, 2011; Price *et al.*, 2014). While athletes typically inhale repetitive doses of salbutamol below the current therapeutic threshold of 1600 µg, the 2015 WADA anti-doping regulations for salbutamol allow inhalation of 1600 µg as a single dose. Therefore, pharmacokinetic data of the maximal allowed dose of 1600 µg salbutamol conducted during exercise in hydrated and dehydrated state in controlled settings are important to evaluate the WADA urinary threshold and decision limit for salbutamol on the list of prohibited substances.

Accordingly, the aim of this study was to investigate the effect of inhaling 800 µg and 1600 µg of salbutamol prior to a 5 km time-trial in temperate and hot environmental conditions in non-asthmatic subjects. Furthermore, the pharmacokinetics of 800 µg and 1600 µg of inhaled salbutamol was examined. It was hypothesised that the inhalation of 800 µg and 1600 µg of salbutamol will not improve 5 km running time-trial performance in non-asthmatic subjects in either temperate or hot environment. Also in order to investigate the impact of inhaling short acting β_2-agonists (salbutamol) up to the WADA upper limit of 1600 µg, on simulated football (soccer) performance, it was hypothesized that inhaling up to 1600 µg salbutamol would have no ergogenic effect on simulated association football (soccer) and repeated sprint performance.

4.2 Methodology

4.2.1 Participants

Prior to the commencing the study ethical approval was obtained from Liverpool John Moores University Local Ethics Committee (ethics No: 09E18GW).

Study 1

Ten male non-asthmatic runners (mean ± SD; age 22.4 ± 4.3 years; stature 179.7 ± 7.0 cm; body mass: 76.6 ± 8.6 kg) were recruited for the study. Prior to testing all participants undertook an EVH test to exclude asthma or EIB. Prior to arrival at the laboratory participants were asked to monitor hydration status with a urine colour chart for 24 hours prior to testing. Participants completed a 5km time-trial in a temperate environment (18°C, 40% Relative Humidity (RH)) and hot environment (30°C, 40% RH) 3 times respectively, following the administration of an inhaled placebo, inhaled 800 µg salbutamol, and inhaled 1600 µg salbutamol in a randomised, cross-over, single-blind fashion:
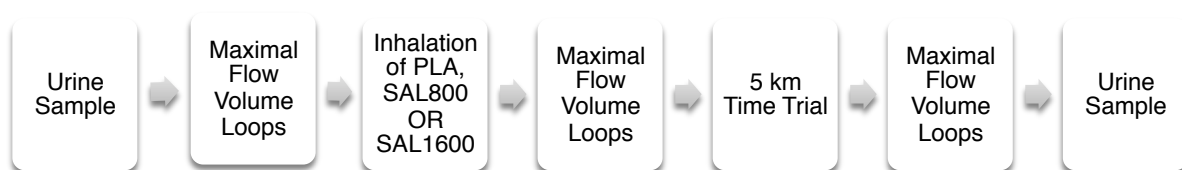
Figure 5. **Schematic of participant protocol on day of each 5 km time-trial**

Temperate (18°C, 40% RH):

Treatment 1: 16 inhalations of placebo (PLA)

Treatment 2: 8 inhalations of 100 µg salbutamol, 8 inhalation of placebo (SAL800)

Treatment 3: 16 inhalations of 100 µg salbutamol (SAL1600)

Hot (30$^{°}$C, 40% RH) environment:

Treatment 4: 16 inhalations of placebo (PLA)

Treatment 5: 8 inhalations of 100 µg salbutamol, 8 inhalation of placebo (SAL800)

Treatment 6: 16 inhalations of 100 µg salbutamol (SAL1600)

Study 2

Seven non-asthmatic male (mean ± SD, age 23 ± 4 years, stature 177.0 ± 4.7 cm, body mass 73.0 ± 4.3 kg) and 6 non-asthmatic female (mean ± SD age 21 ± 1 years, stature 162.3 ± 7.0 cm, body mass 64.0 ± 5.8kg) football players volunteered and provided written informed consent.

The participants visited the laboratory on three separate occasions. Each visit was separated by approximately 6 days. Each visit entailed the assessment of the individual's maximal oxygen consumption (VO_{2max}) using a graded exercise test to volitional exhaustion (Thoden, 1990). The criteria of the British Association of Sport and Exercise Sciences (BASES) were used to ensure that VO_{2max} had been reached (Bird and Davison, 1997). Players were required to complete a 52-minute football specific running protocol (laboratory temperature and relative humidity were 18$^{°}$C and 40%, relatively). The football-specific intermittent protocol devised for the study was performed on a motorized treadmill (Woodway Ergo ELG 55 treadmill; Woodway GmbH, Weil am Rhein, Germany) and consisted of the different

exercise intensities that are observed during football match-play (*e.g.* walking, jogging, cruising, sprinting). Each participant undertook two familiarisation sessions before the trials. During the familiarisation sessions, participants were educated with necessary guidance and trained to use relevant equipment. Fifteen minutes prior the initiation of each football specific run, players inhaled one of the following treatments, via a pocket chamber, in a randomised single blind design:

Treatment 1: 16 inhalations of placebo (PLA)

Treatment 2: 8 inhalations of 100 µg salbutamol, 8 inhalation of placebo (SAL800)

Treatment 3: 16 inhalations of 100 µg salbutamol (SAL1600)

Maximal flow volume loops were recorded at baseline, 10 minutes post-treatment inhalation and 5 minutes post-simulated football match. A urine sample was obtained between 30 and 180 minutes following the Maximal Flow Volume Loops.
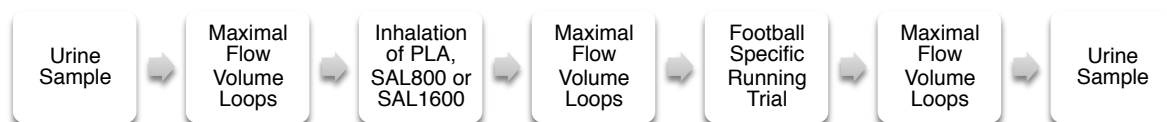
Figure 6. **Schematic of football trial**

4.2.2 Eucapnic Voluntary Hyperpnea (EVH) Test

Participants undertook an EVH test prior to the start of testing to exclude asthma or Exercise Induced Bronchoconstriction (EIB). Three baseline maximal flow-volume loops were measured using a commercially available spirometer (ML3500, Cardinal Health, Basingstoke, UK). Flow-volume values were taken from the flow-volume loop with the best combined Forced Expiratory Volume in 1 second (FEV_1) and Forced Vital Capacity (FVC). Each participant then completed an EVH challenge test (Anderson *et al*. 2001) involving 6 minutes hyperventilation at approximately 85% of maximal voluntary ventilation (30 x baselines FEV_1) pre-determined from a maximal flow volume loop. Maximal flow volume loops were measured at baseline and at 3, 5, 7, 10 and 15min following completion of the challenge. A fall of ≥10% in FEV_1 from baseline was deemed positive.

4.2.3 Baseline Measurement

In order to exclude the possible performance improvement effect from caffeine due to caffeine's half-life as this study discussed earlier, all of the participants were requested to refrain from caffeine consumption before the tests and eat within 2 hours prior to each trial. Considering the time range of caffeine's half-life for healthy adults, we informed the participants not to take any caffeine within 24 hours prior to the tests. After arriving at the laboratory, all of the participants were required to complete a pre-trial questionnaire before the measurement of body mass (kg) and stature (cm), as well as blood sample taken by finger prick, which including the checklist if they had consume any caffeine within 24 hours, only participants who had not taken any caffeine could proceed to the experiments.

Baseline spirometry was measured three times to record maximal lung function. Following the completion of baseline measurements, inhalation of placebo or salbutamol (800 μg or 1600 μg) was administered in a randomised, cross-over and single-blind fashion. Following a 10-minute post-inhalation period, post inhaler spirometry was then measured, again for three times to record the maximal lung function.

4.2.4 Performance

Study 1: 5 km Time-Trial Performance

On the test day, participants were asked to refrain from caffeine consumption for 24 hours and to refrain from eating within 2 hours of the 5 km time-trial. After arriving at the laboratory participants completed a pre-trial questionnaire and provided written informed consent prior to the measurement of body mass (kg) and stature (cm). Each participant was familiarized to running on a non-motorised treadmill (Woodway Curve, Woodway, USA) prior to initiating the 5 km time-trial. Then under controlled laboratory conditions, participants took 3 trials in temperate conditions (18°C, 40% RH) and 3 trials in hot environment (30°C, 40% RH). Prior to starting the time-trial participants were fitted with a heart rate monitor (Polar RS400; Polar Electro Oy, Kempele, Finland) and connected to a breath-by-breath gas analyser via a facemask (Oxycon Pro, Jager, Wuerzberg, Germany). Over the course of the 5 km time-trial the following were measured: time, average heart rate (HR), oxygen consumption (VO_2), carbon dioxide production (VCO_2), minute ventilation (V_E), respiratory exchange ratio (RER) and rating of perceived exertion (RPE). Two minutes following the completion of the 5 km time-trial capillary blood lactate was measured (Lactate Pro, Arkray KDK, Japan).

Following the completion of baseline measures inhalation of salbutamol (800 µg or 1600 µg) or placebo was administered in a randomised, crossover, single-blind fashion. Following a 20-minute rest period post-inhalation spirometry was obtained. The participants mounted the treadmill and following a 10-minute free warm-up were asked to begin the 5 km time-trial. During the time-trial RPE, HR, and 500 m split time was measured. During the time-trial the participants were able to observe the distance they had covered, however, no feedback related to the time, speed, or HR was provided. During the time-trial participants were not allowed to drink however, on completion they were allowed to drink water *ad libitum* to provide urine samples within 3 hours of completion, in line with WADA guidelines. Post 5 km time-trial spirometry was measured. Each participant was asked to repeat the trial three times in a single-blind, randomised design separated by a minimum of 3 days to allow for sufficient recovery.

Study 2: Simulated Football Trial

The participants were then asked to start a 52-minute (45-minute of a half football match-play, plus seven 1-minute stationary rest period to measure HR, RPE, VO_2, RER and the collection of blood sample by finger prick) intermittent football specific trial, which included five low intensity sets (walk and jog; Figure 7) and two high intensity sets (cruise and sprint; Figure 8), in the order of low-low-high-low-low-high-low. High intensity exercise bouts were separated by low intensity recovery. The order of these bouts was devised to replicate the non-cyclical nature of the exercise pattern observed in football. The low set is 6"25 minutes, the highest speed of low intensity phase is 15 km/h; and the high set is 7 minutes, with the highest speed of 23 km/h. The duration of each bout was determined by matching the percent total time for each separate activity pattern during the protocol (after the deduction of the

total time for the treadmill speed changes) to that observed during match-play, base upon the data of Reilly and Thomas (1976). The participants were reminded every time before the speed going to change. The treadmill speeds for each activity in the protocol were based upon the speeds observed for each specific movement category during soccer match-play (Van Gool *et al.*, 1988). The speeds chosen for each activity pattern were as follows: walking 6 km·h^{-1}; jogging 12 km·h^{-1}; cruising 15 km·h^{-1}; and sprinting 21 km·h^{-1}. The speeds for female footballers were 10% slower than male protocol.

During each of the seven 1-minute stationary rest periods, each participant's heart rate was monitored by a pre-fitted heart rate monitor (Polar RS400; Polar Electro Oy, Kempele, Finland) and connected to a breath-by-breath gas analyser via a facemask (Oxycon Pro; Jagear, Wuerzberg, Germany). Over the course of the football match-play the following were measured: average heart rate (HR), oxygen consumption (VO$_2$), respiratory exchange ratio (RER) and rating of perceived exertion (RPE). HemoCue Hb 201+Hemoglobin Analyser and Haemoglobin Hematocrit Centrifuge were used to analyse the blood sample collected by finger prick. A total of seven sets of HR, RPE, VO$_2$, RER and blood lactate were obtained. Body mass and post football run spirometry were then measured at the end of the simulated football trial.
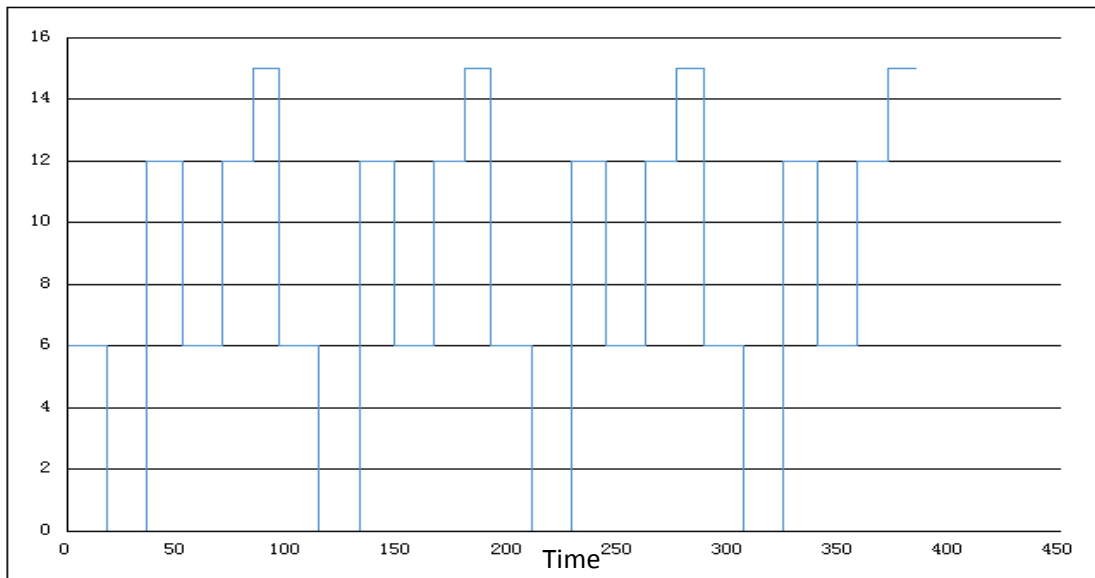
Figure 7. Low intensity exercise block
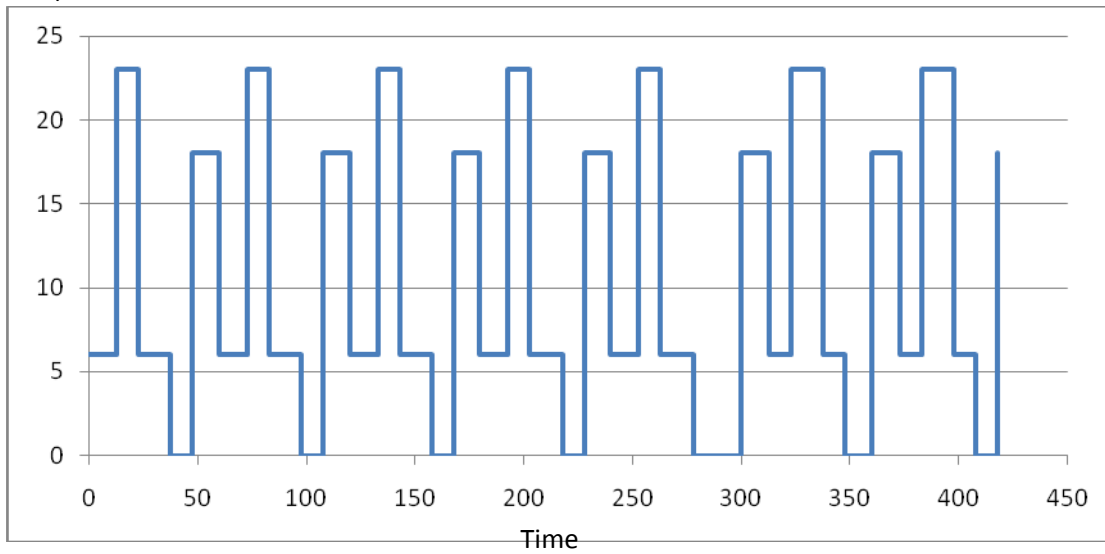
Figure 8. High intensity exercise block

Following the completion of the trial, participants were able to drink water *ad libitum* according to WADA guidelines.

4.2.5 Urine Collection

Prior to the commencement of each trial in these two studies, subjects were asked to provide a urine sample in order to void themselves of urine, after which placebo or salbutamol was administered as described above. Subjects were instructed to collect the first sample of urine passed following completion of the laboratory-based tests in line with in-competition anti-doping procedures as outlined in the World Anti-Doping Code International Standard for Testing 2012. Urine samples were provided by participants between 30 and 180 minutes following the completion of the trails. Consumption of water during this period was encouraged *ad libitum* to ensure diuresis. From the sample provided by each subject a 20 mL aliquot of urine was stored at -80°C until analysis. Urinalysis to quantify salbutamol enantiomers was performed using the analytical method outlined by Berges *et al.* (1999) which involves a solid-phase clean-up procedure followed by a chiral HPLC separation and fluorescence detection (see General Methods).

4.2.6 Urinalysis

All urinalysis was performed at HFL Sport Science (Fordham, UK), an independent drug surveillance laboratory and former WADA-accredited laboratory. Sample preparation involved the addition of 200 ng of salbutamol-D3 (NMI) as an internal standard to 1 mL of urine. Following the addition of 2 mL of 0.1 M phosphate buffer pH 6.8 and 100 μL of E. Coli enzyme (β-glucuronidase) solution the mixture was incubated overnight at 37°C. Strata XC 60 mg solid phase extraction cartridges (Phenomenex, Macclesfield, UK) were conditioned with 3 mL of methanol followed by 3 mL of reagent grade water. Following centrifugation at 3500 rpm for 5 minutes the samples were applied to the cartridges. The cartridges were then washed with 3 mL of 0.1 M acetate buffer pH 9.0 followed by 3 mL of

reagent grade water, 3 mL of 0.1 M HCl, 3 mL of methanol and 3 mL of diethyl ether. The cartridges were then dried for 5 minutes under vacuum and samples were eluted into glass vials with two, 1 mL of basic drug elution solvent (160 mL ethyl acetate, 34 mL propan-2-ol and 6 mL 34% ammonia solution). Samples were then evaporated to dryness at ambient temperature using a centrifugal vacuum concentrator (Genevac Ltd, Ipswich, UK) and reconstituted in 10 μL of isopropanol followed by 200 μL of basic reconstitution solution (495 mL of 0.1 acetic acid mixed with 5 mL Benzyldimethylphenyl Ammonium). Samples were centrifuged at 3000 rpm for 10 minutes prior to LCMS submission. Samples were injected onto a Thermo Scientific Accela HPLC system coupled to a Thermo Scientific LTQ Orbitrap Discovery Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Chromatographic separation was performed on a Waters Atlantis T3 column (2.1 x 100 mm, particle size 3 um; Waters Ltd, Elstree, UK) at 35°C. The mobile phase was a gradient system of 0.1% acetic acid aqueous solution containing uracil (300 ng·mL^{-1}) and 0.1% acetic acid in acetonitrile containing uracil (300 ng·mL^{-1}) set at a flow rate of 0.4 mL·min^{-1}.

The urine salbutamol concentrations reported correspond to the sum of the free and glucuronide conjugates. The samples were analysed over the calibration range of 10 to 2000 ng·mL^{-1}. Samples with salbutamol concentrations greater than the upper limit of quantification were diluted with blank human urine prior to analysis. The lower limit of quantification was accepted as the lowest standard on the calibration curve (10 ng·mL^{-1})

4.2.7 Statistical analysis

Following the completion of 5 km time-trial and associated football performance under each condition, statistical analyses were done using the statistical software program SPSS (SPSS, Inc., Chicago, IL), and a two-way analysis of variance (ANOVA) was performed to compare differences in heart rate, oxygen consumption, minute ventilation, respiratory exchange ratio, rating of perceived exertion, blood lactate, forced expiratory volume in one second, forced vital capacity, peak expiratory flow rate and forced expiratory flow between 25 and 75% of forced vital capacity (study 1); differences in sprint time, peak power, peak velocity, heart rate, blood lactate, oxygen consumption, minute ventilation, respiratory exchange ratio, rating of perceived exertion (study 2). Normally distributed continuous variables are expressed as mean (SD). Statistical significance for this test was set at a p-value of ≤0.05. An effect size was also calculated to estimate the magnitude of the difference between groups. This provides a way to describe the meaningfulness of the differences, especially when the sample size is small. The size of the effect was classified according to the system proposed by Cohen (1988), where an effect size of 0.3 represents a small effect, one of 0.5 represents a moderate effect and one of 0.8 or above represents a large effect. All data was tested with Mauchly's test of sphericity. The primary variable is the amount of salbutamol inhaled, being nil, 800 µg or 1600 µg. A total number of ten non-asthmatic males and thirteen non-asthmatic males and females completed all trials in Study 1 and Study 2 respectively.

4.3 Results

Study 1: 5 km Time-Trial Performance

Throughout all trials participants reported no side effects from inhalation of up to 1600 µg inhaled salbutamol. Due to a number of measurements errors associated with three participants which excluding them from analysis. Accordingly, the data from 7 participants were analysed. All participants were free from chest infection for at least 4 weeks prior to assessment; they were not taking any medication and there were no other health or medical contradictions to them taking part in the study as confirm by information provided on a physical activity readiness questionnaire. All participants were actively engaged in endurance running training (>45 minutes continuous running) at least 3 times per week and were members of a competitive team. All participants were free from asthma and bronchial hyperresponsiveness confirmed by the presentation of a negative Eucapnic Voluntary Hyperpnoea (EVH) challenge (Anderson et al., 2001).

Performance trial in moderate environment (18°C, 40% RH)

All seven participants completed all three 5 km time-trial. No significant difference was noted for overall completion time between trials (see Figure 9). No significant difference was observed over each 1 km split between conditions (see Figure 10). Furthermore, over each 1 km split no significant difference between conditions for mean: HR; VO_2; VCO_2; V_E; and RER were observed (see Figure 11). There was no significant difference between conditions in the post 5 km time-trial blood lactate values (see Figure 11). At 2, 3 and 4 km RPE was significantly increased under the SAL800 and SAL1600 when compared to PLA (Figure 11d) (See Appendix 1 for results of 1-5 km time-trial).

Figure 9. Individual and mean ± SD 5 km time-trial performance in a moderate environment (18°C, 40% RH) following the inhalation of placebo (PLA), 800 μg of salbutamol (SAL800) and 1600 μg of salbutamol (SAL1600).

Figure 10. Average time in seconds in a moderate environment (18°C, 40% RH) following the inhalation of placebo (PLA), 800 μg of salbutamol (SAL800) and 1600 μg of salbutamol (SAL1600).

a)

b)

c)

d)

e)

f)

Figure 11. Mean ± SD for HR, VO_2, VCO_2, V_E, RPE and Blood Lactate at each 1 km split of the 5 km time-trial (a-e) and post-5 km time-trial blood lactate (f) in a moderate environment (18°C, 40% RH). * = SAL800 and SAL1600 significantly different from PLA.

Performance trial in hot environment (30°C, 40% RH)

All seven participants completed all three 5 km time-trial. No significant difference was noted for overall completion time between trials (see Figure 12). Furthermore, over each 1 km split no significant time difference between conditions was observed (see Figure 13). Average VO$_2$ during the 1st km was significantly greater (p=0.046) during PLA (3.941± 0.490 L·min^{-1}) when compare with SAL800 (3.591 ± 0.544 L·min^{-1}) and SAL1600 (3.516 ± 0.416 L·min^{-1}) trials, however overall mean: HR; VO2; VCO2; and RPE during the 5 km time-trial were not significantly different between trials (see Figure 14). There was no significant difference between conditions in the post 5 km time-trial blood lactate values (see Figure 14; see Appendix 1 for results of 1-5 km time-trial).
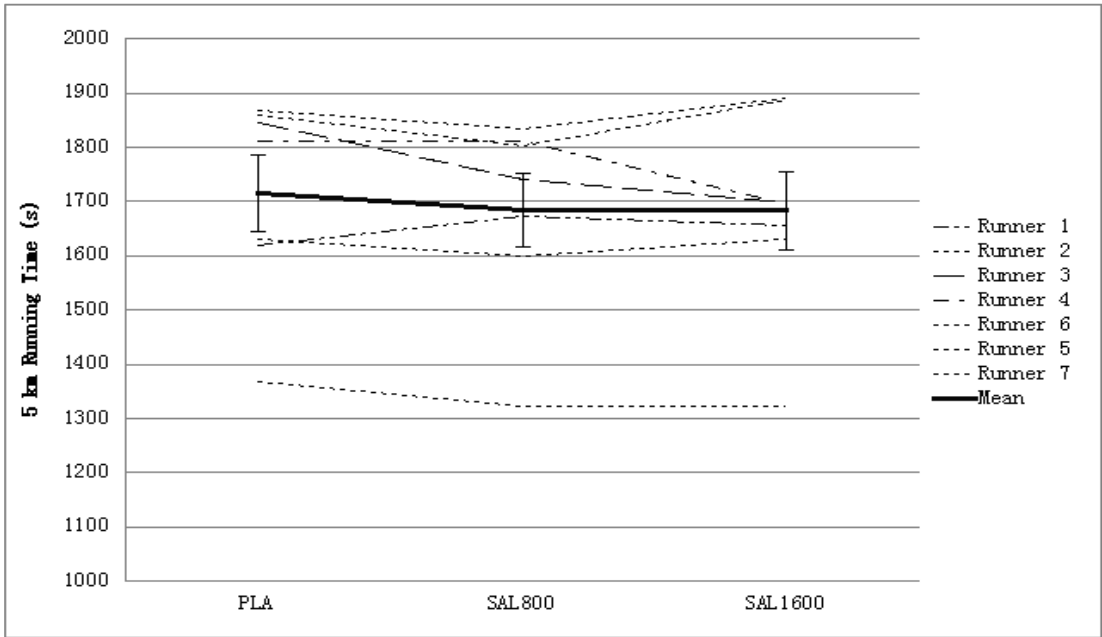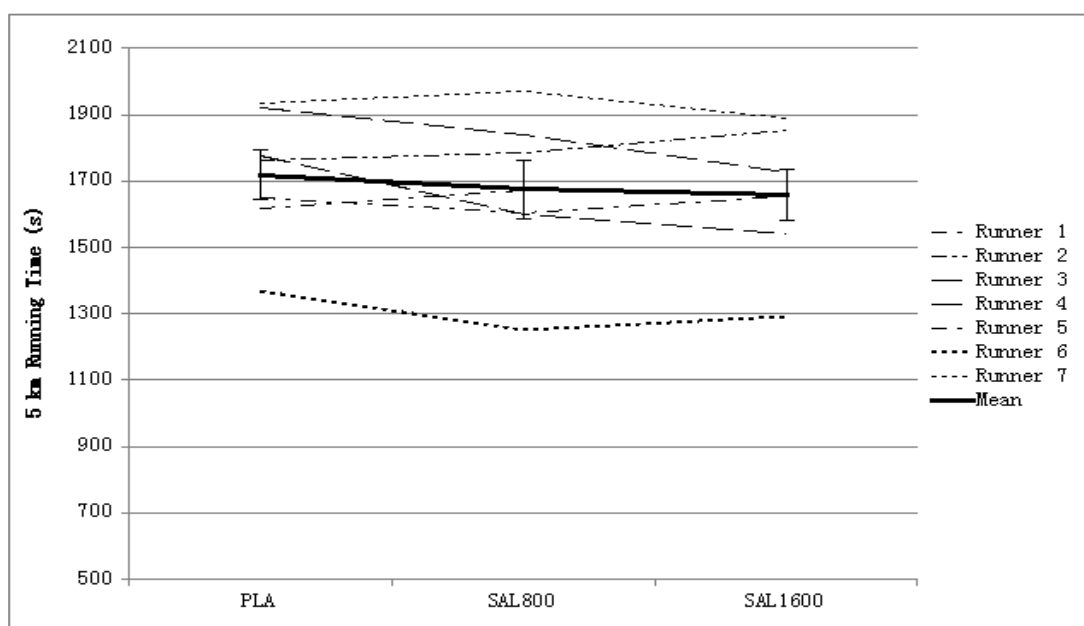

Figure 12. Individual and mean ± SD 5 km time-trial performance in a hot environment (30°C, 40% RH) following the inhalation of placebo (PLA), 800 μg of salbutamol (SAL800) and 1600 μg of salbutamol (SAL1600).
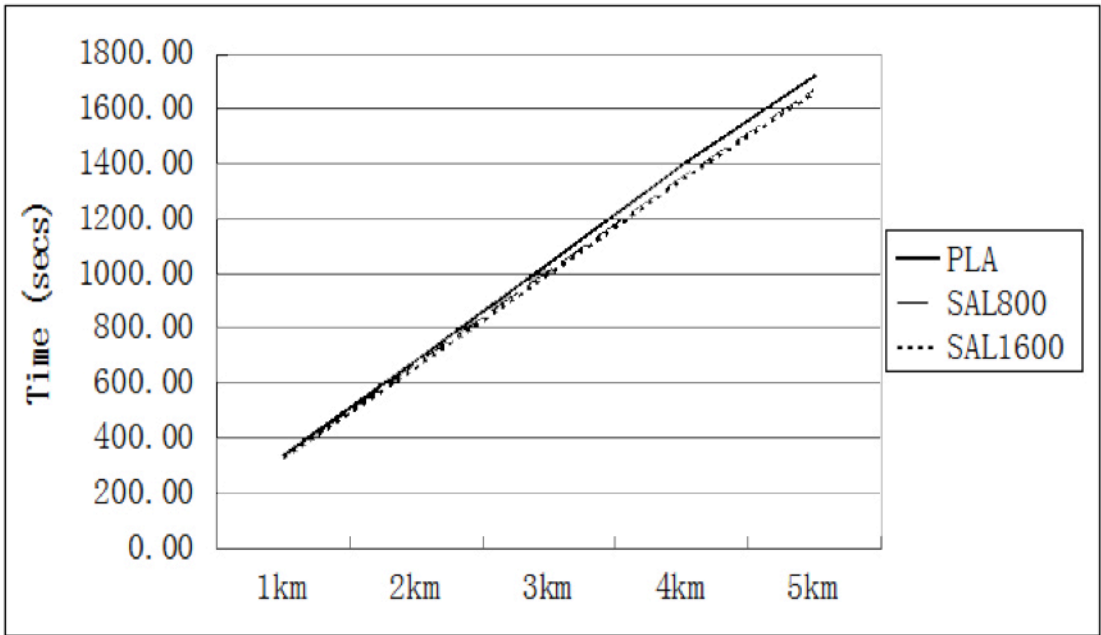
Figure 13. Average time in seconds in a hot environment (30°C, 40% RH) following the inhalation of placebo (PLA), 800 μg of salbutamol (SAL800) and 1600 μg of salbutamol (SAL1600).

a)
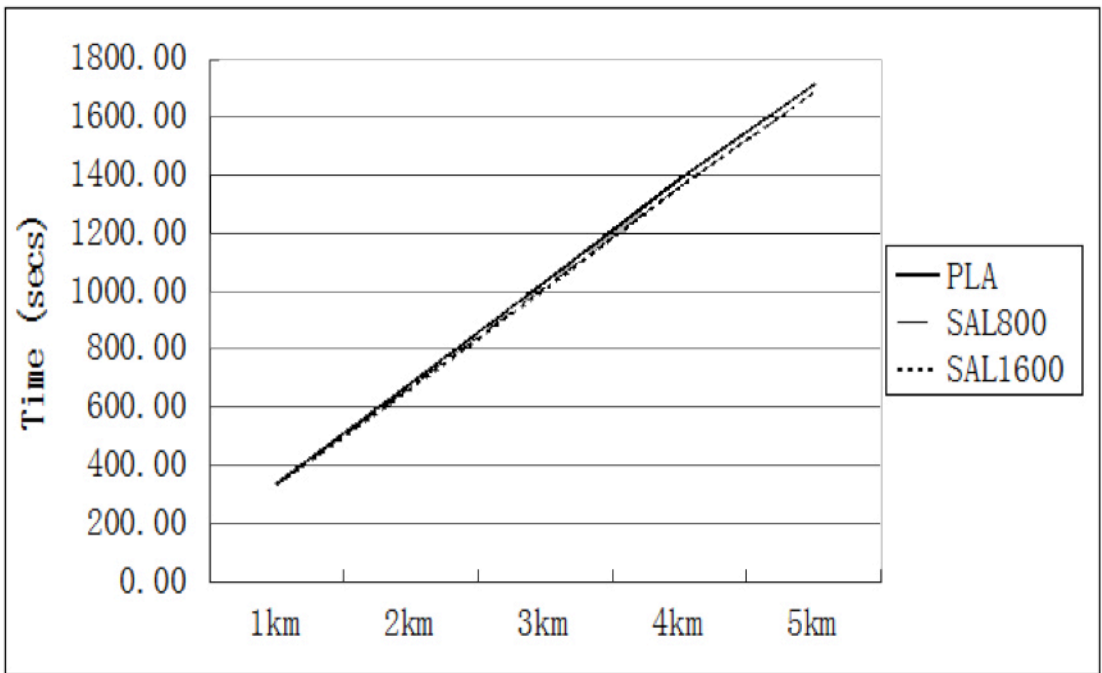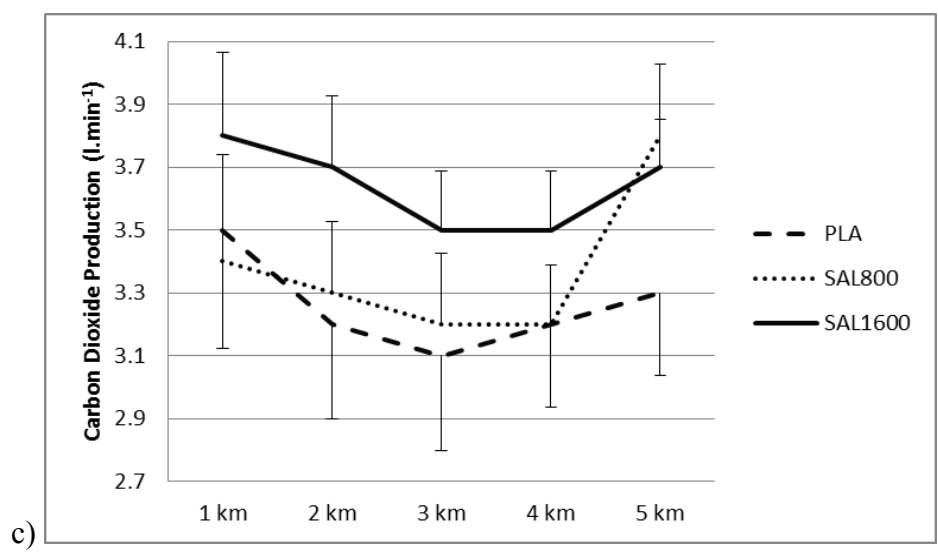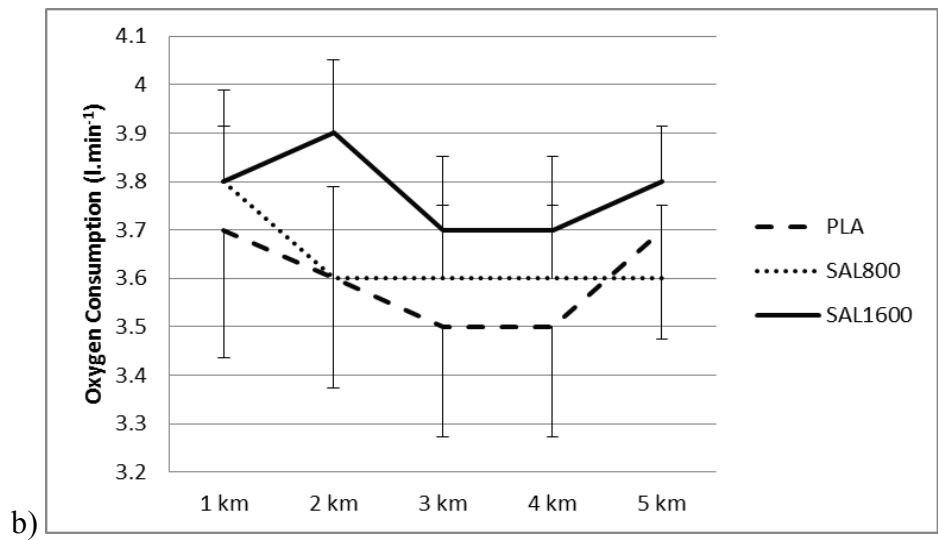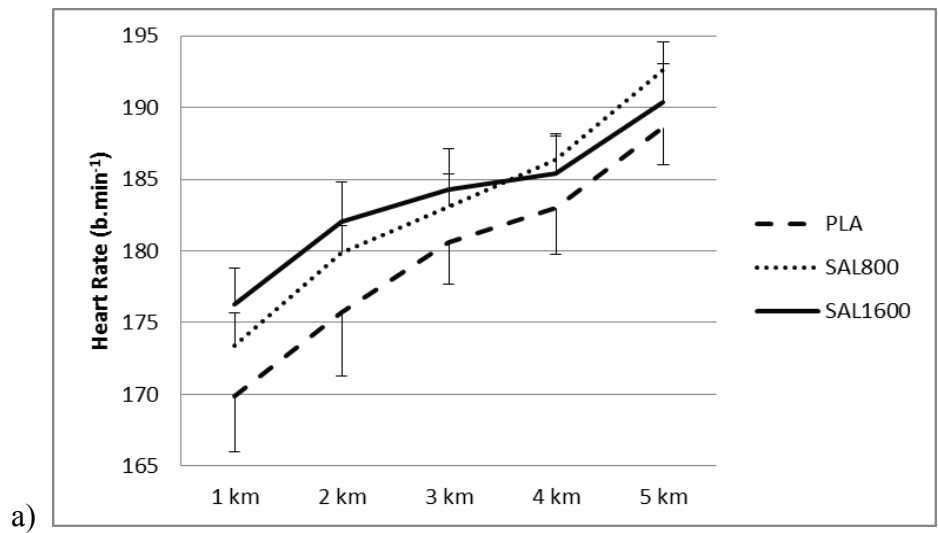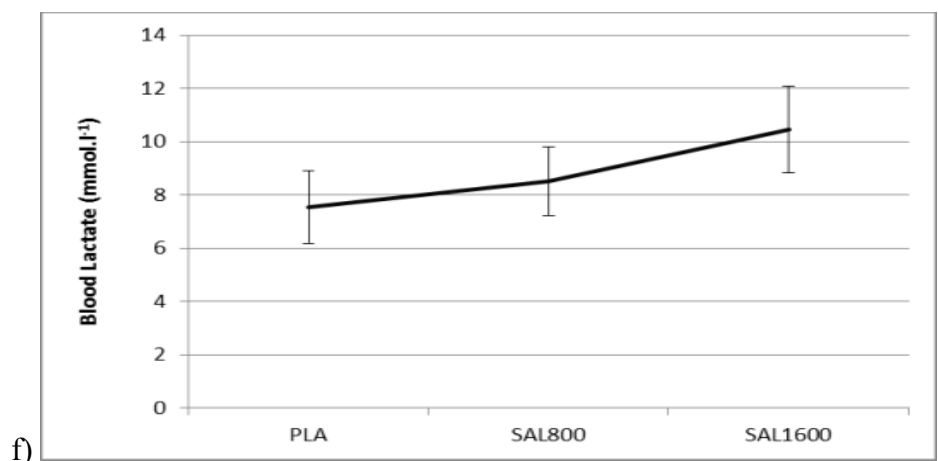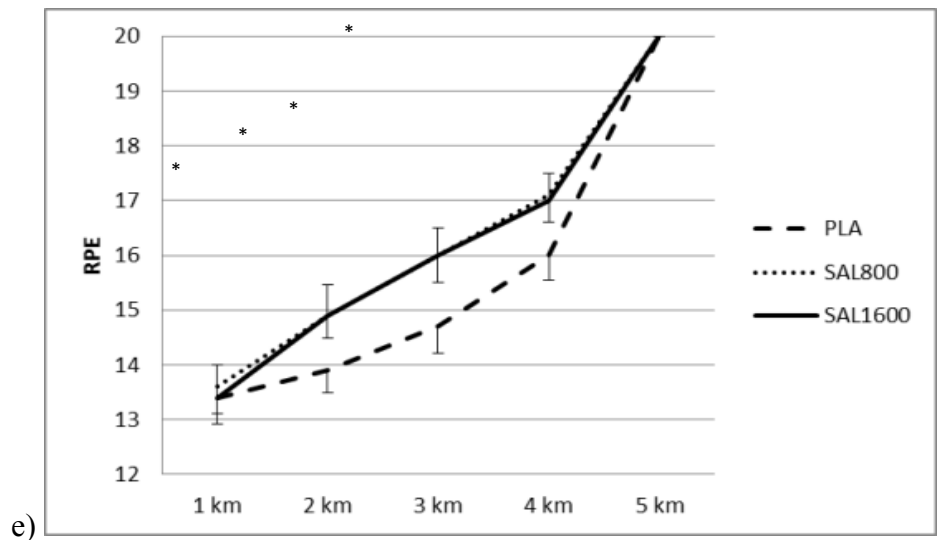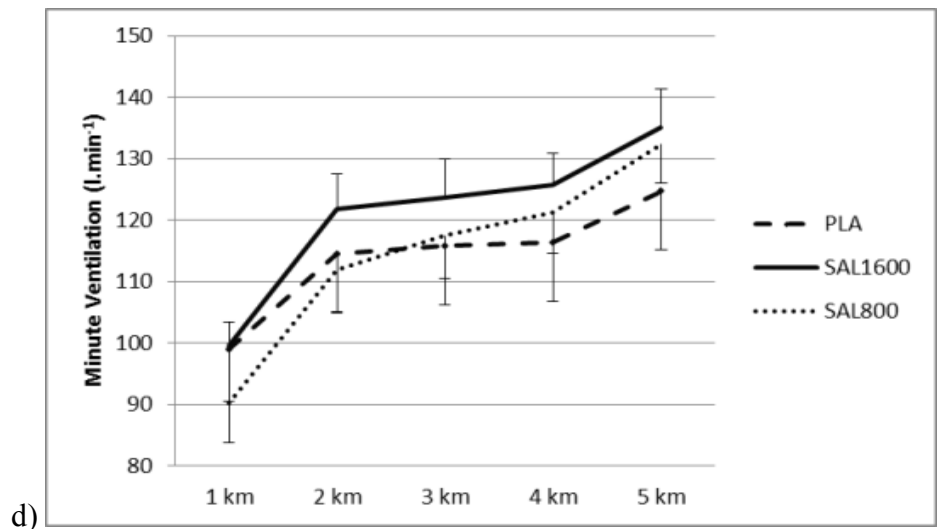

b)

c)

d)

e)
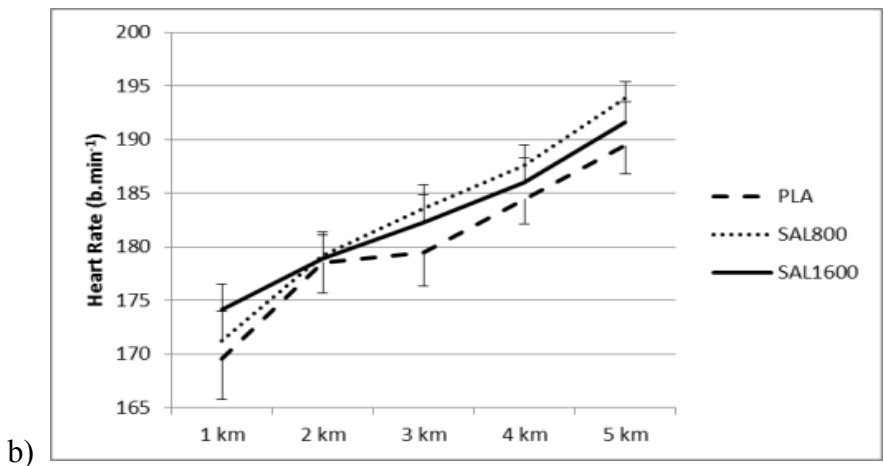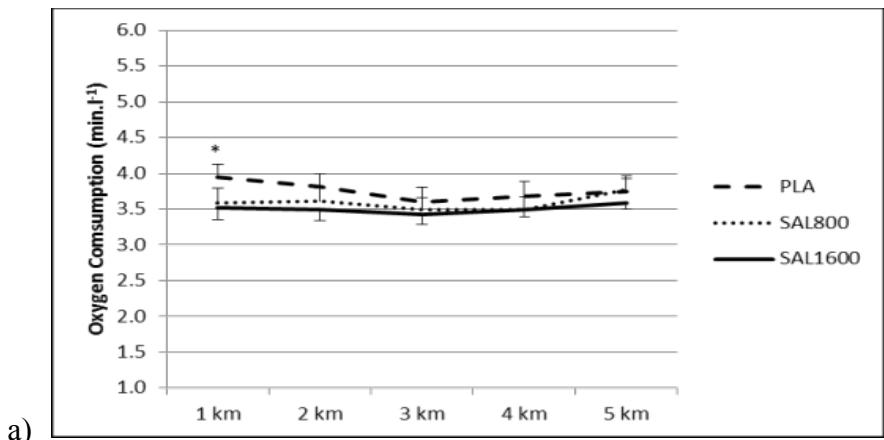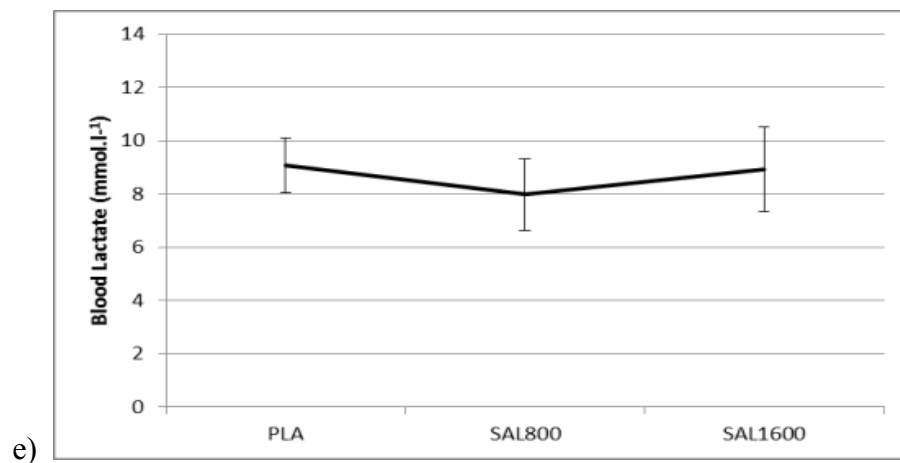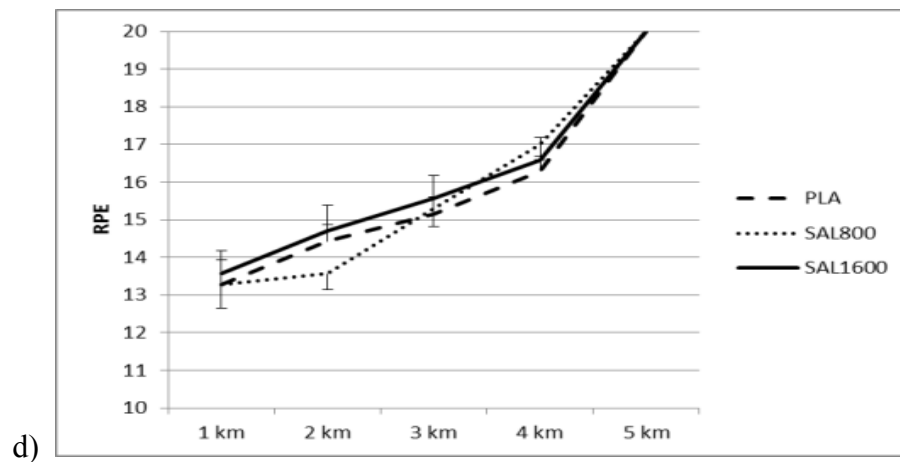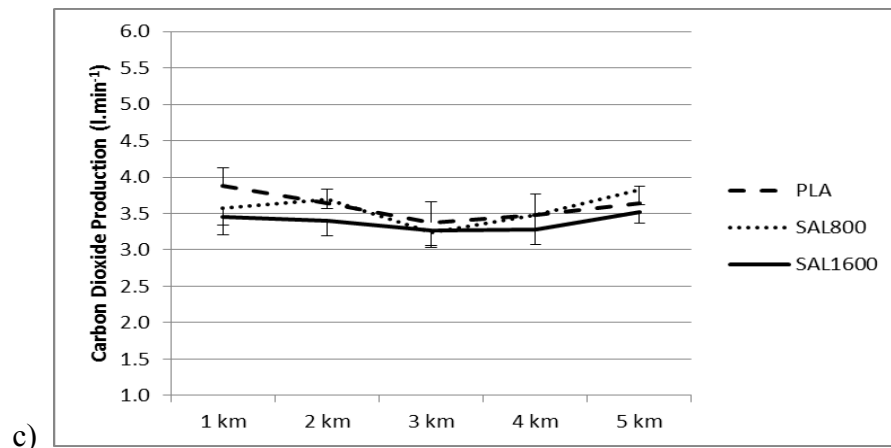
Figure 14. Mean ± SD for HR, VO$_2$, VCO$_2$, and RPE at each 1 km split of the 5 km time-trial (a-d) and post-5 km time-trial blood lactate (e) during 5 km time-trial in a hot environment (30°C, 40% RH).

Lung Function Data:

A significant difference in FEV_1/FVC at baseline was observed following inhalation of 800 µg, and 1600 µg of salbutamol ($p<0.05$).

	FEV1			FVC			FEV1/FVC		
	PLA	SAL800	SAL1600	PLA	SAL800	SAL1600	PLA	SAL800	SAL1600
Baseline	4.57 ± 0.86	4.61 ± 0.82	4.6 ± 0.91	5.37 ± 0.91	5.28 ± 1.01	5.24 ± 0.98	86.42 ± 3.4	87.42 ± 4.57	87.57 ± 3.4
Post-SAL	4.62 ± 0.85	4.68 ± 0.87	4.69 ± 0.94	5.30 ± 0.94	5.25 ± 0.94	5.24 ± 0.95	87.14 ± 3.43	89.14 ±3.97	89.42 ± 4.11
Post-run	4.60 ± 0.92	4.65 ± 0.96	4.67 ± 0.94	5.13 ± 0.99	5.13 ± 1.02	5.09 ± 1.01	89.85 ± 5.39	91 ± 3.82	92 ± 4.65

Table 8. Lung Function Data in moderate temperature (18$^{°}$C, 40% RH) and 3 conditions.

	FEV1			FVC			FEV1/FVC		
	PLA	SAL800	SAL1600	PLA	SAL800	SAL1600	PLA	SAL800	SAL1600
Baseline	4.57 ± 0.88	4.58 ± 0.92	4.63 ± 0.90	5.27 ± 0.98	5.29 ± 0.97	5.30 ± 1.04	87.57 ± 4.39	86.43 ± 4.35	87.29 ± 3.90
Post-SAL	4.58 ± 0.79	4.72 ± 0.90	4.71 ± 0.95	5.32 ± 0.93	5.28 ± 0.99	5.25 ± 1.05	86.43 ± 2.94	89.14 ± 3.53	89.86 ± 3.72
Post-run	4.65 ± 0.94	4.73 ± 0.91	4.69 ± 0.91	5.12 ± 0.95	5.13 ± 0.99	5.17 ± 1.00	90.43 ± 4.61	92.43 ± 4.28	90.86 ± 3.93

Table 9. Lung Function Data in hot condition (30$^{°}$C, 40% RH) and 3 conditions.

Study 2: Football Specific Match-play

All seven males and six females completed the 52 minutes football specific match-play.

Male results

Under each of the three treatments no significant difference was observed in average sprint time, peak power, peak velocity, peak HR or peak lactate in the male players (Figure 14). During the football specific treadmill run male players demonstrated a significantly greater average VO_2 during stage 1 in SAL1600 (1.906 ± 0.174 $L \cdot min^{-1}$) when compared with PLA (1.630 ± 0.119 $L \cdot min^{-1}$; p=0.01) and SAL800 (1.726 ± 0.267 $L \cdot min^{-1}$; p=0.04). HR was significantly greater in SAL1600 when compared to PLA and SAL800 during stages 1 to 5, and 7. V_E was significantly greater in SAL1600 when compared to PLA in all seven stages. Blood lactate values were significantly greater in SAL1600 than PLA during stages 1, 4 and 5. There was no significant difference during any of the stages in VCO_2 or RPE (Figure 15).

There was no significant difference in maximal lung function during the baseline measurements between treatments. At 10 minutes post-inhalation of SAL800 and SAL1600 FEV_1 was significantly greater than PLA (p=0.03). 5 minutes following the football specific run FEV_1 was significantly greater in SAL1600 (p=0.05) and SAL800 (p=0.03) when compared to PLA. FEV_1% was significantly greater (p=0.03) 5 minutes post-trial in SAL800 when compared with PLA (see Table 10).
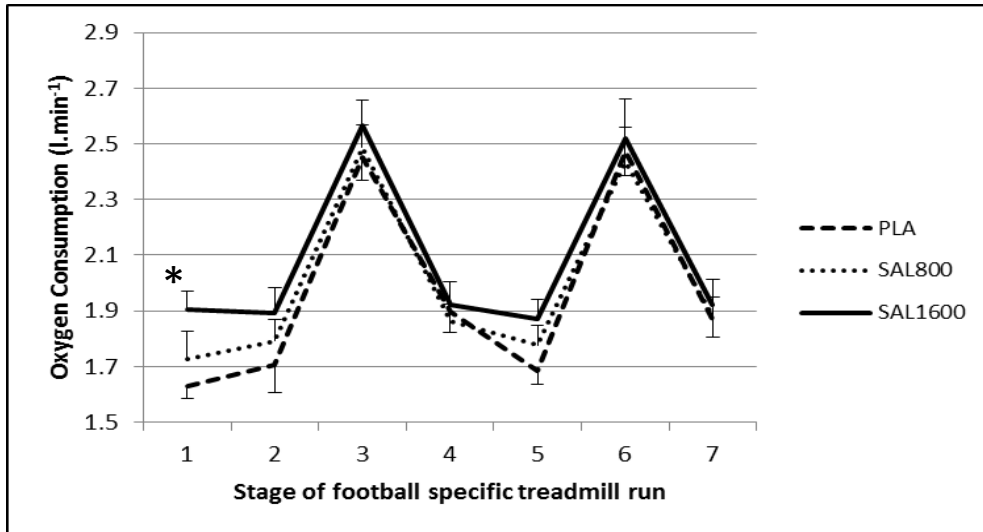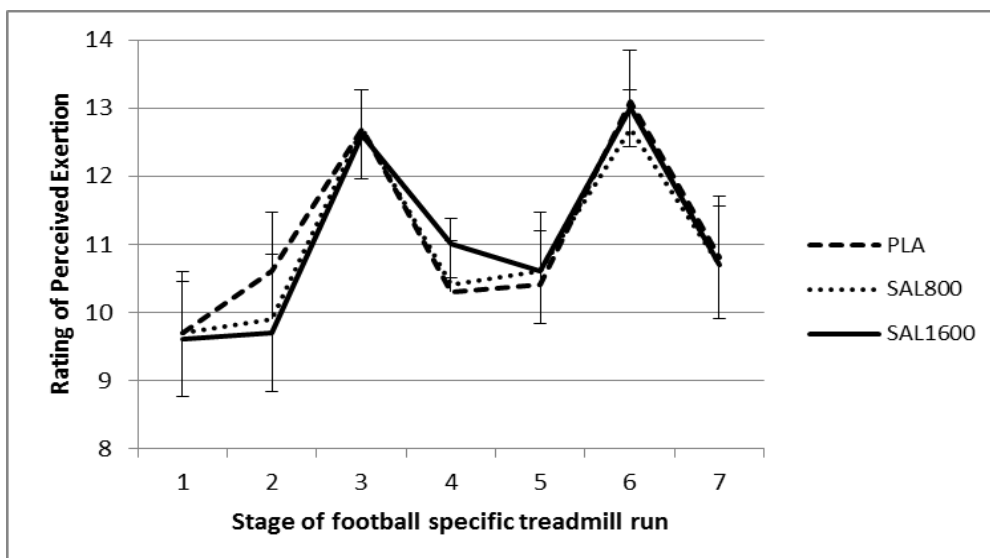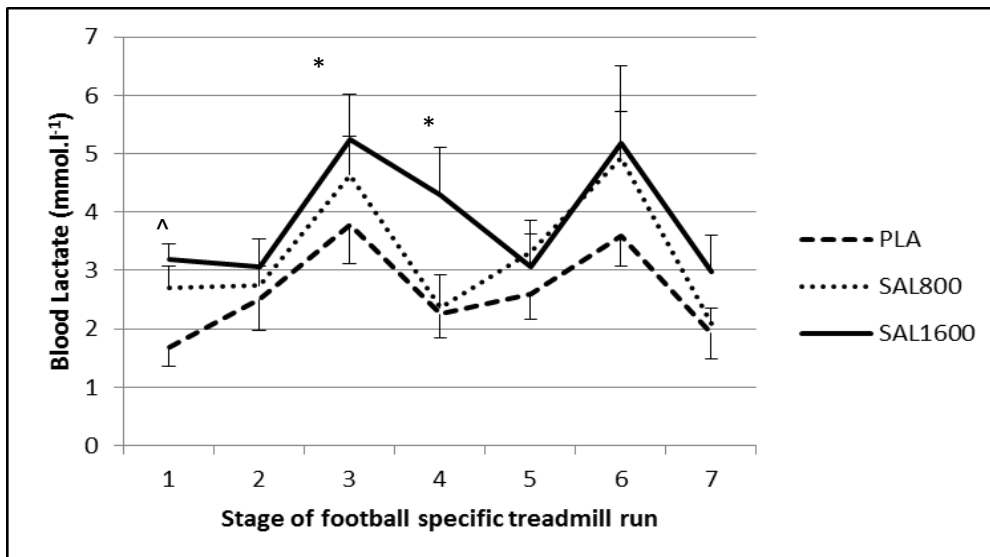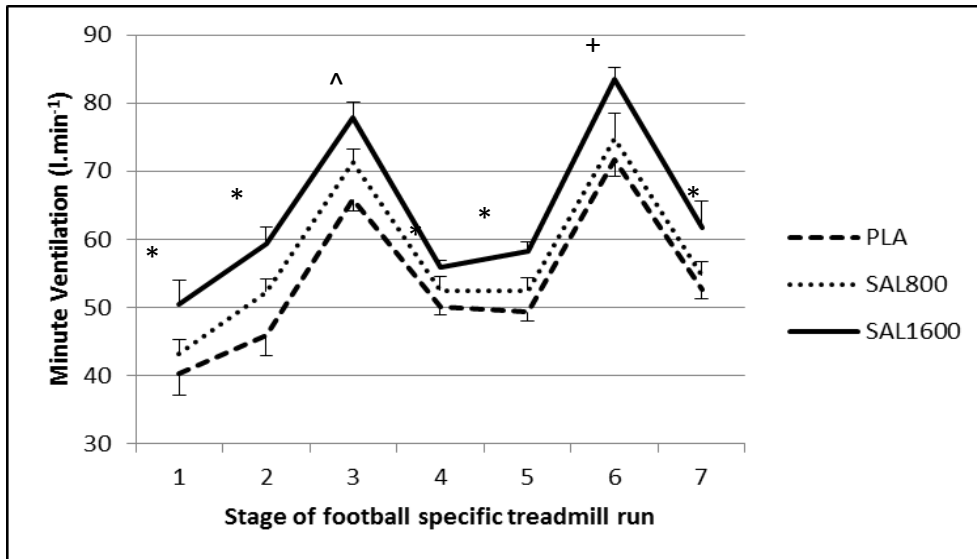
*=SAL1600 and SAL800 significantly greater than PLA

*=SAL1600 significantly greater than SAL800 and PLA

*= SAL1600 significantly greater than PLA

+=SAL1600 significantly greater than PLA

^=SAL1600 significantly greater than PLA and SAL800；

*=SAL 800 significantly greater than PLA

^=SAL1600 and SAL800 significantly greater than PLA

+=SAL1600 significantly great than PLA and SAL800

*= SAL1600 significantly greater than PLA

^ = SAL1600 and SAL800 significantly greater the PLA

Figure 15. VO_2, VCO_2, HR, V_E, blood lactate and RPE over the seven stages of the football specific match-play test following inhalation of Placebo (PLA), 800 μg of salbutamol (SAL800), or 1600 μg of salbutamol (SAL1600) in male players (mean ± SD)

	PLA	SAL800	SAL1600
Baseline			
FEV$_1$ (l)	4.2 ± 0.5	4.3 ± 0.5	4.3 ± 0.5
FEV$_1$ predicted	102.4 ± 11.7	105.2 ± 12.7	105.1 ± 11.5
FVC (l)	4.9 ± 0.59	5.0 ± 0.61	5.0 ± 0.66
FVC predicted	102.1 ± 17.9	106.7 ± 22.9	101.6 ± 19.4
EV$_1$ %	85.6 ± 9.1	84.7 ± 10.3	85.1 ± 7.9
10 minutes post-inhalation			
FEV$_1$ (l)	4.2 ± 0.6	4.4 ± 0.4*	4.4 ± 0.5*
FVC (l)	4.9 ± 0.6	5.0 ± 0.6	5.0 ± 0.6
FEV1 %	84.7 ± 8.8	87.7 ± 6.8	88.3 ± 7.9
5 minutes post-football specific run test			
FEV$_1$ (l)	4.2 ± 0.5	4.4 ± 0.4*	4.4 ± 4.3*
FVC (l)	4.8 ± 0.57	4.8 ± 0.5	5.0 ± 0.6
FEV1 %	87.0 ± 9.8	92.7 ± 8.3*	89.4 ± 8.3

*=significantly ($p<0.05$) greater than placebo (PLA)

Table 10. Maximal Lung Function in Male Football Players following inhalation of Placebo (PLA), 800 μg of salbutamol (SAL800), or 1600 μg of salbutamol (SAL1600) (mean ± SD)

Predicted values from European Coal and Steel Community (Quanjer *et al.*, 1997).

Female results

Under each of the three treatments the female players demonstrated no significant difference in average sprint time, peak power, peak velocity, peak HR or peak lactate (Figure 17). During the football specific treadmill run VO_2, VCO_2, HR and RPE were not significant during any stage of the protocol between treatments. V_E was significantly greater SAL1600 (p=0.02) and SAL800 (p=0.03) when compare to PLA during stage one. During stages 5, 6 and 7, V_E was significantly greater in SAL1600 when compared to PLA (Figure 16).

Maximal lung function did not differ between treatments at baseline, 10 minutes after inhalation of treatment or 5 minutes after the completion of the football specific run (see Table 11).

a)

b)

c)

d)

e)

f)
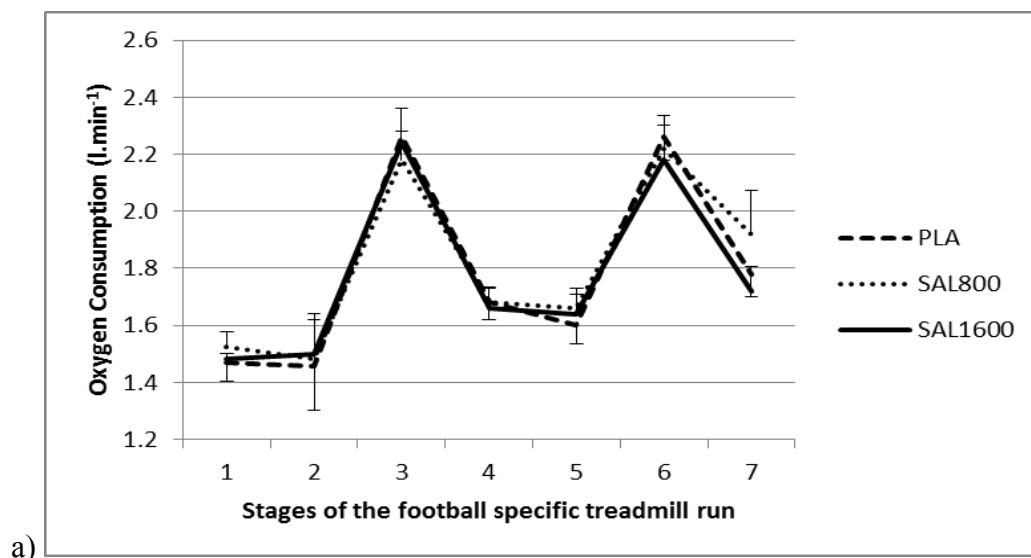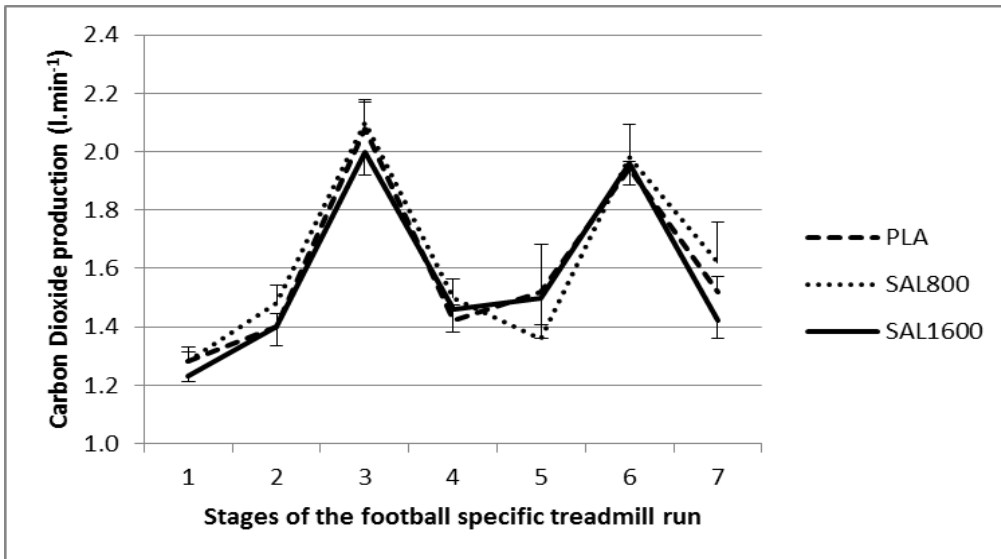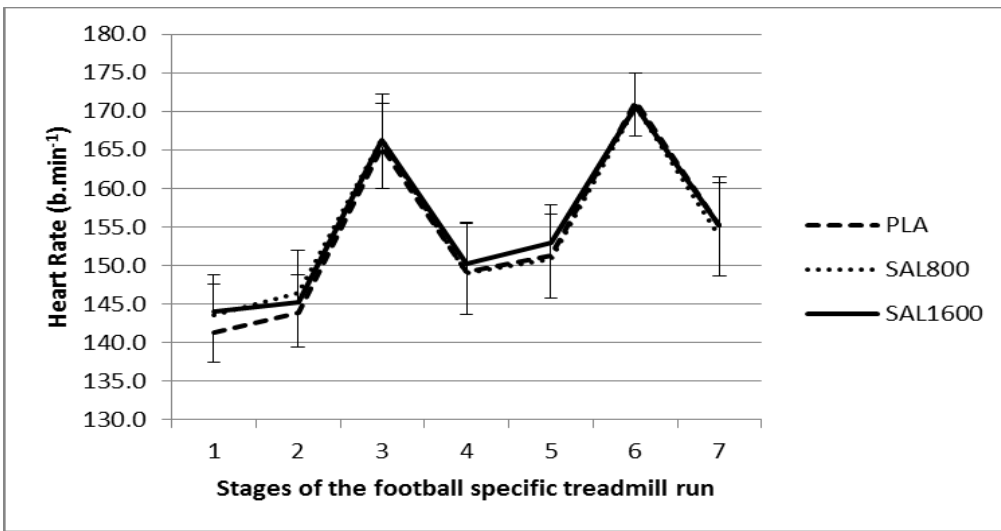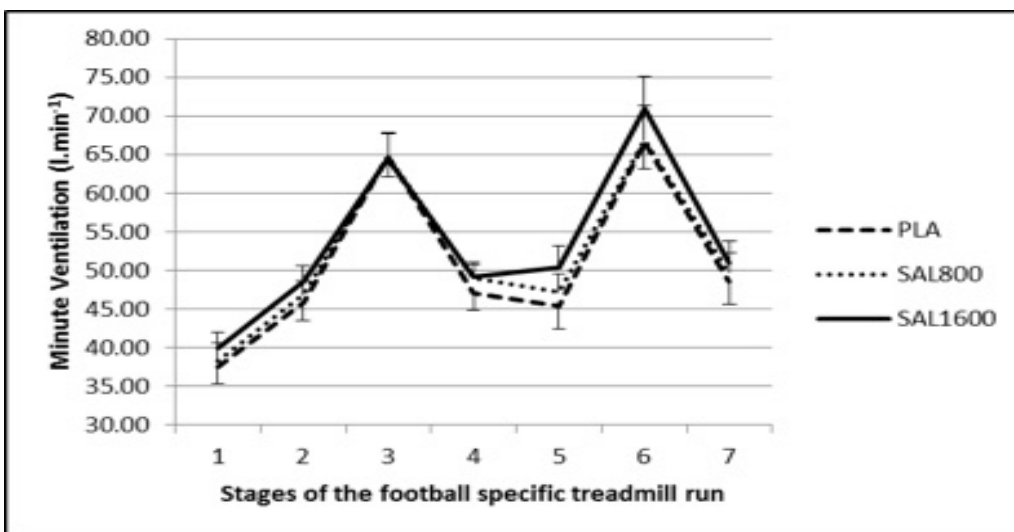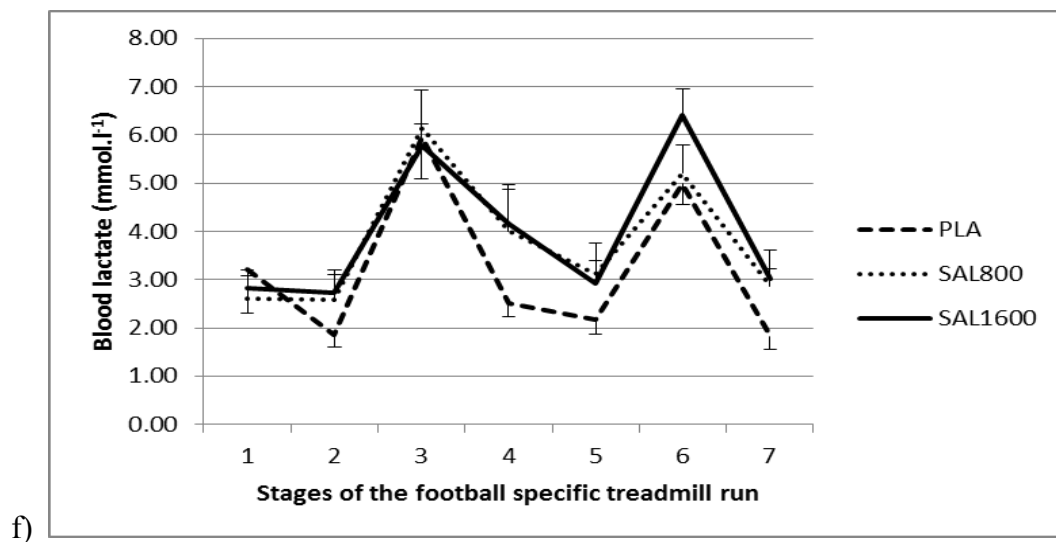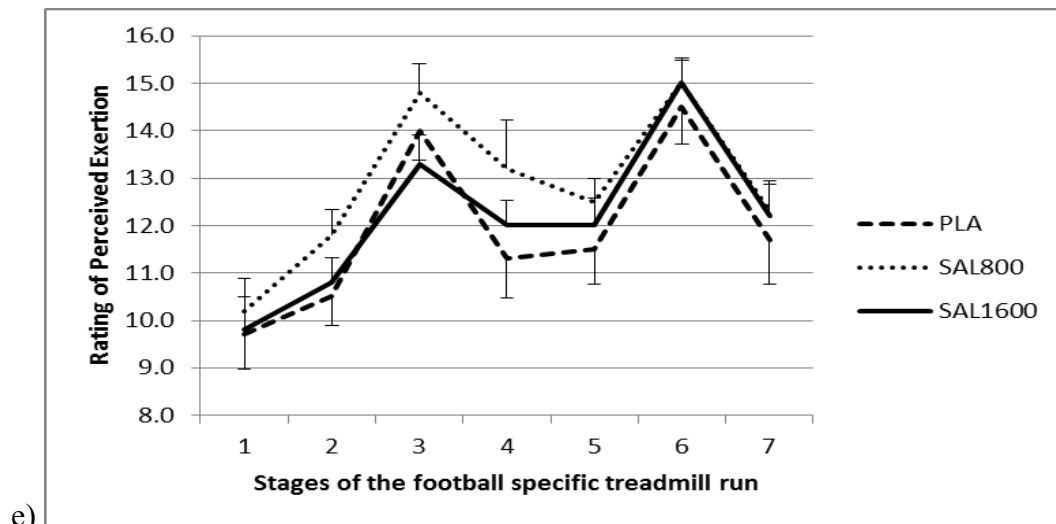
*= SAL1600 significantly greater than placebo and SAL800

^= SAL1600 sig greater than PLA
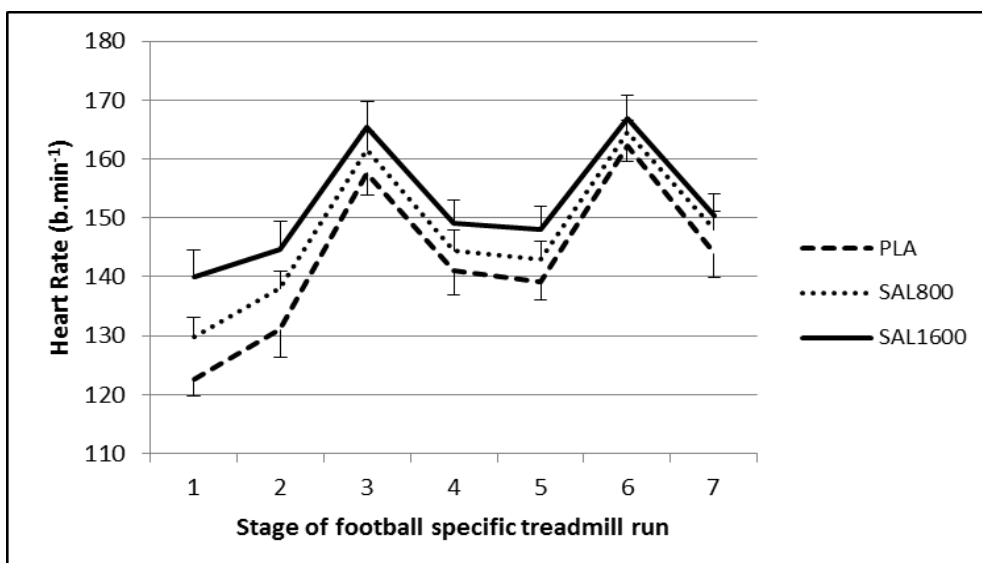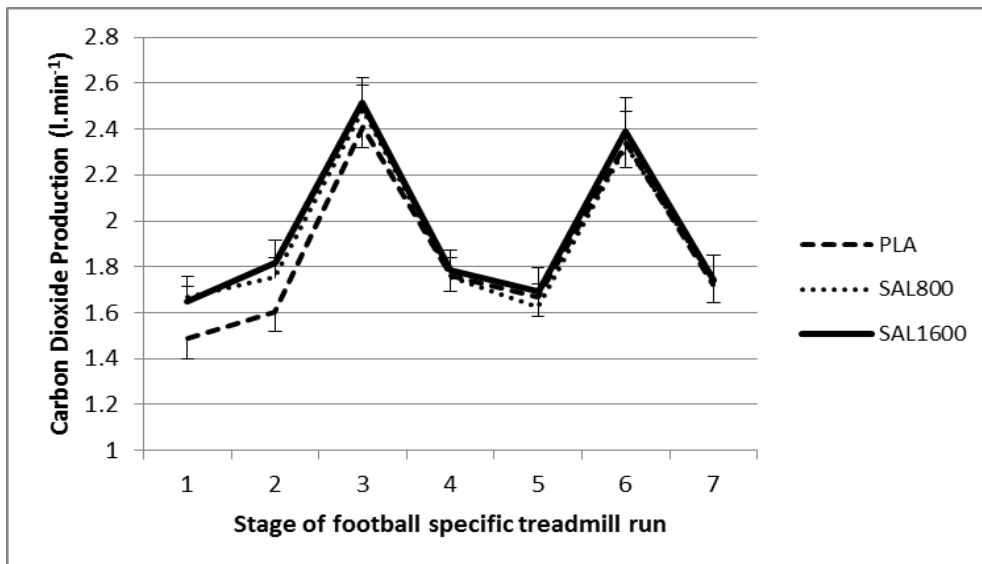
Figure 16. VO_2, VCO_2, HR, V_E, blood lactate and RPE (a-f) over the seven stages of the football specific match-play (mean ± SD) following inhalation of Placebo (PLA), 800 μg of salbutamol (SAL800), or 1600 μg of salbutamol (SAL1600) in female players.

	PLA	SAL800	SAL1600
Baseline			
FEV_1	3.4 ± 0.2	3.4 ± 0.3	3.4 ± 0.3
FEV_1 predicted	103.5 ± 5.5	102.7 ± 9.0	102.0 ± 9.1
FVC	3.8 ± 0.3	3.8 ± 0.4	3.8 ± 0.3
FVC predicted	101.3 ± 5.9	100.0 ± 7.8	98.8 ± 7.9
FEV_1 %	89.3 ± 1.4	89.5 ± 2.1	90 ± 1.9
10 minutes post-inhalation			
FEV_1	3.5 ± 0.3	3.5 ± 0.2	3.5 ± 0.3
FVC	3.9 ± 0.4	3.8 ± 0.3	3.8 ± 0.3
FEV1 %	89.7 ± 2.3	92.2 ± 2.2	92.3 ± 2.1
5 minutes post-football specific run test			
FEV1	3.4 ± 0.3	3.5 ± 0.2	3.5 ± 0.3
FVC	3.7 ± 0.4	3.8 ± 0.3	3.8 ± 0.4
FEV1 %	92.7 ± 3.6	93.3 ± 3.3	93.3 ± 3.3

Table 11. Female maximal lung function following inhalation of Placebo (PLA), 800 μg of salbutamol (SAL800), or 1600 μg of salbutamol (SAL1600) (mean ± SD)

Predicted values from European Coal and Steel Community (Quanjer *et al*., 1997).

Urine Analysis

Study 1

Following inhalation of 800 μg of Salbutamol and a 5 km time-trial under temperate (18°C; 40% RH) and hot (30°C; 40% RH) ambient conditions mean ± SD urine concentrations were 122.96 ± 69.22 ng·mL^{-1} and 138.83 ± 98.11 ng·mL^{-1}, respectively (see Figure 17). Following inhalation of 1600 μg of salbutamol and a 5 km time-trial, mean ± SD urine concentrations were 574.06 ± 448.17 ng·mL^{-1} and 270.32 ± 183.90 ng·mL^{-1} under temperate and hot ambient conditions, respectively (Figure 17). Whilst there were no significant differences between drug urine concentrations under either condition there was a high degree of inter-individual variation following the inhalation of 1600 μg of salbutamol (Figure 18). Only one sample reached the WADA upper limit of 1000 ng·mL^{-1} (1190 ng·mL^{-1} (in the temperate environment) however; the sample failed to reach the decision limit of 1200 ng·mL^{-1}.
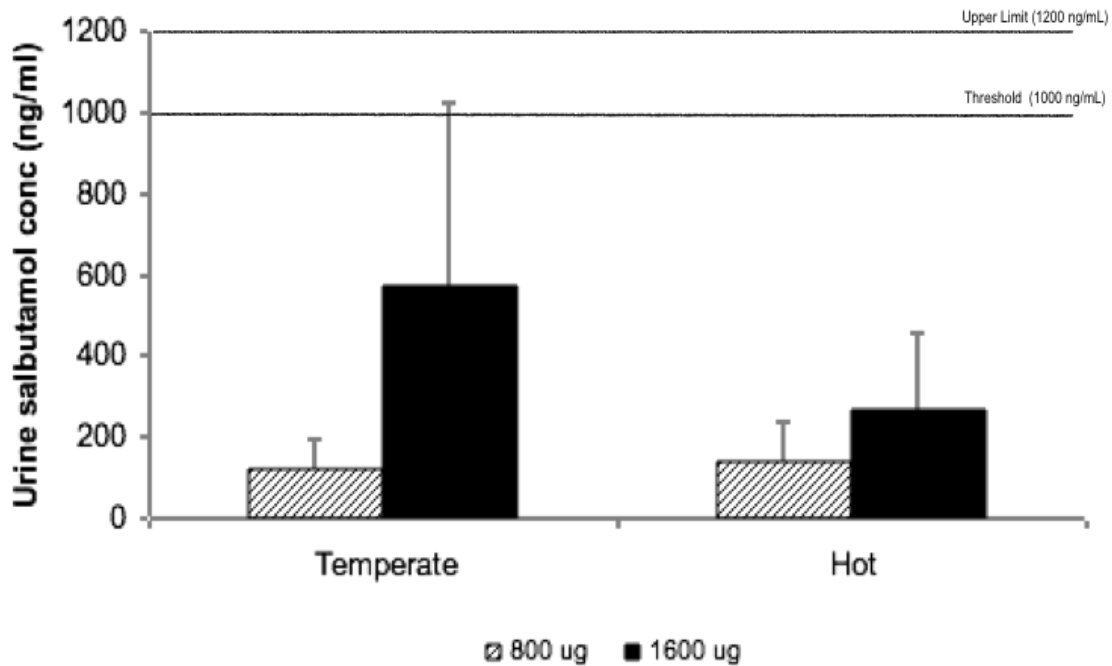
Figure 17. Mean (± SD) urine SAL concentration (µg·mL^{-1}) post 5 km time-trial under temperate (18$^{°}$C; 40% RH) and hot (30$^{°}$C; 40% RH) conditions following inhalation of 800 µg (SAL800) or 1600 µg of salbutamol (SAL1600).
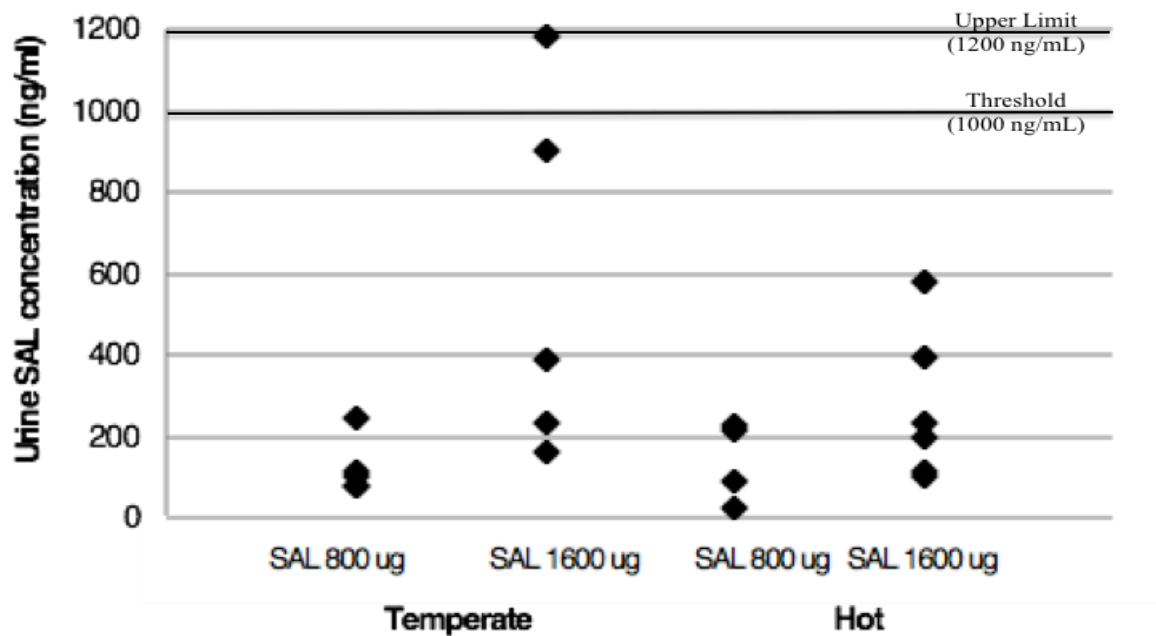
Figure 18. Urine SAL concentration (ng·mL^{-1}) post 5 km time trial under temperate (18$^{°}$C; 40% RH) and hot (30$^{°}$C; 40% RH) conditions following inhalation of 800 µg (SAL800) or 1600 µg of salbutamol (SAL1600).

Study 2

Following inhalation of 800 μg and 1600 μg of salbutamol and completion of a football-specific running protocol under temperate (20°C, 40% RH) ambient conditions the cohort reported mean (± SD) urine drug concentrations of 250.91 ± 234.51 ng·mL^{-1} and 804.02 ± 568.46 ng·mL^{-1}, respectively. Whilst mean (± SD) urine drug concentrations following inhalation of 800 μg of salbutamol was 301.47 ± 294.47 ng·mL^{-1} in males compared to 180.12 ± 102.15 ng·mL^{-1} in females there was no significant difference according to gender (see Figure 19). Likewise there were no significant sex differences in urine drug concentrations following inhalation of 1600 μg of salbutamol in male 739.24 ± 549.21 ng·mL^{-1} and female 879.58 ± 633.14 ng·mL^{-1} participants (see Figure 19). Nevertheless there was a high degree of inter-individual differences in urine drug concentrations amongst male and female participants (see Figure 20). Whilst mean urine drug concentrations did not breach the WADA urinary threshold for salbutamol, two male and two female subjects recorded urine drug concentrations that exceeded the threshold (1000 ng·mL^{-1}), of which, two cases were above the WADA decision limit set at 1200 ng·mL^{-1}.
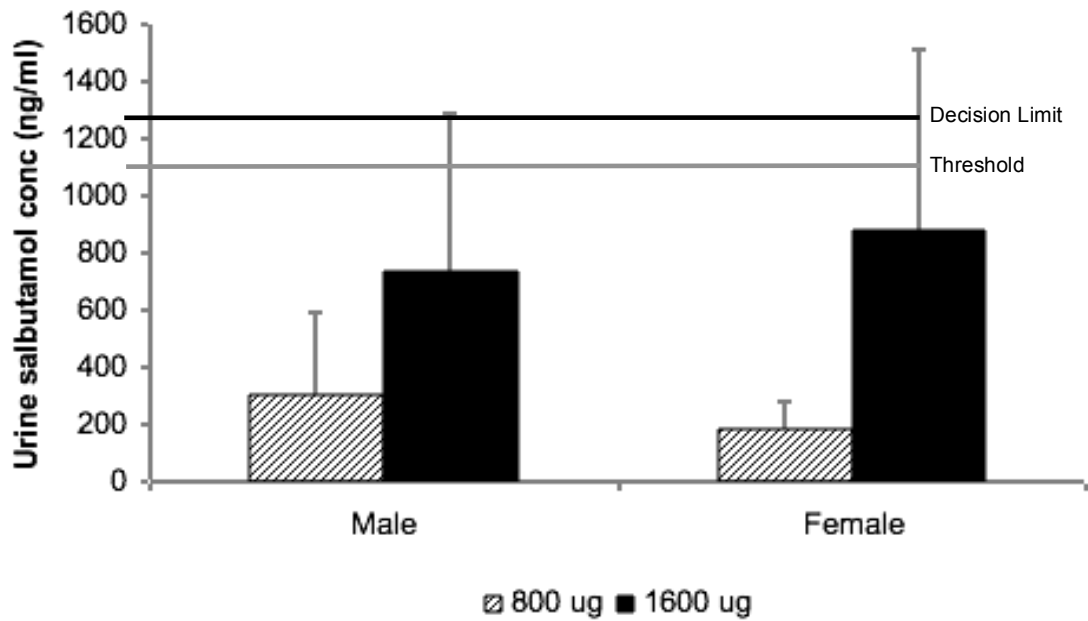
Figure 19. Mean (± SD) urine SAL concentration (ng·mL^{-1}) post football-specific running protocol in temperate (20°C, 40% RH) environment following inhalation of 800 μg or 1600 μg SAL in male and female players.
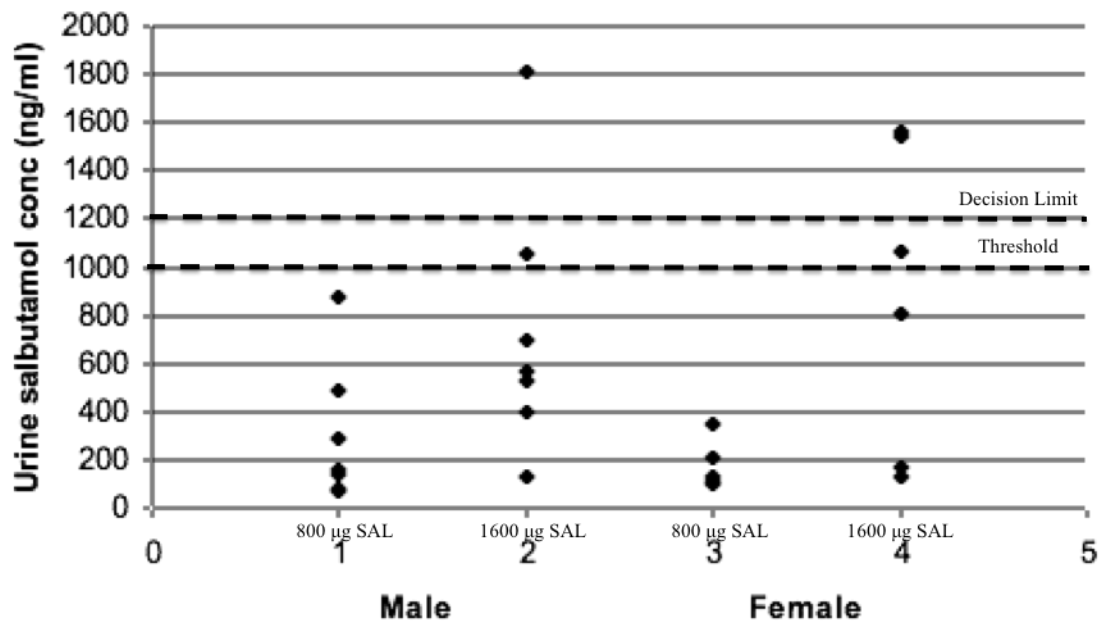
Figure 20. Individual urine SAL concentration (ng·mL^{-1}) post football-specific running protocol in temperate (20°C, 40% RH) environment following inhalation of 800 μg or 1600 SAL in male and female players.

4.4 Discussion

This is one of the first studies to examine the impact of inhaled salbutamol at a dose of 1600 μg versus 800 μg and placebo on time-trial endurance running performance, as well as on simulated association football performance in male and female players. The results from the current study suggest that inhaling up to 1600 μg of salbutamol 15 minutes prior to a 5 km time-trial does not result in any performance improvement or change in physiological function; inhaling up to 1600 μg of salbutamol 15 minutes prior to a football specific treadmill performance test does not result in any sprint performance improvement in either male or female players.

Furthermore, this study is also one of the first studies to examine the salbutamol elimination in urine following inhalation of salbutamol at a doses of 1600 μg and 800 μg and competitive endurance performance in moderate environment (18°C; 40% RH) and hot environment (30°C; 40% RH); the pharmacokinetics of inhaled salbutamol at a dose 1600 μg and 800 μg following a simulated association football performance in male and female players.

The current urinary threshold imposed by WADA is intended to enable differentiation between the use of oral and inhaled salbutamol and also approved therapeutic use and misuse. Oral use is associated with performance enhancement since it typically represents doses in the region of 10 times that of inhaled use. Nevertheless there has been limited research to examine this association. From an endurance exercise perspective only (Collomp *et al*., 2000) has demonstrated enhanced performance whereby short-term oral administration of salbutamol (12 mg/day for 3 weeks) improved time to exhaustion during sub-maximal

cycling exercise. Caruso *et al*. (1995) and Martineau *et al*. (1992) have demonstrated an increase in muscle strength following prolonged oral administration of salbutamol. Indeed protein synthesis and muscle hypertrophy have been shown to be an effect of β_2-agonist use in animal models, particularly long acting β_2-agonists such as Clenbuterol. However, research is clearly required to examine further the claim that oral administration has a positive impact on sports performance.

There exists some ambiguity in terms of the therapeutic use of inhaled salbutamol. Whilst the recommended salbutamol maximal dosing regimen is 100 µg to 400 µg up to four times daily, it is typically prescribed *pro re nata* (PRN; when required) which may add to the confusion. Individuals encouraged administering salbutamol PRN may dose over and above the maximal recommended daily dose of 1600 µg either intentionally or inadvertently, however in both instances individuals intent to dope for performance enhancement purposes may be nil. Such circumstances may lead to the current threshold being unintentionally breached and thus bring about an adverse analytical finding (AAF). Clearly individuals administering inhaled salbutamol up to, and above the 1600 µg dose indicates uncontrolled asthma. Desensitisation or tolerance is experienced by those regularly administering inhaled salbutamol, and it not only increases the risk of unsuccessful treatment in an emergency but also increases the likelihood of further overdosing in an attempt to control EIB.

The current study demonstrates that the possibility of a urinary salbutamol concentration above the current threshold following therapeutic use is possible. However, whilst the threshold as stated by the Prohibited List is 1000 ng·mL^{-1} (WADA, 2013), according to the International Standards for Laboratories Technical Document (WADA, 2010) salbutamol

should only be reported as an AAF when detected at a concentration greater than 1200 ng·mL^{-1}, a level referred to as the 'Decision Limit'. On this basis the urinalysis of the current study would not warrant any sample to be reported as an AAF. This finding was true for a hot (30°C, 40% RH) as well as a temperate environment (18°C, 40% RH). The range of salbutamol concentrations in this study are similar to Sporer *et al.* (2008) who reported urine concentrations of salbutamol up to 800 ng·mL^{-1} 60 minutes post time trial following inhalation of 800 μg salbutamol. Elers *et al.* (2012) reported urine salbutamol concentrations peaked between 0-4 hours. They reported peak salbutamol concentration was 1057 ng·mL^{-1} following 800 μg inhaled salbutamol. Nevertheless, in this study the inter-individual variation was high in both temperate and hot environments and combined with the low subject numbers caution is advised and future studies should aim to examine the impact of high dose salbutamol (1600 μg) administration on urine concentration following endurance performance to establish the likelihood of the Decision Limit being breached. In line with current anti-doping practice the current study did not normalize drug concentrations for urine specific gravity. Elers *et al.* (2012) demonstrated that when urine samples are corrected for specific gravity no urine samples following inhalation of 800 μg salbutamol breached the WADA Prohibited List threshold of 1000 ng·mL^{-1}. Normalising urine samples for specific gravity may be considered by WADA consider in the future.

Previous studies that have focused on inhaled salbutamol have generally focused on non-specific performance trials such as exercise time to exhaustion or physiological markers of performance (Decorte *et al.*, 2008; Sporer *et al.*, 2008). Some previous studies have demonstrated no improvement following acute inhalation of up to 800 μg of salbutamol on running time to exhaustion, VO$_{2max}$, peak power, 20 km cycling time-trial and total work during a 30 s Wingate test (Pluim *et al.*, 2011). A study conducted by Koch *et al.* (2013)

demonstrated no performance enhancement in 10 km cycling time trial performance following 400 μg inhaled salbutamol in athletes with and without a positive EVH challenge. The data in this study complements previous research by focusing on running time-trial performance, football performance in male and female players and adds to the current body of knowledge by reporting on the current upper WADA limit of 1600 μg per day of inhaled salbutamol. Further to our data, a recent study by (Elers *et al.*, 2011) suggested that inhaling an acute dose of up to 400 μg of salbutamol resulted in no improvement in cycling time to exhaustion or oxygen uptake kinetics. Accordingly, from a performance perspective the current WADA upper limit of 1600 μg per day appears appropriate for endurance events given the absence of improvement in performance in non-asthmatic athletes.

The main action of inhaled salbutamol is to act as a bronchodilator to reverse the bronchoconstriction of airway smooth muscle. This results in the asthmatic airway becoming dilated leading to reduced airway resistance, leading to improvements in V_E and exercise performance (Haverkamp *et al*., 2007). It has been suggested that inhaled salbutamol may result in a performance improvement by causing a significant bronchodilation in the airways of non-asthmatics leading to an improved V_E and increased oxygen uptake during exercise. In this study we observed a non-significant 0.1 Litre improvement in FEV_1 10 minutes post-salbutamol inhalation, which did not result in an improvement in V_E during the 5 km time-trial following inhalation of 800 μg or 1600 μg of salbutamol when compared to placebo. Previous studies have demonstrated non-significant improvements in FEV_1 of 0.2 Litre following inhalation of 800 μg, which also did not result in in greater V_E or improved endurance performance (Decorte *et al*., 2008). Therefore there is no evidence available that up to 1600 μg of inhaled salbutamol results in significant bronchodilation, improved V_E or improved endurance performance.

The aim of the present study was to devise a laboratory-based exercise protocol representative of football match-play work rates, to find out whether inhaled salbutamol has any improvement effects on football match performance on both males and females, and if the upper limit dosage according to WADA regulations is reasonable. Few studies have directly assessed oxygen consumption during games, as the collecting procedures interfere with normal play. A more usual approach when determining the aerobic contribution to energy expenditure during match-play is to monitor heart rate and subsequently estimate energy expenditure from the individual hear rate-VO_2 relationship. Mean values of 70-75% VO_{2max} have been attributed to football using such procedures (Reilly, 1990).

It is well documented that inhaled salbutamol, despite the dosage or the medication involved, do not have any performance enhancing effects in healthy subjects (Carlesen *et al*., 1997; Fleck *et al*., 1993; Freeman *et al*., 1989; Heir *et al*., 1995; Larsson *et al*., 1997; McKenie *et al*., 1983; Meeuwisse *et al*., 1992; Morton *et al*., 1996; Sandsund *et al*., 1998; Sporer *et al*., 2008; Stewart *et al*., 2002). Thus, only two studies have demonstrated a statistically significant effect of a single inhaled therapeutic dose of salbutamol (Bedi *et al*., 1988; Signorile *et al*., 1992). By our knowledge, this is one of the first studies to report the acute effects on performance in male non-athletes without asthma after a single, inhalant administered therapeutic dose of salbutamol. Both Collomp *et al*. (2005) and Van Baak *et al*. (2000) have previously shown an improvement both in time to exhaustion and (supra)maximal workload in healthy male athletes after orally administered salbutamol, but the subjects were not highly endurance trained.

One potential mechanism for salbutamol to have ergogenic properties may be related to its ability to act as a potent bronchodilator. Even in non-asthmatic individual, salbutamol has the ability to increase airway caliber, resulting in a measurable increase in airway function (Carlsen et al., 1997; Goubault et al., 2001; Heir % Stemshaug, 1995; Meeuwisse et al., 1992; van Baak, et al., 2004). Theoretically, this may lead to enhanced alveolar ventilation and/or a reduced work of breathing, thereby increasing available oxygen for working muscles. However, previous reports have shown that during physical activity, salbutamol does not have an accumulative effect on the normal bronchodilatory response to exercise (Carlsen et al., 1997; Goubault et al., 2001; Heir % Stemshaug, 1995; Meeuwisse et al., 1992; van Baak et al., 2004), nor does it reduce respiratory resistance (Pichon et al., 2005). Theoretically, it is unlikely that the bronchodilatory action of salbutamol and other β_2-agonists has an ergogenic effect in healthy non-athletes, because bronchoconstriction is not a performance-limiting factor. What should be remembered, though, is that several studies have demonstrated that elite endurance athletes have a huge demand to the pulmonary diffusing capacity, as the pulmoneus transit time is reduced to 1/3 second or less (Dempsey & Wagner, 1999; Harms et al., 2000; Miyachi & Tabata, 1992). Additionally, through up-regulation of the sodium-up-regulation of the sodium-driven clearance of alveolar fluid, and consequently a reduced risk of pulmonary oedema, the use of β_2-agonists may result in an improvement in the oxygen transfer from the alveoli to the arterial blood in elite endurance athletes with extreme demands to the oxygen transport system.

Our current findings, the applicability of football-specific test results to sport performance enhancement is questionable. The validity of a test to be representative of performance is an important factor when evaluating the ergogenic effects of a treatment (Hopkins, 1999). There

were no significant changes in terms of VO_{2max} under the three experimental conditions in male players; however, it does show a significant VO_{2max} difference during stage 1 between SAL1600 and PLA. The result indicates that inhaled salbutamol at 1600 μg dosage may improve vital capacity, thus, the amount of inhaled salbutamol may enhance athletes' oxygen uptake during football match at the very beginning, with an immediate short-period effect that may improve player's performance, when the match starts during the low intensity exercise stage. Further more, when focusing on heart rate, we observed significantly greater result in 1600 μg salbutamol than 800 μg and placebo during stage 1 to 5, and 7; V_E with 1600 μg salbutamol intake is also significantly greater than Placebo in all stages; 1600 μg inhaled salbutamol has much greater effect on blood lactate values compare to Placebo in stage 1, 4 and 5; the results have indicated that 1600 μg inhaled salbutamol can possibly improve non-asthmatic male football players' endurance performance during the match, which is differ from the previous studies, therefore, further investigate with bigger amount of male participants would be needed to prove the reliability of this result.

In female players' experimental group, this study did not find any statistically significant changes in VO_2, VCO_2, HR, RPE and blood lactate values between treatments. However, during stage 1, V_E with both 1600 μg and 800 μg inhaled salbutamol have significant difference compare to Placebo; and in stage 5, 6, and 7, 1600 μg inhaled salbutamol has significant difference compare to Placebo. These results indicate that for female, no matter how much salbutamol they took, it may always has positive effect on player's vital capacity, therefore improvement on performance endurance is possible at the beginning of the match when the exercise is in low intensity; deeper insight, 1600 μg inhaled salbutamol could improve the female players' performance towards to a later stage and last for a while till the

end of the test. Therefore, the present study cannot support the suggestion of no effect on simulated association football (soccer) performance for both male and female players.

One of the proposed ergogenic mechanisms for inhaled salbutamol is a significant bronchodilation in non-asthmatic athletes resulting in an improved V_E during exercise and increase in oxygen uptake. In the present study an improvement in FEV_1 10 minutes post-salbutamol inhalation was observed in male players (0.1 Litre) but no improvement in female players. The improvement in FEV_1 in male players of 0.1 Litre following inhalation of SAL800 and SAL1600 was significantly ($p=0.03$) greater than PLA. Regardless of FEV_1 improvements following salbutamol inhalation we observed increases in V_E for a given work load during the stages of the football specific treadmill run test in both male and female players. Previous studies have demonstrated non-significant improvements in FEV_1 of 0.2 Litre following inhalation of 800 μg, which did not result in greater V_E or improved endurance performance (Decorte *et al.*, 2008). The low participant number could explain our observed significant increase in V_E as one participant may significantly impact on the group mean. It remains clear that the changes did not impact on player sprint performance however; the differential FEV_1 response between genders is unclear and requires further investigation.

Previous research has attempted to examine the excretion of salbutamol administered by different routes including oral and inhalation (Pichon *et al.*, 2006; Elers *et al.*, 2012) and across a range of acute doses from 200 μg and 800 μg (Sporer *et al.*, 2008) and repeated doses over several hours (Elers *et al.*, 2011). The current research is the first to examine the urinary excretion of an acute, high dose in comparison with an intermediate dose amongst male and female association football (soccer) players. Whilst the findings did not suggest any

significant differences according to sex there was a large variation between individuals at the various doses. This large inter-subject variation confirmed the likelihood that individuals inhaling acute, high doses of salbutamol may exceed the current WADA urinary threshold of 1000 ng·mL^{-1}. Indeed the current research also shows that following such a dosing regimen may also result in urine salbutamol levels in excess of 1200 ng·mL^{-1}. This concentration is significant since it represents a level referred to by WADA as the 'Decision Limit' above which a sample would be classified as an adverse analytical finding (WADA, 2010) and warrant further investigation.

The maximal recommended dosing regimen for salbutamol clearly acts as an upper limit for those requiring immediate relief of asthma symptoms. Individuals who regularly use high doses of salbutamol or exceed these recommendations clearly demonstrate poor management of their condition. There lies the problem from an anti-doping regulatory perspective when the evidence to support any ergogenic properties is not present and the likelihood of poor control of asthma and EIB are high. From a sports medicine perspective improved care may constitute better diagnosis, management and education amongst athletes.

4.5 Conclusion

Study 1

This study demonstrated no improvement in 5 km running time-trial performance following the inhalation of up to 1600 μg of salbutamol in non-asthmatic athletes in moderate or hot environments. This would suggest that the current WADA guidelines, which allows athletes to inhale up to 1600 μg is sufficient to avoid pharmaceutical induced performance

enhancement. However, such high doses not only suggest poor management of asthma but also mean that an athlete may be at risk of contravening the current urinary threshold.

Applicability for doping control and advantages over existing methods:

- There is no improvement in performance following the inhalation of up to 1600 μg of salbutamol in non-asthmatic athletes in temperate or hot environments.

    o The current WADA guidelines, which allows athletes to inhale up to 1600 μg is sufficient to avoid pharmaceutical induced performance enhancement.

- Inhalation of 1600 μg may result in a urine concentration above the current WADA limit and decision limit.

    o Further, larger studies are required to corroborate these findings.

Study 2

There is an improvement in performance following the inhalation of up to 1600 μg of salbutamol in non-asthmatic female association football (soccer) players. Furthermore, inhalation of 1600 μg may result in a urine concentration above the current WADA upper limit and decision limit leading to a positive test finding. Data from this study will assist WADA in the implementation of regulations on the use of inhaled short acting β_2-agonist and assist in the resolution of contested doping violations.

Applicability for doping control and advantages over existing methods.

- There is a possible improvement in performance following the inhalation of up to 1600 μg of salbutamol in non-asthmatic male and female association football (soccer) players.

    o The current WADA guidelines, which allows athletes to inhale up to 1600 μg whether is sufficient to avoid pharmaceutical induced performance enhancement is still questionable according to the result from this study.

- Inhalation of 1600 μg may result in a urine concentration above the current WADA limit and decision limit.

    o Further, larger studies are required to corroborate these findings.

4.6 Limitations

One of the limitations to these two studies is the variability in actual dose inhaled. Whilst the use of a chamber aimed to reduce this limitation it remains possible that some participants with low urine concentration inhaled lower doses of salbutamol. In addition future work should investigate whether there is a relationship between body weight and the urinary concentration of salbutamol. A lighter athlete may be at a greater risk of breaching the threshold when administering high doses compared to a heavier athlete. Such findings would have implications to the care athletes receive in the future.

Because of the time, location, region and funding issues, not many participants' data were valid for these studies and the total number of the samples was very limited. An increase in the number of participants may provide more robust findings. Further study is required in a

larger number of participants including a range of gender and ethnicity to ensure an avoidance of Type II error. A power calculation would suggest that to detect a change in 1% in sprint performance a sample size of 48 participants would be required. Study 1 only focused on the male participants. Since male and female may respond differently to the higher doses of salbutamol inhalation, future studies should examine on female athletes. However, certain improvement has been made to study 2, by introducing female participants in the football trial, even though the results hasn't shown any significant difference, yet the range of people have been enlarged. The same situation is related to the issue of ethnicity for both studies, which may also have an impact on the findings. Furthermore, the participants were not elite endurance athletes. Elite athletes could not be included in this study as they would have been at risk of a doping violation. Therefore we cannot claim our results directly represent the effects of inhaled salbutamol in elite endurance athletes.

CHAPTER V

STUDY 3

THE IMPACT OF ETHNICITY, GENDER

AND DEHYDRATION STATUS ON THE

PHARMACOKINETICS OF SHORT ACTING

B$_2$-AGONISTS

5.1 Introduction

Inhaled short acting β₂-agonists are commonly used in the treatment of asthma, exercise induced bronchoconstriction (EIB) and airway hyperresponsiveness (AHR). The principle role of inhaled short acting β₂-agonists is to act as a bronchodilator to reverse the bronchoconstriction of airway smooth muscle. This results in the asthmatic airway becoming dilated leading to reduced airway resistance and improvements in minute ventilation (V_E). It has been suggested that inhaled salbutamol may result in a performance improvement by causing a significant bronchodilation in the airways of non-asthmatic individuals leading to an improved V_E, increased oxygen uptake and exercise performance (Haverkamp *et al*. 2007), however, there is limited evidence to suggest that inhaled doses of β₂-agonist (200 to 800 µg) have a significant ergogenic effect. The small numbers of studies that do exist have focussed on endurance performance and have reported no performance effect of up to 800 µg of inhaled short acting β₂-agonist (Pluim *et al*., 2011).

Whilst there appears to be no ergogenic effect from inhaled salbutamol at low doses (200 to 800 µg), the World Anti-Doping Agency (WADA) recommends a daily upper limit is 1600 µg (~16 inhalations of a standard salbutamol inhaler; WADA, 2012). A small number of studies have reported an absence of an ergogenic effect of high dose inhaled short acting β₂-agonist (up to 4000 µg; Elers *et al*., 2012) however; concern has been raised that inhalation of 1600 µg in a single episode may result in urine concentrations close to the WADA upper limit of 1000 ng·mL⁻¹ and, therefore, result in an adverse analytical finding (AAF).

A small number of recent studies have examined the impact of inhaling the WADA daily upper limit on urine concentrations. These studies have focussed on multiple dosing regimens such as 4 x 400 µg of salbutamol as opposed to a single high dose (Elers *et al.*, 2011). Whilst athletes are usually prescribed 200-400 µg of inhaled salbutamol they are often instructed to use their inhaler *pro re nata* (*i.e.* on an as needed basis) that could be interpreted as a clearance to inhale unlimited amounts of salbutamol to combat respiratory symptoms. Individuals encouraged to administer salbutamol *pro re nata* may dose over and above the maximal recommended daily dose of 1600 µg either intentionally or inadvertently, however in both instances an individual intent to dope for performance enhancement purposes may be nil. Such circumstances may lead to the current threshold being unintentionally breached and thus bring about an AAF.

A number of cases have been reported in the literature where athletes have tested positive following inhalation of high doses of short acting β_2-agonists. Recently, a Rugby League player escaped a doping violation after he inhaled in excess of 1600 µg over the course of a match and then tested positive in the post-match anti-doping test. The player's defense was based on the prescription on an 'as needed basis' with no guidance on an upper limit to its use. Accordingly, in practice 16 inhalations in a short period of time prior to competition may occur in poorly controlled, less informed, as well as potentially unscrupulous athletes and yet still working within the recommended limit stated on the WADA 2012 Prohibited List. Because of the potential ergogenic effects of β_2-agonists, particularly with oral doses, unauthorized use of salbutamol is closely monitored through doping control. Even for athletes possessing a TUE, a urine concentration of nonsulphated salbutamol (cSAL) greater than 1000 ng·mL^{-1} is considered an adverse analytical finding, which can result in suspension. This cutoff point has been questioned of late, with recent reports of positive test

results using inhaled therapeutic doses, all with urine concentrations well over 1000 ng·mL^{-1} after exercise (McKenzie, 2004; Schweizer *et al*., 2004). Although the majority of urine samples reported in the literature rarely exceed 500 ng·mL^{-1} (Pichon *et al*., 2006; Ventura *et al*., 2000), it has been suggested that with individual variations in dose, changes in hydration status after competition, and the ability to absorb, metabolize, and excrete salbutamol, the possibility for elevated concentrations exists (McKenzie, 2004). The percentage of salbutamol exist in urine is due to the absorption ability of the subjects, therefore in this study, the participants were not restricted from intake any fluid before the test and the first sample of urine, but they are not allowed to drink any more fluid during the test until the last sample has been collected. In addition to the inhalation of high, single bolus doses of short acting β$_2$-agonists the impact of dehydration through sweating associated with high intensity exercise in hot environments may have a profound effect on urine concentration however, limited data exists to support this hypotheis.

WADA impose global regulations and as yet little is known of the impact of ethnicity on the pharmacokinetics of inhaled short acting β$_2$-agonist and their subsequent appearance in the urine. To date, no studies have examined the impact of dehydration and ethnicity on the pharmacokinetics of short acting β$_2$-agonists. Accordingly, the global purpose of this study was to contribute to the understanding of the impact of dehydration and ethnicity following the inhalation of short acting β$_2$-agonists at doses up to and including the maximal dose (1600 µg) as stipulated on the 2012 WADA Prohibited List.

5.2 Methods

Prior to the commencing the study ethical approval was obtained from Liverpool John Moores University Local Ethics Committee (ethics no: 09E18GW). Eighteen male and 14 female athletes (9 Caucasian males, 9 Caucasian Females, 2 Afro-Caribbean males, 2 Afro-Caribbean females, 6 Asian males and 4 Asian females) were recruited for the study. All participants were free from asthma, EIB and AHR confirmed by no previous history of disease and presenting with a negative Eucapnic Voluntary Hyperpnoea (EVH) challenge (Anderson *et al.*, 2001). Participants were allowed to drink water *ad libetum* prior and post to testing, but not during the test. The presence of salbutamol in urine is associated with the absorption rate of the individual subjects, therefore in this study, the participants were not restricted from consuming fluid before the test and providing the first sample of urine, but they are not allowed to drink any more fluid during the test until the last sample had been collected. Body weight is measured after their intake of water, but prior to the start of the trials. All participants were free from chest infection for at least 4 weeks prior to assessment; they were not taking any medication and there were no other health or medical contradictions to them taking part in the study as confirm by information provided on a physical activity readiness questionnaire. All participants were physically active (30 minutes of organised exercise at least 3 times per week).

Naked body mass (BM) was measured at baseline following an initial urine sample and inhalation of low (800 μg) or high (1600 μg) dose salbutamol. Body mass was then measured after every 20 minutes of self-selected exercise in a hot, controlled environment (35°C, 40% relative humidity) until the target weight loss (2% or 5%) was achieved. Pre- and post-

dehydration haematocrit and haemoglobin were measured for the derivation of blood volume. Participants were required to complete 4 trials as follows:

- 2% reduction in body mass (BM), low dose (800 μg) inhaled short acting β₂-agonist (2% BM 800 SAL)

- 2% reduction in BM, high dose (1600 μg) inhaled short acting β₂-agonist (2% BM 1600 SAL)

- 5% reduction in BM, low dose (800 μg) inhaled short acting β₂-agonist (5% BM 800 SAL)

- 5% reduction in BM, high dose (1600 μg) inhaled short acting β₂-agonist (5% BM 1600 SAL)
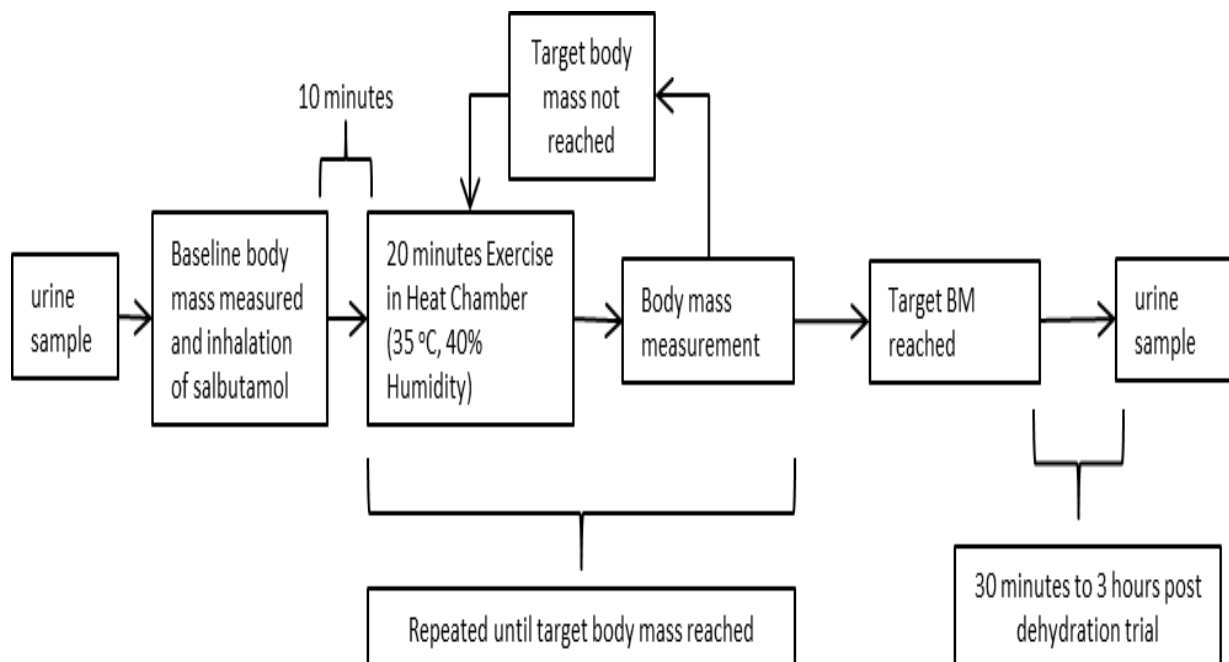
Figure 21. Time-line schematic of dehydration trial.

Exercise in the Heat Chamber

The heat chamber (T.I.SS Environmental Extremes; Peak Performance Chamber; Hampshire, UK) was set at a constant 35°C and 40% relative humidity. Participants were asked to exercise in heat chamber at a self-selected pace for 20 minutes using a motorised treadmill (Woodway Ergo ELG 55 treadmill; Woodway GmbH, Weil am Rhein, Germany), cycling ergometer (Corival; Lode Holding Company B.V. Medical Technology, Groningen, the Netherlands) or rowing ergometer (Concept 2 Rowing; Model D, USA). Participants could change mode of exercise during this 20 minutes if they desired. Following 20 minutes athletes exited the environmental chamber to measure BM. If BM had not reached the target (2% or 5%) participants re-entered the heat chamber and completed another 20 minutes bout of self-paced exercise. Participants repeated this process until they reached their target BM. Participants were instructed to stop 2% BM trial after 2 hours of exercise if they had not achieved target BM. Participants were instructed to stop 5% BM trial after 4 hours of exercise if they had not achieved target BM. Water consumption during the trials was kept to an absolute minimum. Once participants had reached the target weight they were able to drink fluid *ad libitum*.

Urine collection

Prior to the commencement of each trial subjects were asked to provide a urine sample in order to void themselves of urine, after which salbutamol was administered as described above. Subjects were provided with water after the test, in order to be ensure urination within three hours after dehydrated, and they were instructed to collect the first sample of urine passed following completion of the laboratory-based tests in line with in-competition anti-doping procedures as outlined in the World Anti-Doping Code International Standard for

Testing 2012 (the sample could be collected at home and handed in the next day as long as it was within 3 hours post-test, the subjects did not need to stay in the laboratory until they had urinated). Consumption of water during this period was encouraged *ad libitum* to ensure diuresis. From the sample provided by each subject a 20 mL aliquot of urine was stored at -80°C until analysis.

Urinalysis

All urinalysis was performed at HFL Sport Science (Fordham, UK) an independent drug surveillance laboratory and former WADA-accredited laboratory.

Sample preparation involved the addition of 200 ng of salbutamol-D$_3$ (NMI) as an internal standard to 1 mL of urine. Following the addition of 2 mL of 0.1 M phosphate buffer pH 6.8 and 100 μL of E. Coli enzyme (β-glucuronidase) solution the mixture was incubated overnight at 37°C. Strata XC 60 mg solid phase extraction cartridges (Phenomenex, Macclesfield, UK) were conditioned with 3 mL of methanol followed by 3 mL of reagent grade water. Following centrifugation at 3500 rpm for 5 minutes the samples were applied to the cartridges. The cartridges were then washed with 3 mL of 0.1 M acetate buffer pH 9.0 followed by 3 mL of reagent grade water, 3 mL of 0.1 M HCl, 3 mL of methanol and 3 mL of diethyl ether. The cartridges were then dried for 5 minutes under vacuum and samples were eluted into glass vials with two, 1 mL of basic drug elution solvent (160 mL ethyl acetate, 34 mL propan-2-ol and 6 mL 34% ammonia solution). Samples were then evaporated to dryness at ambient temperature using a centrifugal vacuum concentrator (Genevac Ltd, Ipswich, UK) and reconstituted in 10 μL of isopropanol followed by 200 μL of basic reconstitution solution

(495 mL of 0.1 acetic acid mixed with 5 mL Benzyldimethylphenyl Ammonium). Samples were centrifuged at 3000 rpm for 10 minutes prior to LCMS submission. Samples were injected onto a Thermo Scientific Accela HPLC system coupled to a Thermo Scientific LTQ Orbitrap Discovery Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Chromatographic separation was performed on a Waters Atlantis T3 column (2.1 x 100 mm, particle size 3 um; Waters Ltd, Elstree, UK) at 35°C. The mobile phase was a gradient system of 0.1% acetic acid aqueous solution containing uracil (300 ng·mL^{-1}) and 0.1% acetic acid in acetonitrile containing uracil (300 ng·mL^{-1}) set at a flow rate of 0.4 mL·min^{-1}.

The urine salbutamol concentrations reported correspond to the sum of the free and glucuronide conjugates. The samples were analysed over the calibration range of 10 to 2000 ng·mL^{-1}. Samples with salbutamol concentrations greater than the upper limit of quantification were diluted with blank human urine prior to analysis. The lower limit of quantification was accepted as the lowest standard on the calibration curve (10 ng·mL^{-1}).

Statistical Analysis

That data was considered as a case series. A case series is a type of research study that tracks subjects with a known exposure, such as patients who have received a similar examination. Therefore, the results are compared in between different cases but according to the same individual. Accordingly, data are presented as individual raw data. Sample size was determined for the primary response variable (urine concentrations of salbutamol) in a repeated measures analysis of variance (ANOVA) design. The effect size and standard deviation were chosen based on previous literature investigating urine concentrations of

inhaled salbutamol at rest and after exercise (Elers *et al*., 2012; Sporer *et al*., 2008). To detect differences in the urine concentrations of salbutamol between the two conditions across three different ethic groups, at least 13 subjects should complete the study. With the risk of having drop-outs, there were 18 male and 14 female subjects in this study.

Statistical analyses were done using the statistical software program SPSS (SPSS, Inc, Chicago, Illinois). Normally distributed continuous variables were expressed as mean (SD). Skewed data were presented as median (range). Paired data were compared by Wilcoxon signed rank test. Comparisons of 3 independent groups were done using the Mann-Whitney test. Urinary concentrations were corrected for urine specific gravity before analyses. Where appropriate descriptive statistics (Mean ± SD) for urine salbutamol concentrations were presented and differences according to sex and ethnicity were assessed using independent t-tests and hydration status assessed using the paired t-test. A p-value of <0.05 was accepted as significant.

5.3 Results

All participants were able to achieve a 2% BM loss, however, a number of participants had difficulty in attaining a 5% BM loss during the trials. The range in exercise time to achieve a 2% BM loss was 76.0 ± 28.8 minutes (range 50 – 80 minutes) in Caucasian Males to 102.5 ± 15.0 minutes (80 - 110 minutes) in Afro-Caribbean Males (Table 12). The range in exercise time to achieve a 5% BM loss was 213.8 ± 39.6 minutes (range 140 - 240 minutes) in Caucasian Males to 240.0 ± 0.0 minutes in all other ethnic and gender categories (See Table 12).

	Caucasian Female		Caucasian Male		Asian Female		Asian Male		Afro-Caribbean Female		Afro-Caribbean Male	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
2% BM 800 SAL	105.7 ± 32.07	50 – 140	92.0 ± 37.9	50 – 170	83.8 ± 10.6	50 – 110	80.0 ± 22.7	50 – 110	85.0 ± 7.1	80 – 90	102.5 ± 15.0	80-110
2% BM SAL	96.7 ± 20.7	80 – 130	76.0 ± 28.8	50 – 110	84.3 ± 20.7	50 – 110	76.25 ± 19.2	50 – 110	99.0 ± 26.9	80 – 120	87.5 ± 28.7	50 – 110
5% BM 800 SAL	240.0 ± 0.0	240 – 240	213.75 ± 39.6	140 – 240	240.0 ± 0.0	210 – 240	235.7 ± 11.3	210 – 240	240.0 ± 0.0	240 – 240	230 ± 20.0	200 – 240
5% BM 1600 SAL	233.3 ± 16.3	240 - 240	226.3 ± 26.7	170 - 240	240.0 ± 0.0	240 - 240	240.0 ± 0.0	240 - 240	240.0 ± 0.0	240 - 240	240 ± 0.0	240 - 240

Table 12. Mean (± SD) and range of exercise time (minutes) to achieve target body mass (BM) percentage loss in each trial.

a)

b)

c)

d)

e)

f)
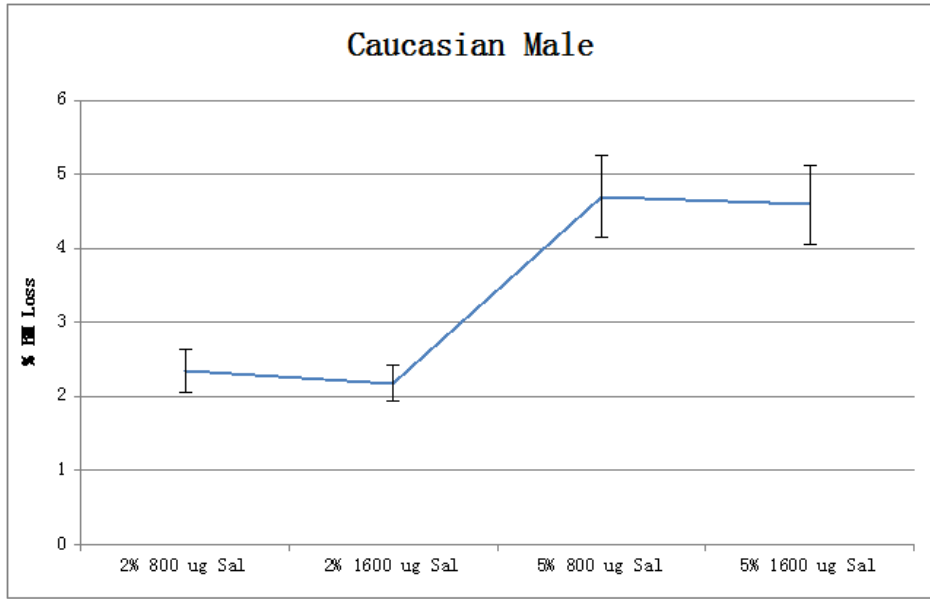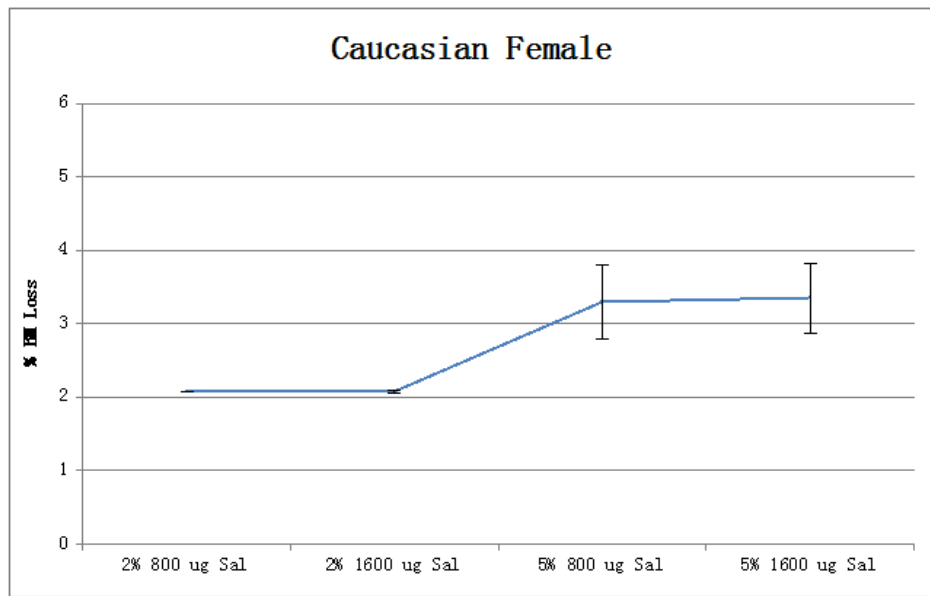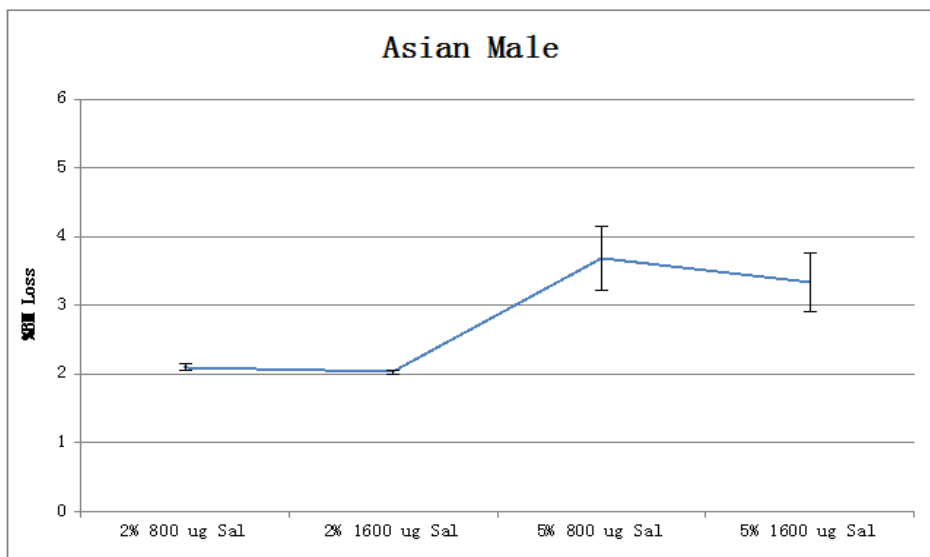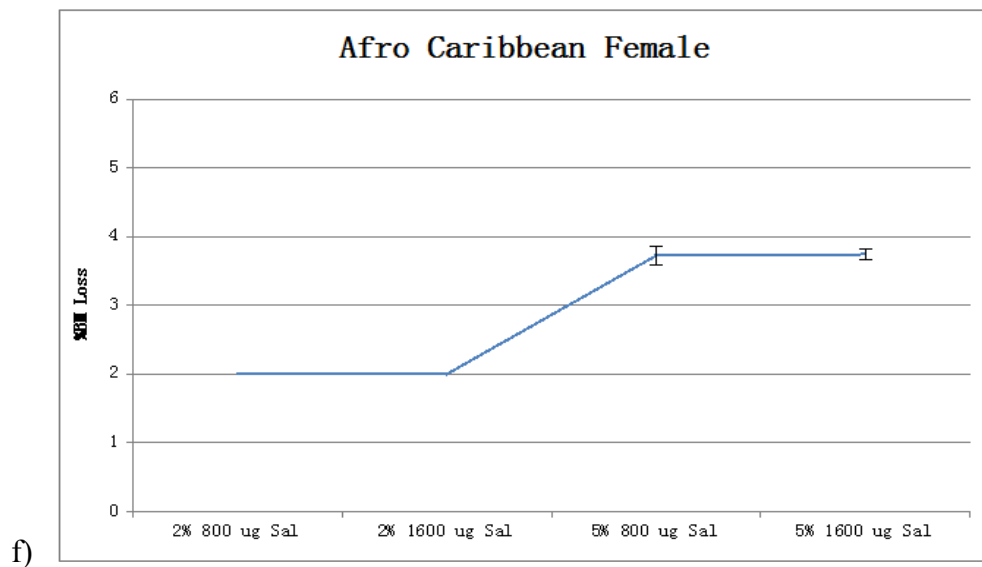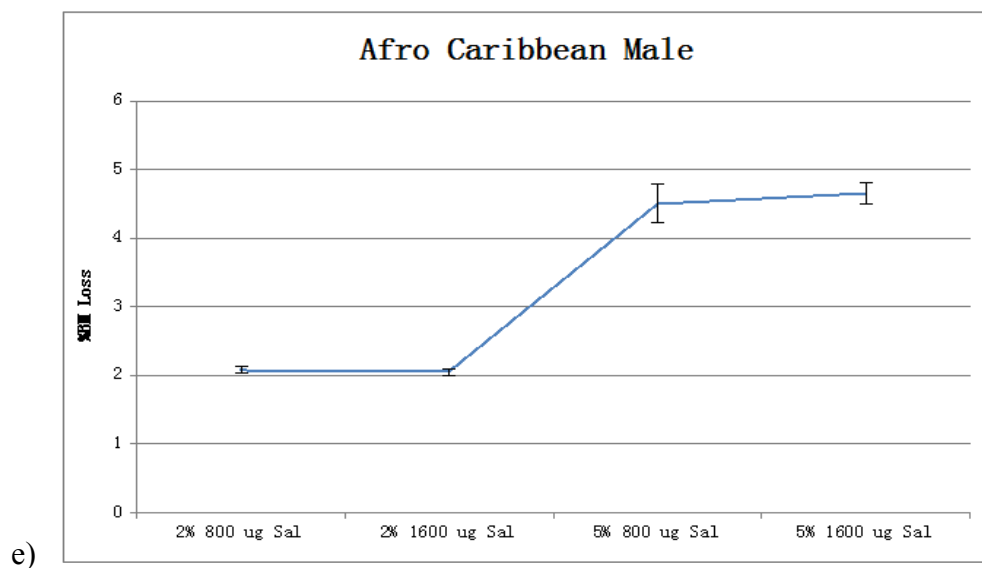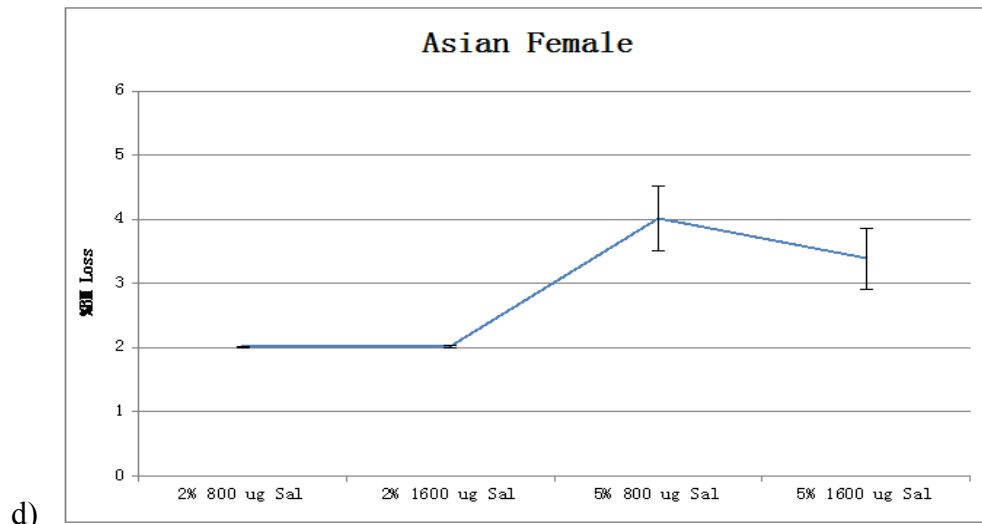
Figure 22. Mean (± SD) %BM loss during each trial for male and female Caucasian, Asian and Afro-Caribbean participants.

Following inhalation of 800 μg of salbutamol and exercise in a heat chamber (35°C and 40% relative humidity) the total cohort reported mean (± SD) urine drug concentrations of 188.35 ± 146.62 ng·mL^{-1} and 363.92 ± 284.84 ng·mL^{-1} under conditions of two and five percent dehydration, respectively. Following inhalation of 1600 μg of salbutamol and exercise under similar conditions the cohort reported mean (± SD) urine drug concentrations of 1055.37 ± 981.03 ng·mL^{-1} and 1059.81 ± 709.33 ng·mL^{-1} under conditions of two and five percent dehydration, respectively. Relative dehydration was shown to have significant difference on the urine drug concentration following inhalation of 800 μg of salbutamol (t_{20} = -2.897, P = 0.009) whereas no significant difference was found according to hydration status following inhalation of 1600 μg of salbutamol. Nevertheless, there was a high degree of inter-individual variation throughout the cohort following administration of 800 μg and 1600 μg salbutamol under both conditions (Figure 23).

Figure 23. Urine drug concentration in all participants following inhalation of 800 μg or 1600 μg salbutamol under dehydration of 2% and 5% of body mass.

This inter-individual variation was explored further in terms of ethnicity (see Figure 24). Following inhalation of 800 μg of salbutamol and exercise in a heat chamber (35°C and 40% relative humidity) the mean (± SD) urine drug concentrations of 160.24 ± 99.78 ng·mL^{-1}, 263.38 ± 255.54 ng·mL^{-1} and 260.35 ± 185.13 ng·mL^{-1} under conditions of two percent dehydration were reported for Caucasian, Asian and Afro-Caribbean groups, respectively. Following inhalation of 800 μg of salbutamol and under conditions of five percent dehydration mean (± SD) urine drug concentrations of 323.36 ± 234.87 ng·mL^{-1}, 209.05 ± 108.41 ng·mL^{-1} and 612.25 ± 388.70 ng·mL^{-1} were reported for Caucasian, Asian and Afro-Caribbean groups, respectively. Following inhalation of 1600 μg of salbutamol and under conditions of two percent dehydration mean (± SD) urine drug concentrations of 841.86 ± 960.92 ng·mL^{-1}, 937.46 ± 494.67 ng·mL^{-1} and 2757 ± 1068.29 ng·mL^{-1} were reported for Caucasian, Asian and Afro-Caribbean groups, respectively. Following inhalation of 1600 μg of salbutamol and under conditions of five percent dehydration mean (± SD) urine drug concentrations of 930.29 ± 532.55 ng·mL^{-1} and 1149.20 ± 884.50 ng·mL^{-1} were reported for Caucasian and Asian groups, respectively. Whilst subject number was particularly low in the Afro-Caribbean cohort, differences in urine drug concentrations were assessed following administration of either 800 or 1600 μg of salbutamol between Caucasian and Asian subjects under conditions of two and five percent dehydration. With 95% confidence, no significant statistical differences were found between ethnic groups (Caucasian and Asian subjects; p value under each condition is between 0.147 and 0.910).

A

B

Figure 24. Urine drug concentration following inhalation of 800 μg or 1600 μg salbutamol under dehydration of 2% (A) and 5% (B) body mass in different ethnic groups (Asian; Caucasian; Afro-Caribbean)

The elimination of varying doses of inhaled salbutamol under conditions of both two and five percent dehydration was also examined according to sex (see Figure 25). Whilst mean (± SD) urine drug concentrations following inhalation of 800 μg of salbutamol under conditions of two percent dehydration was 196.84 ± 162.93 ng·mL^{-1} in males compared to 177.03 ± 127.75 ng·mL^{-1} in females there was no significant difference according to sex. Likewise there were no significant sex differences in urine drug concentrations following inhalation of 800 μg of salbutamol under conditions of five percent dehydration in male (Mean = 473.87 ± 340.85 ng·mL^{-1}) and female (Mean = 242.97 ± 142.04 ng·mL^{-1}) participants. Mean (± SD) urine drug concentrations under conditions of two percent dehydration following inhalation of 1600 μg of salbutamol were 1197.58 ± 1156.35 ng·mL^{-1} and 872.52 ± 694.57 ng·mL^{-1} for male and female participants respectively. Under conditions of five percent dehydration urine salbutamol levels following a dose of 1600 were 1027.81 ± 684.98 ng·mL^{-1} and 1088.24 ± 770.60 ng·mL^{-1} for male and females respectively. Urine drug concentrations under both conditions of hydration status (2% and 5%) were not statistically significant according to sex.
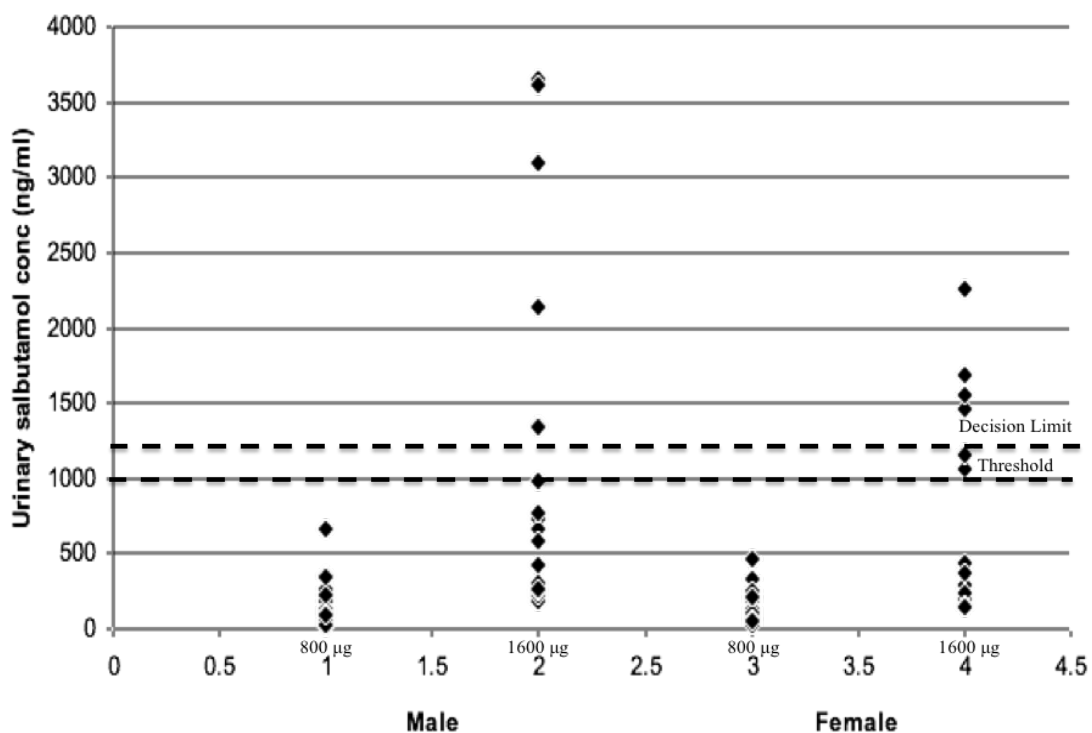
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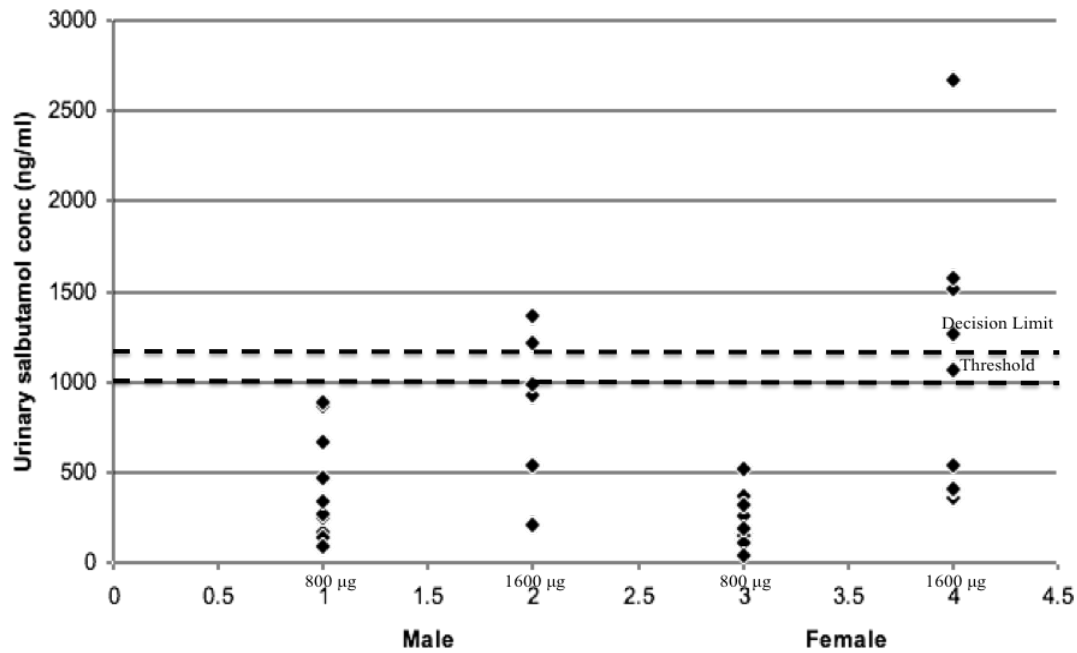
B

Figure 25. Urine drug concentration following inhalation of 800 μg or 1600 μg salbutamol under dehydration of 2% (A) and 5% (B) body mass amongst male and female subjects

Further differentiation of the data set is illustrated in Figure 26 whereby male and female urine drug concentrations are categorised according to ethnic group. Whilst the data set attributed to Afro-Caribbean is particularly small, where possible descriptive statistics for Caucasian and Asian subjects are presented in Table 13. Therefore, the only significant difference found in these data was the urine concentration under each condition following inhalation of 800 μg of salbutamol, despite gender or ethnicity; whereas no significant difference when observed following inhalation of 1600 μg of salbutamol. As for inter-individual, no significant differences were found between genders or ethnicities.
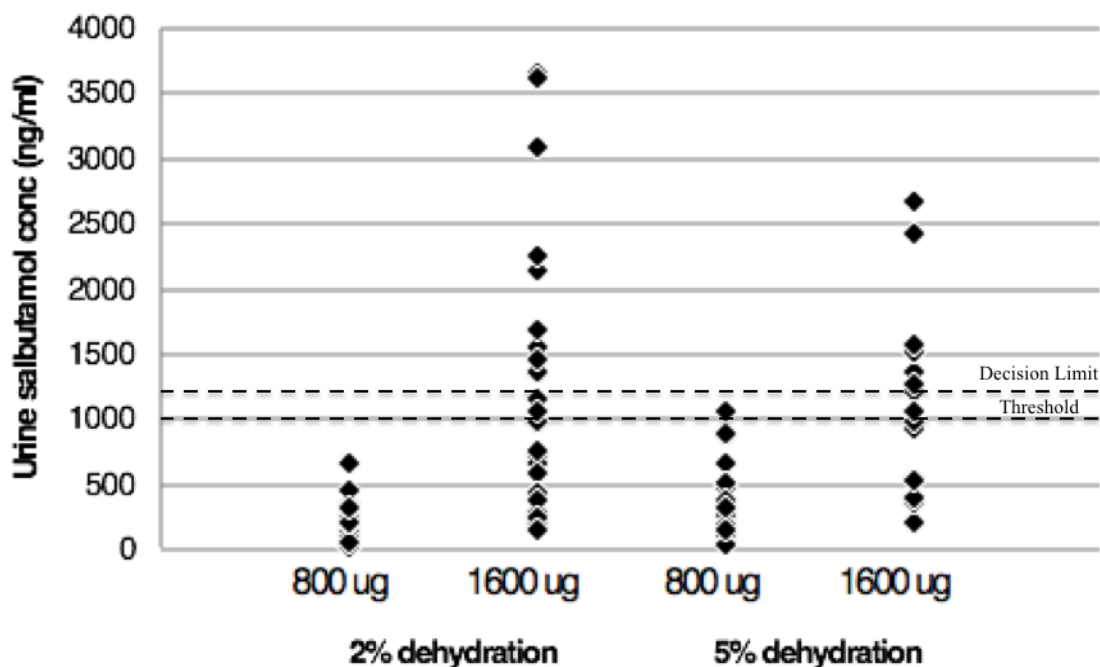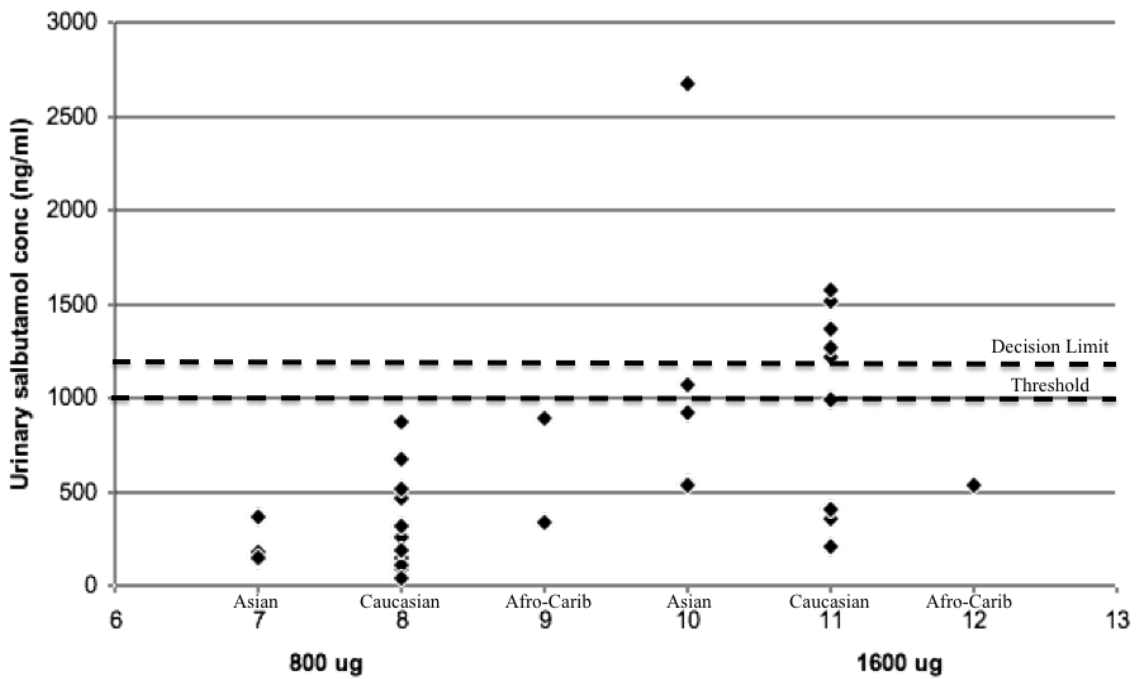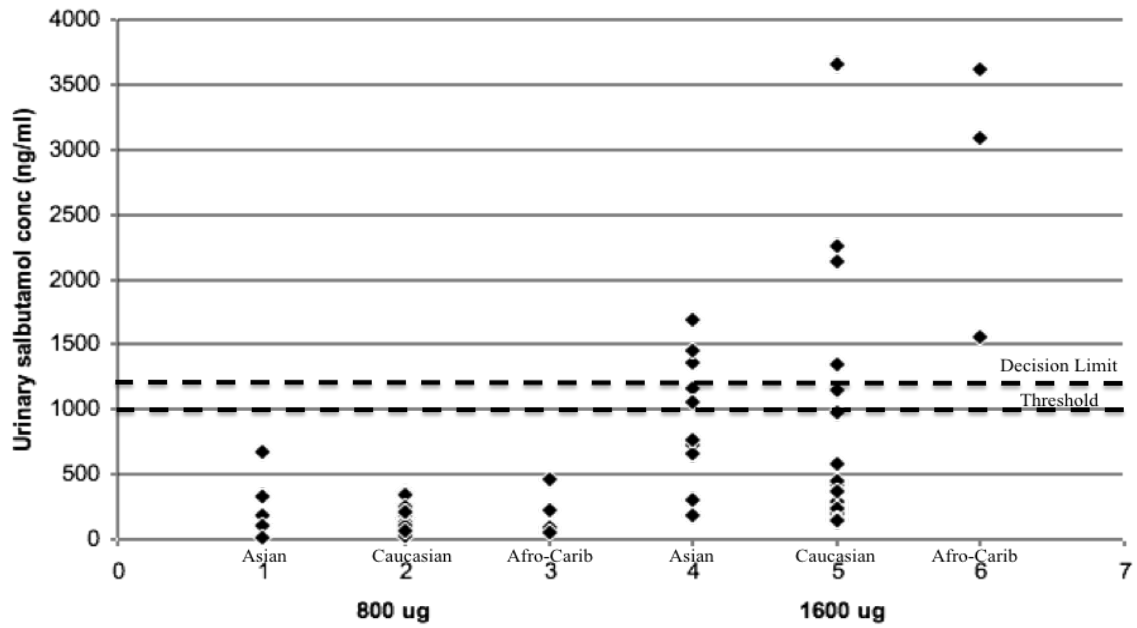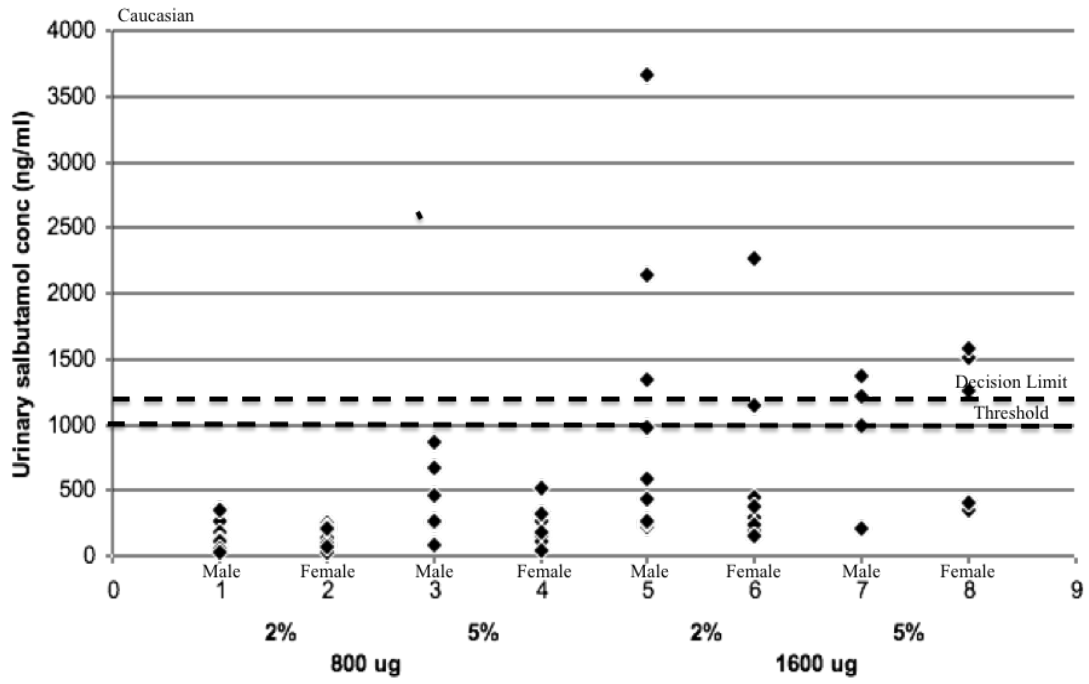
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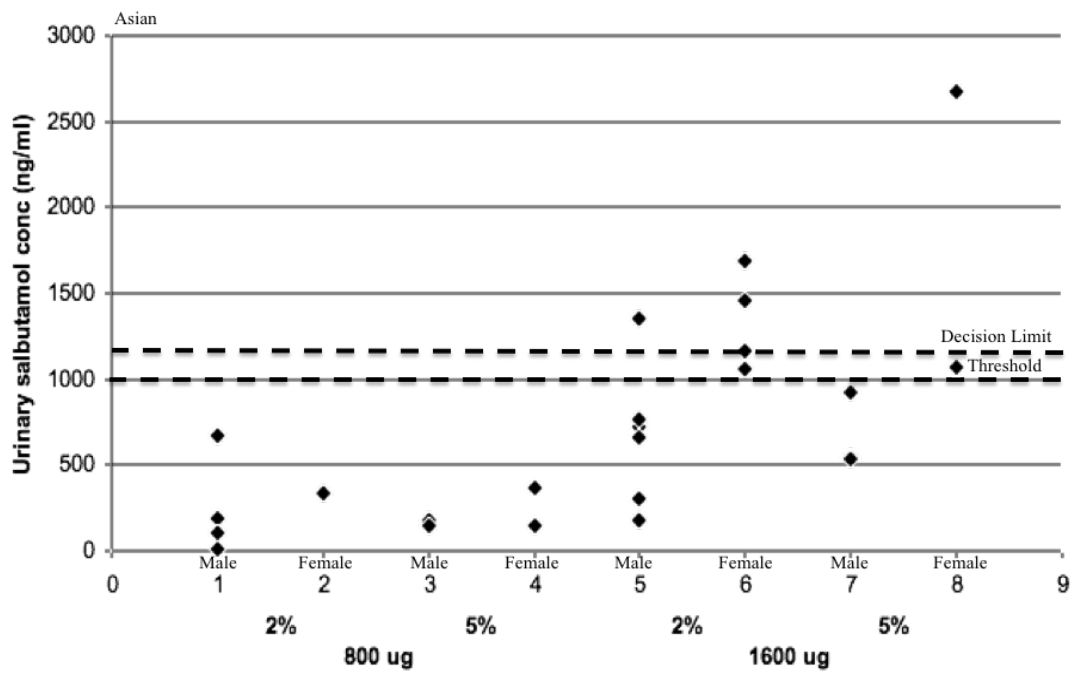
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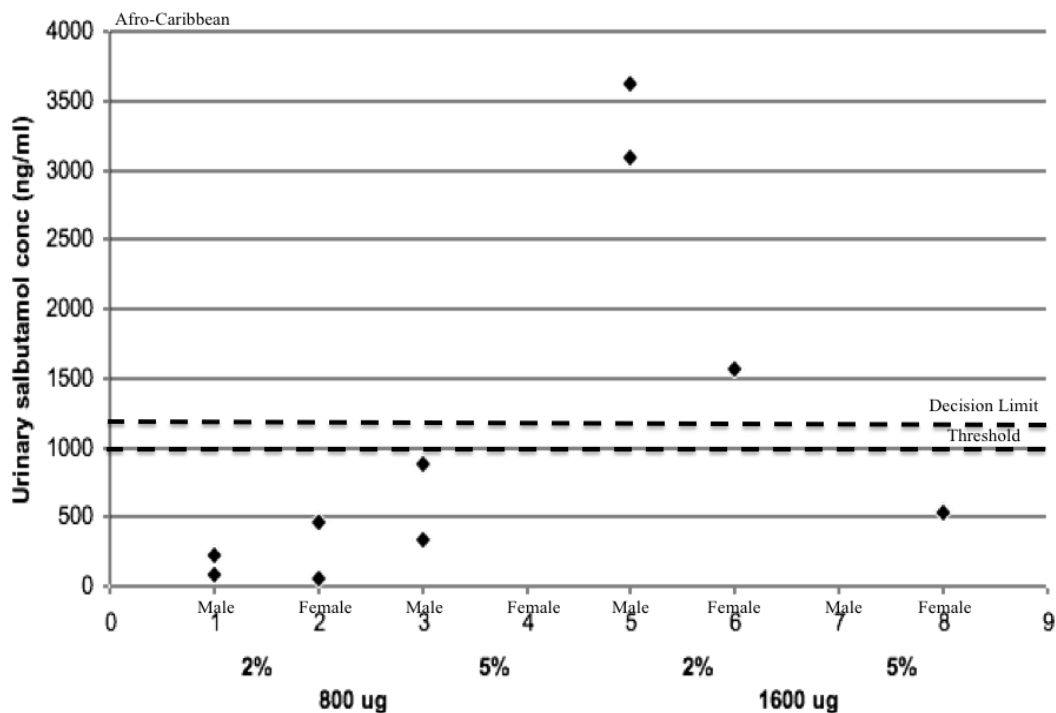
C

Figure 26. Urine drug concentration following inhalation of 800 μg or 1600 μg salbutamol under dehydration of 2% and 5 % body mass amongst male and female Caucasian (A), Asian (B) and afro-Caribbean (C) subjects.

	Caucasian Female		Caucasian Male		Asian Female		Asian Male	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
2% BM 800 SAL	142.2	± 78.34	178.29	± 119.53	-	-	246.60	± 291.88
2% BM 1600 SAL	586.52	± 700.63	1097.20	± 1150.89	1343.93	± 286.06	666.48	± 413.60
5% BM 800 SAL	238.76	± 149.48	436.17	± 292.27	-	-	-	-
5% BM 1600 SAL	919.53	± 592.20	946.43	± 515.51	-	-	667.97	± 221.23

Table 13. Mean and ± SD urine drug concentrations (ng·mL^{-1}) following inhalation of 800 μg or 1600 μg salbutamol under dehydration of 2% and 5 % body mass amongst male and female Caucasian and Asian subjects.

5.4 Discussion

This is the one of the first studies to investigate the impact of inhalation of up to 1600 μg of Salbutamol concomitant to a 5% BM loss through dehydration on urine salbutamol concentration. Our study demonstrates that following the inhalation of 1600 μg as a single dose resulted in several AAFs, hence exceeding the decision limit of the current WADA regulations that impose a urinary drug threshold of 1200 ng·mL^{-1} for salbutamol (WADA, 2015). This concentration is significant since it represents a level referred to by WADA as the 'Decision Limit' above which a sample would be classified as an adverse analytical finding (AAF; WADA, 2015) and warrant further investigation. Whilst our findings do not suggest significant differences according to sex or ethnic origin there is a large variation between individuals at the various salbutamol doses and %BM loss. This large inter-subject variation confirms the likelihood that individuals inhaling acute, high doses of salbutamol and exercising in conditions that may results in BM loss, may present with an AAF.

The current WADA urinary threshold (1000 ng·mL^{-1}) and decision limit (1200 ng·mL^{-1}) are based upon research that was able to provide a distinction between 1600 μg inhaled salbutamol (8 x 200 μg doses over a 24 hour period) and 20 mg oral salbutamol (Ventura *et al*., 2000). This research demonstrated that inhaled cumulative doses up to 1600 μg of salbutamol were unlikely to result in a urine concentration above 500 ng·mL^{-1}, whereas 20 mg oral salbutamol would result in a urine salbutamol concentration above 1400 ng·mL^{-1} (Ventura *et al*., 2000). Others have added to this research by attempting to examine the excretion of salbutamol administered by different routes including oral and inhaled (Pichon *et al*., 2006; Elers *et al*., 2012) and across a range of acute doses from 200 μg and 800 μg (Sporer *et al*., 2008) and repeated doses over several hours (Elers *et al*., 2011). None of this

previous research investigating inhaled salbutamol doses within the WADA limit of 1600 µg over a 24 hour period has demonstrated it is possible to exceed 1000 ng·mL^{-1}. However, the previous research has not examined acute doses up to 1600 µg. A recent case report suggested a urine salbutamol concentration in excess of 1000 ng·mL^{-1} may be possible with acute, high dose administration of inhaled salbutamol within the recommended daily dose (1600 µg; Schweiser *et al*., 2004).

The purpose of the current WADA regulations that impose a urinary drug threshold of 1000 ng·mL^{-1} for salbutamol (WADA, 2012) is to distinguish legitimate therapeutic use from misuse. Supporting such a threshold are maximal dosing recommendations of 1600 µg per day (WADA, 2012), with recommended dosing regimens between 100 and 400 µg up to four times daily. Our study adds to the previous existing data by asking participants to inhale the entire WADA 24 hour limit of 1600 µg inhaled salbutamol in a single dose. The rationale behind this is that athletes are regularly prescribed salbutamol *pro re nata* (when required). Individuals encouraged to administer salbutamol *pro re nata* may dose over and above such recommendations, for therapeutic purposes, either intentionally or inadvertently. In such cases athletes are at risk of exceeding the WADA threshold and in doing so, facing possible anti-doping sanctions. Given the results of this study it may be appropriate to educate athletes and support staff around the use of salbutamol and provide guidelines on the misuse (inhaled doses >800 µg) of salbutamol that may result in salbutamol levels exceeding the 1000 ng/mL threshold value.

Similar to previous studies (Elers *et al*., 2011; Elers *et al*., 2012; Sporer *et al*., 2008; Pinchon *et al*., 2006; Ventura *et al*., 2000) we observed a high variability between individuals across

gender and ethnicity. Individual inhalation techniques and different deposition of the drug and thereby bioavailability might explain some of the variation between subjects. We did try to reduce this by requiring participants to inhale salbutamol via a pocket chamber to increase the dose of salbutamol that was inhaled into the lower airways.

Other than under the 800 μg drug condition there was no significant difference in urine drug concentrations according to individuals' hydration status equivalent to either 2% or 5% loss in body mass. Nevertheless it is clear from the current work that hydration status *per se* is a critical factor in relation to doping control and the likelihood of approaching or indeed breaching the current urinary threshold for salbutamol. In line with current anti-doping practice the current study did not normalised drug concentrations for urine specific gravity. The results would however suggest that WADA consider introducing such analysis in an attempt to negate the impact on hydration status on doping control tests.

While the administered dose of 1600 μg salbutamol as a single inhaled dose used in the present study exceeds that considered as normal therapeutic treatment, athletes are often medically advised to inhale the amount that is needed to relieve asthma symptoms *pro re nata* (McKenzie & Fitch, 2011; Price *et al*., 2014). Atheles using salbutamol may inhale repetitive doses prior to a warm-up and again before and after competition (Price et al., 2014; Elers *et al.*, 2011). Indeed, doping case reports have shown that athletes inhale salbutamol repetitively during competition (Elers *et al.,* 2011; Schweizer *et al*., 2004). Athletes should as such be aware that supratherapeutic inhalation of the maximal allowed for salbutamol may increase the risk of presenting with a urine sample that exceeds the decision limit for salbutamol in doping control. Although doping cased of salbutamol were only reported 11

times in 2013 (World Anti-Doping Agency, 2015), it is important that athletes are not at risk of a false positive doping control test after inhalation within the current anti-doping regulations. As shown in Figure 25 of this study, large inter-individual differences exist in the urine excretion of salbutamol, while some individuals may exceed the decision limit for salbutamol when 1600 µg is inhaled as a single dose. Timing of urine sampling also has an impact on the concentration of salbutamol, with most of the AAFs observed within the first 4 hours of sampling (Figure 25). Importantly, athletes that present an AAF of salbutamol in doping control are given the opportunity to prove that the AAF was due to therapeutic use through a pharmacokinetic study. Lung function is another factor may affect the absorption of salbutamol (Schmekel, *et al*., 1992; Khazaeinia, *et al*., 2000; Lipworth, 1996). Exercise also affects blood distribution (Khazaeinia *et al*., 2000; Kamimori *et al*., 1990), as cardiac output is re-directed to exercising muscles, and secondly that water will be driven from plasma to the muscles and the interstitial space surrounding the muscles (Costill & Fink, 1974; Harrison *et al*., 1975). Nevertheless, given the large inter-individual difference in the urine salbutamol concentration observed in the present and previous studies after oral and inhaled administration (Eler et al., 2010; Hostrup *et al*., 2014; Elers *et al*., 2012; Elers *et al*., 2011), it does not appear that blood samples are suitable for doping control purposes in the case of salbutamol.

In conclusion 2% BM loss and greater concomitant with acute inhalation of 1600 µg of salbutamol may result in a urine concentration above the current WADA upper limit and decision limit leading to a false positive test finding. Although 1600 µg of inhaled salbutamol as a single dose exceeds that considered as normal therapeutic treatment, athletes using salbutamol should be made aware of this risk. This finding is independent of gender or ethnic origin. Data from this study will assist WADA in the implementation of regulations on the

use of inhaled short acting β$_2$-agonist and assist in the resolution of contested doping violations.

Applicability for doping control and advantages over existing methods.

- A fluid loss of 2% body mass or greater concomitant with acute inhalation of 1600 μg of Salbutamol may result in a urine concentration above the current WADA upper limit and decision limit leading to a positive test finding, irrespective of gender or ethnic origin.

  o The current WADA guidelines, which allows athletes to inhale up to 1600 μg daily may result in a positive test when fluid loss of 2% body mass or greater . The current WADA upper limit and decision limit should be re-assessed in light of these findings

5.5 Limitations

A limitation to our study is the variability in actual dose inhaled. Whilst the use of a chamber aimed to reduce this limitation it remains possible that some participants with low urine concentration inhaled lower doses of salbutamol. In addition future work should investigate whether there is a relationship between body weight and the urinary concentration of salbutamol. A lighter athlete may be at a greater risk of breaching the threshold when administering high doses compared to a heavier athlete. Such findings would have implications to the care athletes receive in the future. Another limitation is with a relatively

wide group of ethic individuals, the number of participants within Afro-Caribbean and Asian groups were very small; this is due to the difficulties of recruitment. Small number of data in each group may affect the generality of the results. Furthermore, it may affect the comparison results across different groups. Future studies should look to recruit a larger number of subjects across ethic groups in order to improve the reliability of the data. The subjects were not elite athletes also limited the result of the present study. Nonetheless, previous pharmacokinetic studies found no difference in the urine concentrations of salbutamol between athletic and non-athletic subjects or between asthmatics and non-asthmatics after oral and inhaled administration (Elers *et al.*, 2011; Elers *et al*., 2012). Therefore, the present findings may be applicable for WADA.

CHAPTER VI

STUDY 4

THE ERGOGENIC EFFECT OF CAFFEINE

& THEOBROMINE ON 3 KM RUNNING

PERFORMANCE

6.1 Introduction

A number of nutritional products have been used in the treatment of asthma. Of note, caffeine and theobromine are commonly employed in asthma treatment associated with their influence on respiratory smooth muscle leading to bronchiodilation. In addition to their use as nutraceuticals caffeine and theobromine have been examined for their performance enhancing potential, caffeine to a far greater extent compared with theobromine.

Caffeine, a non-specific adenosine receptor antagonist, is a bitter, white crystalline purine, a methylxanthine alkaloid, and is closely related chemically to the adenine and guanine contained in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). It peaks in the blood 30-40 minutes after ingestion of a 72 mg dose (Mumford *et al.*, 1996). Caffeine can be found in a variety of food sources including: coffee, tea, soft drinks, chocolate, and kola nuts.

Schirlitz first evidenced the possible ergogenic effect of caffeine in laboratory in 1930 (cited in Jacobson and Kulling, 1989), and some early findings (Ive *et al.*, 1979; Costill *et al.*, 1977) have examined the role of caffeine in enhancing athletic performance across a range of sports, using different doses as well as controlling for other conditions. Since these early findings research in this field has expanded rapidly leading to a large body of evidence supporting caffeine as a powerful ergogenic aid (Jenkens *et al.*, 2008; Mc Naughton *et al.*, 2008; Desbrow *et al.*, 2012; Gonzalez, 2012).

Caffeine was on the WADA banned list from 1962 to 1972 and then 1984 till 2003 when it was placed on the monitored list in order to monitor the possible potential of misuse in sport. It was then removed from the Prohibited List because many experts believe it to be omnipresent in beverages and food and that having a threshold might lead to athletes being sanctioned for social or dietary consumption of caffeine (Prohibite List, 2012). Furthermore, caffeine has only been demonstrated to be ergogenic at doses lower than those that result in a urine concentration of 12 μg/mL (Graham, 2011). In addition, urinary caffeine concentrations can very considerably because it is metabolized at very different rate in individuals (Fenster *et al.*, 1998), and does not always correlate to the dose ingested.

Theobromine is a caffeine derivative and metabolite found primarily in chocolate. It is highly fat soluble; peaking in the plasma 1-2 hours after ingestion (Mumford *et al.*, 1996). An adenosine receptor antagonist, theobromine appears to have equal affinity for A_1 compared to A_{2A} receptors, while caffeine shows a slightly lower affinity for A_1 receptors (Macht & Dettmer, 2006). Theobromine has one-tenth the stimulant effect of caffeine, but with a longer half-life in the body (Mumford, 1994). Theobromine is widely consumed in the diet and is currently used in metabolism-enhancing sporting supplements for its stimulant effects (Pearce *et al.*, 2012). Furthermore, theobromine is not prohibited or restricted in any form by the WADA.

Very few studies have investigated the behavioral effects of theobromine, and thus no clear conclusions can yet be made about its psychoactive profile. Although two early reports found null mood effects from theobromine (Brunk *et al.*, 1973; Dorfman & Jarvik, 1970), a more recent study by Mumford *et al.* (1994) showed that 5 out of 7 subjects were able to

discriminate a high dose of theobromine (560 mg) from a placebo or caffeine dose. The combination of caffeine (19 mg) and theobromine (250 mg) in capsules increased the self-reported mood construct "energetic arousal", and improved cognitive function as measured with a simple reaction time test (Smit, 2004) compared to placebo capsules.

An increasing amount of evidence in the last decade has showed that theobromine has psychoactive actions in humans and effect on blood pressure that are qualitatively different from those of caffeine (Mitchell *et al*., 2011; Baggott *et al*., 2013, Mitchell *et al*., 2011). One possible reason for the conflict in the effects of caffeine and theobromine could be their different half-life. In recent years, theobromine is starting to be widely studied to examine common and differential mechanisms with caffeine.

This study examined the impact of caffeine and theobromine compared with placebo on endurance exercise in a group of non-asthmatic recreational athletes. It was hypothesized that caffeine and theobromine would have no improvement effect on 3 km running performance in non-asthmatic athletes.

6.2 Methods

Subjects

Prior to the commencing the study, ethical approval was obtained from Liverpool John Moores University Local Ethics Committee (ethics approval code: 12/SPS/042). Ten male athletes (mean ± SD; age 22.4 ± 4.3 years; height 175.9 ± 3.9 cm; body mass 74.5 ± 9.9 kg)

were recruited for the study. All participants were free from asthma and EIB and AHR confirmed with no previous history of disease and presenting with a negative Eucapnic Voluntary Hyperpnea (EVH) challenge (Anderson *et al*., 2001). All participants were free from chest infection for at least 4 weeks prior to assessment; they were not taking any medication and there were no other health or medical contradictions to them taking part in the study as confirm by information provided on a physical activity readiness questionnaire. All participants were actively engaged in endurance running training (>45 minutes continuous running) at least 3 times per week. Participants were asked to refrain from ingesting foodstuffs containing methylxanthines, such as caffeine (coffee) and theobromine (chocolate) for 24 hours prior to testing due to the half-life of caffeine and theobromine.

Each of the participants was required to complete three, 3 km time-trial. Trials were conducted in the same laboratory, on the same treadmill (Woodway Curve, Woodway, USA) and under similar temperature and humidity environment (18°C; 40% RH). During their first visit, participants completed a pre-trial questionnaire and provide written informed consent prior to the measurement of body mass (kg) and stature (cm). Fifteen minutes prior the initiation of each 3 km time-trial participants consumed one of the following in a randomized, single-blind fashion: placebo (sugar pill); caffeine (6 mg/kg body mass); or theobromine (6 mg/kg body mass).

3 km Running Time-Trial

Participants were familiarized to running on a non-motorised treadmill (Woodway Curve, Woodway, USA) prior to initiating the 3 km time-trial. Familiarisation runs took place over a

distance of 3 km on at least two occasions. Paticipants progressed to the recorded 3 km time-trial once they felt comfortable pacing themselves on the non-mortorised treadmill over the 3 km distance.

Each time-trial was conducted under controlled laboratory conditions in temperate of $18^{\circ}C$, humidity of 40% RH environment. Participants completed three 3-km running time-trial following the administration of a placebo, caffeine (6 mg/kg BM) or theobromine (6 mg/kg BM) in a randomized, cross-over, single-blind fashion. The caffeine, theobromine and placebo were adminstered 30 minutes prior to the start of the time trial. Prior to starting the time-trial participants were fitted with a heart rate monitor (Polar RS400; Polar Electro Oy, Kempele, Finland) and connected to a breath-by-breach gas analyser via a facemask (Oxycon Pro, Jagear, Wuerzberg, Germany). Over the course of the 3 km time-trial the following were measured every 500 meters during each trial: average heart rate (HR), oxygen uptake (VO_2), carbon dioxide production (VCO_2), minute ventilation (V_E), respiratory exchange ratio (RER), and rating of perceived exertion (RPE). Two minutes following the completion of the 3 km time-trial capillary blood lactate was measured (Lactate Pro, Arkray KDK, Japan). Spirometry was used to measure lung function pre- and 20 minutes post-administration of placebo, caffeine or theobromine. Following a 10 minutes free warm-up, participants were asked to begin the 3 km time-trial. During the time-trial all participants were able to observe the distance covered, however, no feedback related to time, speed, or heart rate (HR) was provided. In addition to completion time, Spirometry was obtained immediately after each time-trial. Participants were encouraged to complete the time-trial as fast as possible with prizes offered to the five fastest times. During the time-trial consistent positive encouragement was given to each participant.
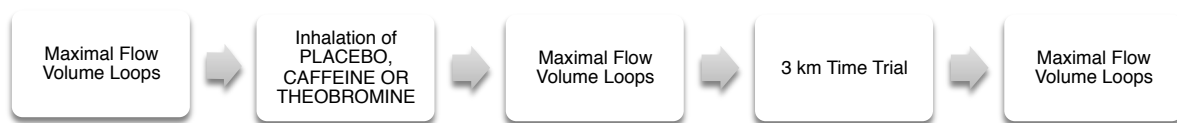
Figure 27. **Schematic of participant protocol on day of each 3 km time-trial**

Eucapnic Voluntary Hyperpnea (EVH) Test

In-line with the previous studies in this thesis and to maintain consistency across studies, participants were free from asthma evidenced by an EVH test prior to the start of testing to exclude asthma or Exercise Induced Bronchoconstriction (EIB). Three baseline maximal flow-volume loops were measured using a commercially available spirometer (ML3500, Cardinal Health, Basingtoke, UK), 10 minutes after inhalation of placebo, caffeine and theobromine and then 10 minutes post 3 km time-trial. On each occasion maximal flow volumes were measured according to the European Respiratory Society criteria (Miller *et al*., 2005). Flow-volume values were taken from the flow-volume loop with the best combined Forced Expiratory Volume in 1 second (FEV_1) and Forced Vital Capacity (FVC) calculated. Each participant then completed an EVH challenge test (Anderson *et al.*, 2001) involving 6 minutes hyperventilation at approximately 85% of maximal voluntary ventilation (30 x baselines FEV_1) pre-determined from their maximal flow volume loop. Maximal flow volume loops were measured at baseline and at 3, 5, 7, 10 and 15min following completion of the challenge. A fall of >10% in FEV_1 from baseline was considered positive.

Statistical analysis

Statistical analyses were performed using the statistical software program SPSS (SPSS, Inc., Chicago, IL), and a one-way analysis of variance (ANOVA) was performed to compare differences in time, heart rate, oxygen consumption, minute ventilation, respiratory exchange ratio, rating of perceived exertion. A paired t-test was performed to compare differences in blood lactate. Normally distributed continuous variables are expressed as mean (± SD). Statistical significance for this test was set at a p-value of <0.05. An effect size was also calculated to estimate the magnitude of the difference between groups. This provides a way to describe the meaningfulness of the differences, especially as the sample size is small. The size of the effect was classified according to the system proposed by Cohen (1988), where an effect size one of 0.2 represents a small effect, one of 0.5 represents a moderate effect and one of 0.8 or above represents a large effect. All data was tested with Mauchly's test of sphericity. The primary variable is the active ingredient consumed, being nil, 6 mg/kg BW caffeine or 6 mg/kg BW theobromine.

6.3 Results

All ten participants successfully completed all three 3 km time-trial. The mean (± SD) body mass and stature of the athletes were 74.5 (± 9.9) kg and 175.9 (± 3.9) cm, respectively. All participants were free from chest infection for at least 4 weeks prior to assessment; they were not taking any medication and there were no other health or medical contradictions to them taking part in the study as confirm by information provided on a physical activity readiness questionnaire. All participants were free from asthma and bronchial hyperresponsiveness evidenced by a negative Eucapnic Voluntary Hyperpnea (EVH) test. All participants were actively engaged in endurance running training (>45 minutes continuous running) at least 3

times per week. Throughout all trials participants reported no side effects from consumption of caffeine or theobromine.

3km Time-Trial Performance

No significant difference was noted for overall completion time between trials ($p = 0.844$; see Figure 28). In addition to completion time, heart rate (HR), oxygen uptake (VO_2), Rating of Perceived Exertion (RPE), minute ventilation (V_E), were assessed every 500 meters during each trial following intake of placebo, caffeine or theobromine; blood lactate was assessed pre- and post-test (Figure 29). Overall mean: time ($p = 0.133$); HR ($p = 0.676$); VO_2 ($p = 0.492$); V_E ($p = 0.344$); and RPE ($p = 0.774$) during the 3 km time-trial were not significantly different between trials (see Figure 29a-e). There was no significant difference between treatments in the post 3 km time-trial blood lactate values ($p = 0.897$; see Figure 29f).

Of note, performance following the administration of caffeine (6 mg/kg) showed significant time improvement from the 2 km recording point of the time-trial ($p<0.05$), which was further increased throughout the time-trial (see Figure 29a). Theobromine showed a relatively slower enhancement of performance time, however towards to the end of the test, theobromine showed a bigger difference compare to caffeine (see Figure 29a). Furthermore, over each 500 metres split of the time-trial: from 1 km to the end, HR was significantly increased under theobromine ($p<0.05$), but no difference was found under caffeine for each recording point (see Figure 29b); V_{O2} was significantly increased under caffeine from 1 km to the end, the increase occurred under theobromine was at 1.5 km (see Figure 29c).

	0.5km	1km	1.5km	2km	2.5km	3km
PLA	199.0 ± 21.6*	395.8 ± 36.7	610.7 ± 51.7*	833.7 ± 71.6	1057.6 ± 95.2*^	1260.6 ± 110.7*^
CAF	181.5 ± 17.76*	375.8 ± 29.6	579.1 ± 44.2*	798.0 ± 82.0	986.5 ±78.7*	1168.7 ± 78.1*
THEO	185.9 ± 16.3	383.8± 29.3	587.2 ± 43.7	798.5 ± 51.4	990.4 ± 61.4^	1176.5 ± 75.2^

Table 14. Time (s) per 500 meters following the Administration of Placebo, Caffeine and Theobromine. * = Caffeine *vs*. Placebo (p<0.05), ^ = Theobromine *vs*. Placebo (p<0.05), ϕ = Caffeine *vs*. Theobromine (p<0.05).
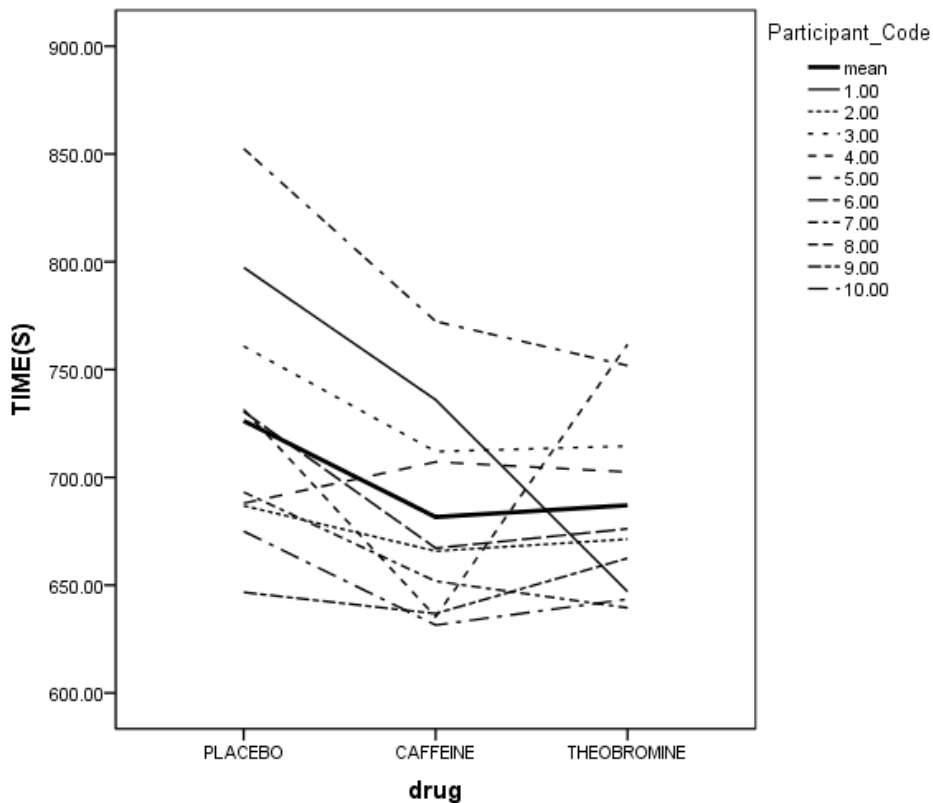
Figure 28. Individual and mean ± SD 3 km time-trial performance under each treatment.
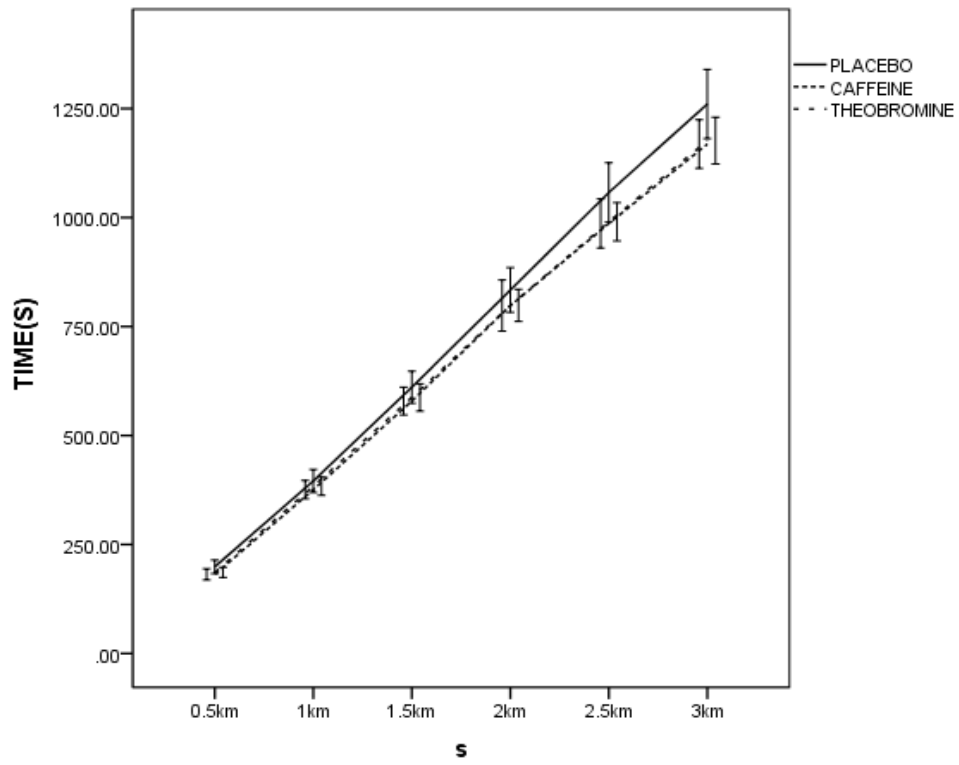
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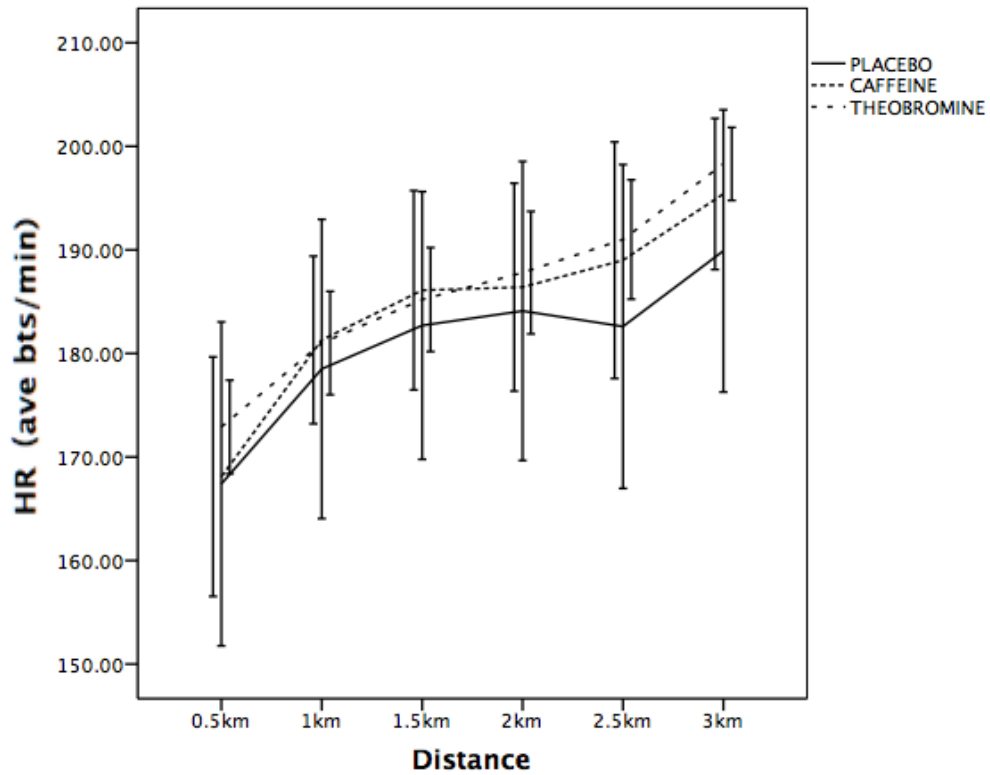
b)
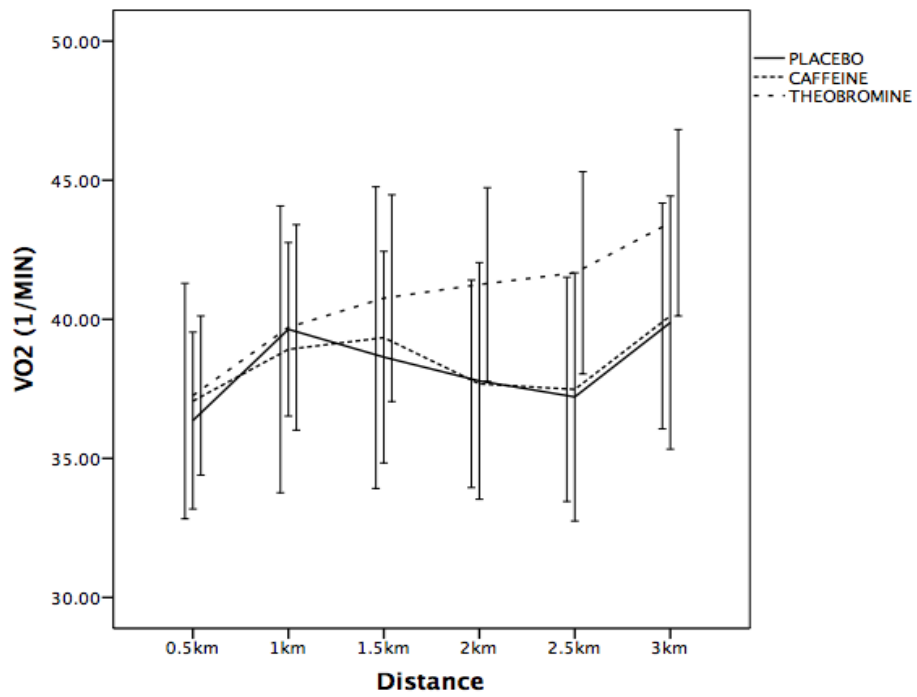
c)
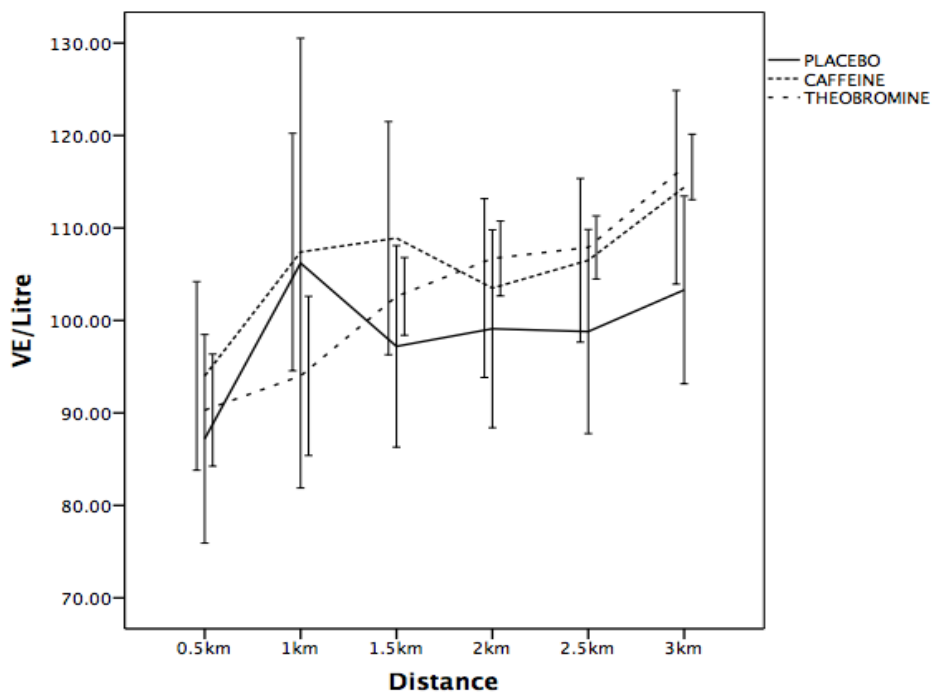
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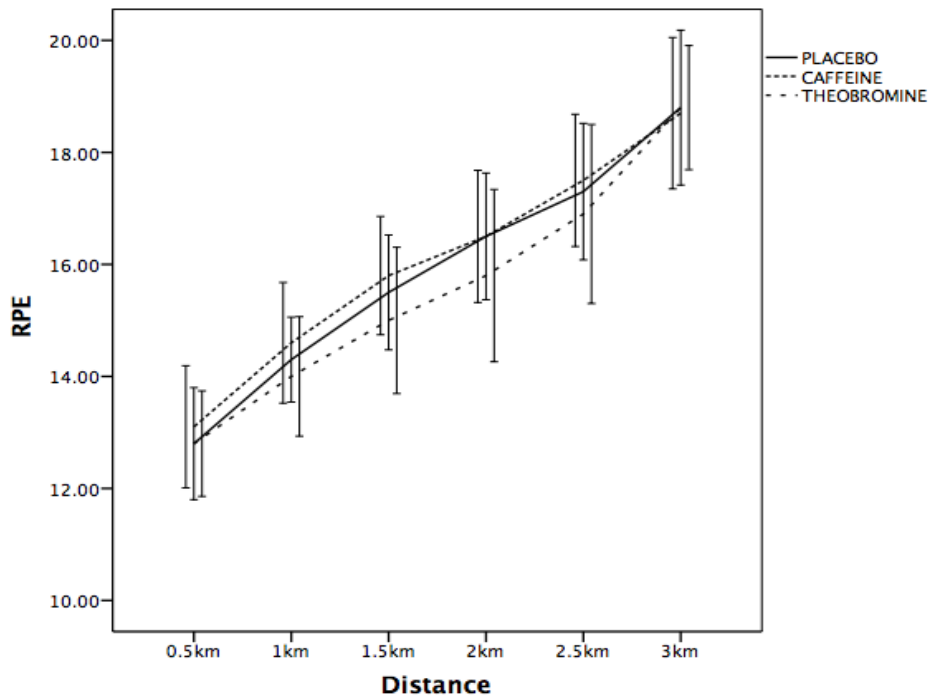
e)
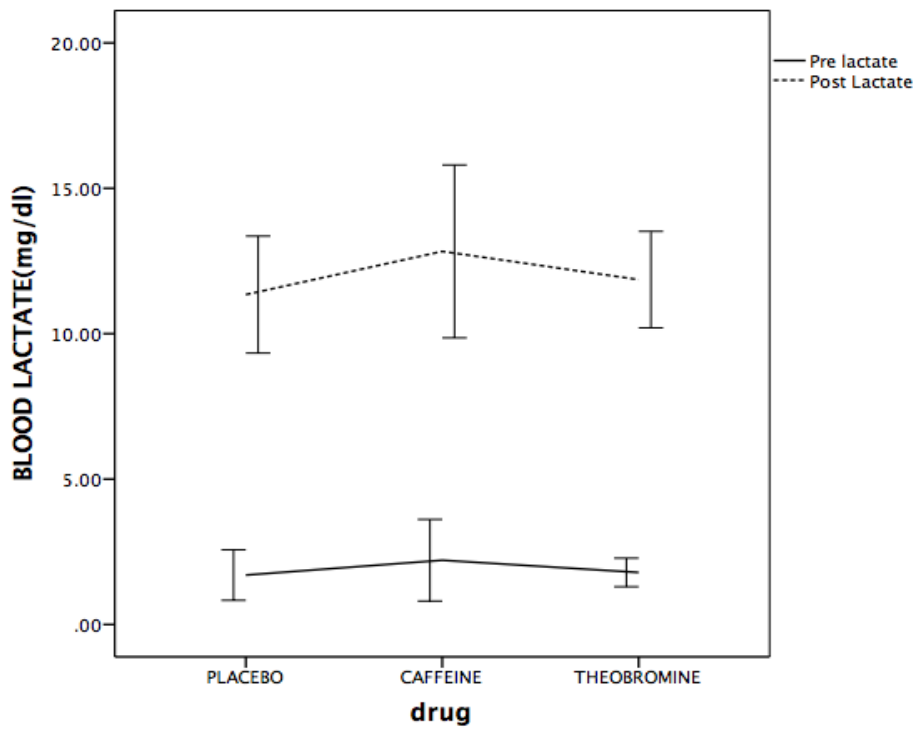
f)

Figure 29. Mean ± SD for time, HR, VO$_2$, V$_E$, RPE and blood lactate at each 500 m point during the 3 km time-trial following the administration of placebo, caffeine or theobromine.

Lung Function

There was no significant difference in FEV_1 (p=0.060) between placebo, caffeine and theobromine at any time point. A significant difference was observed when comparing post-run FVC (Litres) following the administration of placebo and theobromine (p=0.015). A significant difference was also observed between pre- and 20 minutes post-administration of theobromine (p=0.025). This, in turn, showed a significant difference in comparing post-run FEV_1/FVC (%) following the administration of placebo and theobromine (p < 0.05) and a significant different between pre- and 20 minutes post-administration of theobromine (p < 0.05).
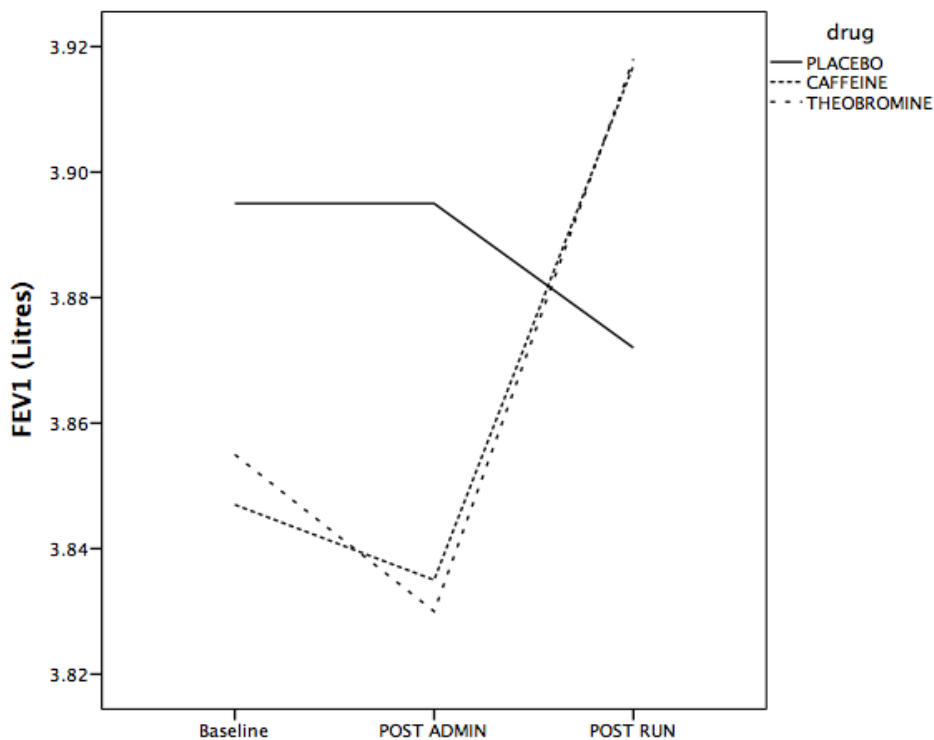
Figure 30. **FEV1 at Baseline, Post Administration and Post Run.**
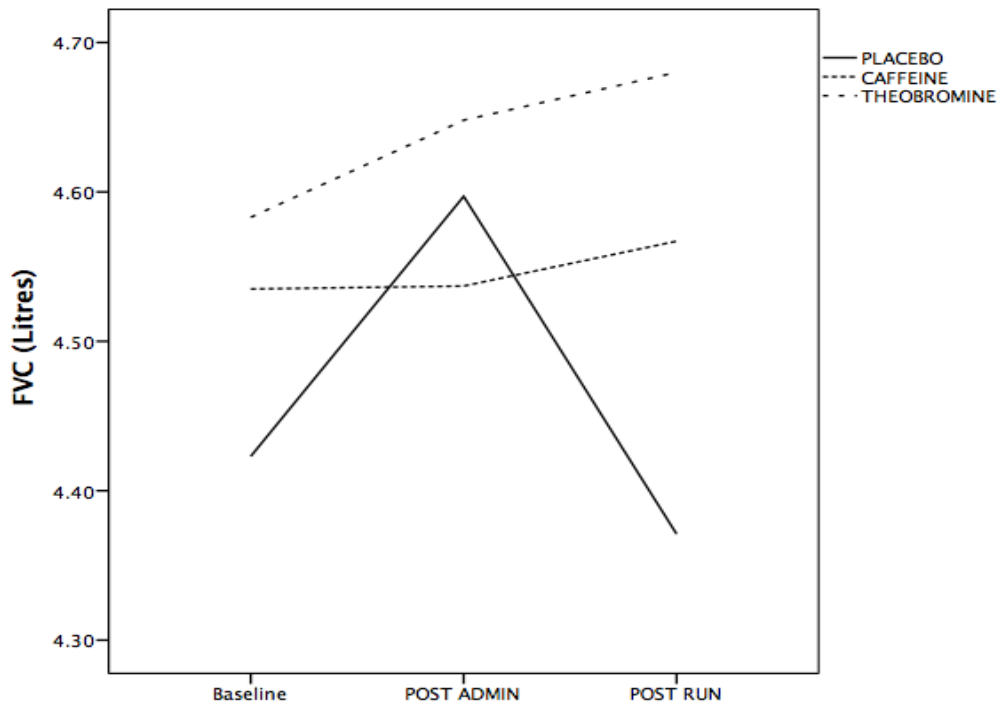
Figure 31. FVC at Baseline, Post Administration and Post Run.
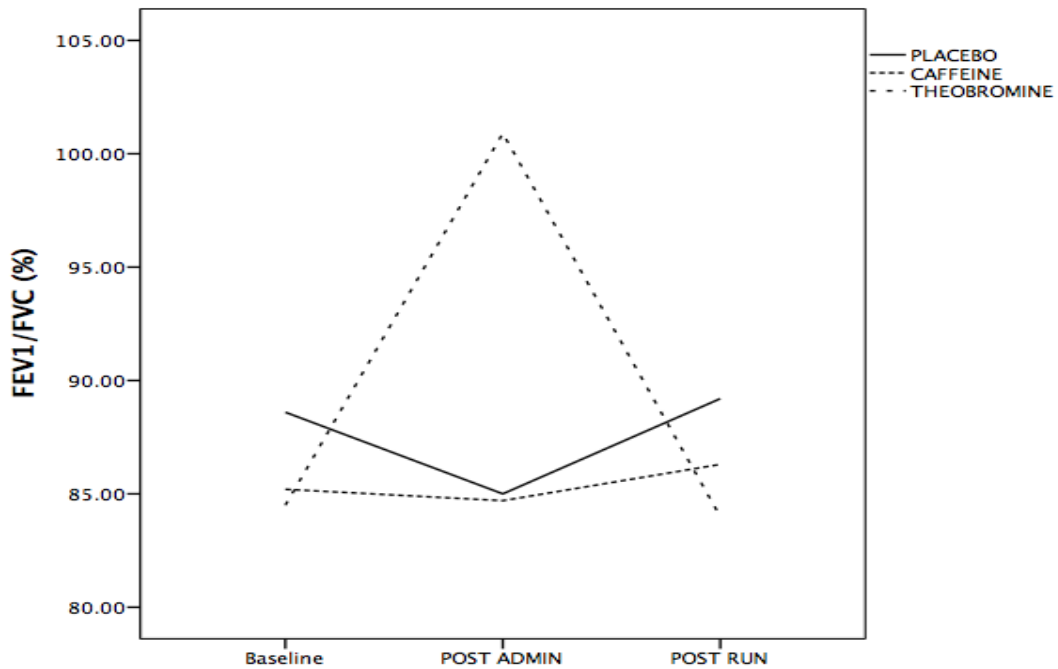
Figure 32. FEV1/FVC at Baseline, Post Administration and Post Run.

6.4 Discussion

The purpose of this study was to explore the effects of caffeine or theobromine consumption on 3 km time-trial running performance in non-asthmatic recreational athletes compared with placebo. The findings of this study suggest that both caffeine and theobromine have no significant effect on 3km time-trial performance at a dose of 6 mg/kg BM. However, a small improvement was observed with both caffeine and theobromine towards to the end of each trial, with caffeine's impacting performance earlier than theobromine.

Caffeine with a concentration > 12 μg/mL in the urine was considered doping, and was on the WADA banned list from 1962 to 1972 and then 1984 untill 2003 when it was placed on the monitored list in order to monitor the possible potential for misuse in sport. In opposition to this move by WADA, caffeine has been demonstrated to be ergogenic at doses lower than those that result in a urine concentration of 12 μg/mL, and higher doses appear to reveal no additional performance enhancing effect (Graham, 2011). A large number of athletes tested positive for caffeine during the second banned period. The sanctions ranged from warnings up to 2-year suspensions, although suspensions usually were only 2 to 6 months. According to WADA, one of the reasons caffeine was removed from the Prohibited List was that many experts believe it to be omnipresent in beverages and food and that having a threshold might lead to athletes being sanctioned for social or dietary consumption of caffeine (Prohibited list, 2012). Furthermore, urinary caffeine concentrations can very considerably because it is metabolized at very different rate in individuals (Fenster *et al*., 1998), and does not always correlate to the dose ingested.

Ivy *et al.* confirmed the ergogenic effect of caffeine on athlete's performance as early as 1979, reporting that total work output was increased after the ingestion of 250 mg caffeine. Since some early findings about caffeine could be a powerful ergogenic aid, the research in this field has expanded rapidly. Researchers tested the ergogenic effect of caffeine on different types of sports, using different doses as well as controlling for other conditions. Jenkins *et al.* (2008) found that the cycling performance was significantly improved in 13 cyclists following the administration of low doses of caffeine (2-3 mg/kg) compared with placebo conditions. A more recent study by Desbrow *et al.* (2012) investigated this effect following the ingestion of two different doses of caffeine, 3 mg/kg and 6 mg/kg, using a randomised, placebo controlled, double-blind design. The results demonstrated a significant improvement in cycling performance with a dose of 3 mg/kg of caffeine, but no additional improvement was observed with of the higher caffeine intake (6 mg/kg).

Of note, the present study investigated the ingestion of 6 mg/kg of caffeine, and found no significant difference in overall time-trial performance compared to placebo. However, there was a significant difference towards to the end of the time-trial recording point till the end of the test (the final 1 km), suggesting that the comsumption of 6 mg/kg caffeine may have a positive impact on endurance exercise, but only at the end of the exercise, for a relatively short period. One possible explanation why the improvement appeared at such late stage was because the timing of caffeine ingestion was too close to the beginning of the test (30 minutes), most previous studies administered it at least one hour prior of the test (Costill *et al.*, 1978; Graham *et al.*, 1998).

Of note, similar to the results in the present study, there are a small number of studies reporting no ergogenic effect on work performance when participants received a high dose of caffeine, including endurance performance (Cameron *et al.*, 1990; Tarnopolsky, 2008; Davis & Green, 2009; Mora-Rodriguez *et al.*, 2012; Butts, 1985; Grossman & Sutton, 1985; Goniewicz *et al.*, 2013; Ryder, 1994; Martinsen & Sundgot-Borgen, 2012). Perkins and Williams (1975) performed an exercise to exhaustion under four different conditions: placebo; caffeine doses in 4 mg/kg; 7 mg/kg; and 10 mg/kg. The results demonstrated no significant difference in time to exhaustion between different doses compared with placebo. Butts and Crowell (1985) made a similar conclusion in time to exhaustion at 75% VO_{2max} with a dosage of 300 mg caffeine.

Not only does caffeine impact endurance, it has been reported to benefit cognitive function and fine motor skills (Foskett *et al*., 2009). Accordingly, caffeine is commonly used by athletes as an ergogenic aid due to its associated reduction in fatigue, and enhancement of concentration and alertness (Paluska, 2003). Trained athletes benefit from a moderate dose of 5 mg/kg (Woolf *et al*., 2008), although lower doses of caffeine (1.0-2.0 mg/kg) may improve performance (Cox *et al*., 2002). Another reason explains why caffeine is widely used in competitive activities might be its small but significant paregoric effect (Derry *et al.*, 2012), possibly mediated by augmenting plasma endorphin concentrations (Grossman & Sutton, 1985). It is also defined that caffeine reduces the rate of perceived exertion during exercise (Doherty & Smith, 2005), proposing that athletes are able to undertake higher intensities but do not perceive this effort to be different from placebo conditions.

Mattew *et al*. (2013) demonstrated that theobromine responses differed according to dose with limited subjective effects at 250 mg and negative mood effects at higher doses. They also observed a dose-dependent increased in heart rate. Accordingly, their study concluded that theobromine at normal intake ranges may contribute to the positive effects on exercise performance, but at higher intakes the performance effects become negative. In the present study, theobromine showed a significant increase in heart rate at 1 km of time-trial whereas no heart rate increase under caffeine; and an improvement on performance from 2 km in time-trails, which is later than that observed with caffeine (from 1.5 km). The results of the present study suggest that the ingestion of caffeine results in a greater rapid enhancement on running performance than theobromine. The slower action of theobromine compared with caffeine may be associated with theobromine acting as a weaker analogue of caffeine (Becker & Simons, 1985). This suggested that caffeine is more efficient in performance enhancement than theobromine should be viewed with caution given the limited scope of this study. Longer distances, higher doses and variable times of ingestion prior to exercise are required to further explore this hypothesis.

According to the report from Mumford *et al.* (1994), caffeine increased subjective alertness in subjects, whilst theobromine did not, and Mitchell *et al.* (2011) for the first time found that theobromine did not act as an incentive alone or combined with caffeine, however, it may have a tension-raising effect. In conclusion, caffeine has strong alerting effects with inappreciable contribution from theobromine; caffeine produced ergogenic effects at early time points, while theobromine decreased calmness at a later time point; caffeine may have more CNS-mediated effects on alertness and theobromine may be acting mostly via peripheral physiology. Further investigations examining the combination of caffeine and theobromine are indicated.

The performance improvement contributions of theobromine are less clear and its psychoactive effects appear subtle compared to caffeine. Mumford (1994) postulated that theobromine is one tenth as potent as caffeine. Pharmacological assays also confirm that theobromine is less active than caffeine. Furthermore, individual variation in sensitivity to caffeine and theobromine is likely to play a role in the ergogenic efficacy of these compounds. Similar to caffeine, the ingestion of lower doses of theobromine may result in performance improvement, however at higher doses may causes a negative effect.

6.5 Conclusion

The results of the present study suggested that the consumption of caffeine and theobromine at a high dose of 6 mg/kg BM have no improvement on 3 km time-trial running performance in non-asthmatic athletes. However, theobromine appeared to enhance performance during the later stages of the time-trial to a similar magnitude as caffeine (6 mg/kg BM), with the ingestion of caffeine resulting in a more rapid enhancement of running performance than theobromine. The present study suggests theobromine abuse may provide an ergogenic effect, which propses a field of future research to examine whether an upper limit of use for theobromine should be proposed on WADA list.

6.6 Limitation

One of the limitations to this study was the small sample size. An increase in the number of participants may provide more robust findings. Furthermore, the present study focused on male participants. Since male and female may respond differently to the caffeine or theobromine inhalation, future studies should examine on female athletes. The issue of

ethnicity is exists in this study, which may have an impact on the findings. Of note, the participants were not elite endurance athletes. Therefore, the results of the present study may not directly represent the effects of caffeine or theobromine in elite endurance athletes. There is no test for combining consumption of caffeine and theobromine together for the athletes while doing the 3 km time-trial, therefore this study failed to find out the inter-relationship between caffeine and theobromine, only the differences. For future study, should examine the combination of caffeine and theobromine to examine possible interactions on performance.

CHAPTER VII

GENERAL DISCUSSION

7.1 Implications

Exercise-induced bronchoconstriction (EIB) is a pulmonary disorder that result in a narrowing of the airways and a measurable reduction in lung function. This limitation may have a negative effect on exercise performance for athletes in comparison with their unaffected competitors (Kindermann *et al*., 2007). Short-acting β_2-agonists are the most commonly prescribed medication for the acute control of EIB. In 2002, The International Olympic Committee (IOC) established the requirement for athletes to present evidence of current asthma, EIA, EIB or AHR through the therapeutic use exemption (TUE) process. These regulations, guided by the IOC Medical Commission, were based on health not doping (performance enhancing) concerns for athletes in light of a marked increase in the notification by athletes for the use of inhaled short acting β_2-agonist from 3.7% in Atlanta, 1996, to 5.7% in Sydney, 2000 (Fitch *et al*., 2008). A study conducted by Dickinson *et al.* (2005) provided support for the health justification of adding inhaled short acting β_2-agonist to the prohibited substances list in the Sydney, 2000 and Athens, 2004 Great Britain Olympic Team. Their data demonstrated that the establishment of a TUE for inhaled short acting β_2-agonist had no impact on the proportion of Team GB presenting with asthma, EIA, EIB or AHR (*c.* 21% at both Olympic Games) however; it identified a number of athletes with false positive diagnoses and athletes who had not been previously identified. Accordingly, it was concluded that the requirement of demonstrable evidence through the TUE process improved the quality of care for athletes. Furthermore, data from others (Dickinson *et al*., 2006a; Dickinson *et al*., 2006b; Anderson *et al*., 2003; Rundell *et al*., 2004; Parsons *et al*., 2007) has demonstrated the improved diagnostic sensitivity and specificity of objective, bronchial provocation tests of airway function as part of the TUE process.

Of the four β_2-agonists (salbutamol, terbutaline, formoterol, and salmeterol) allowed, salbutamol is most commonly used, and there is growing concern that non-asthmatic athletes are using inhaled salbutamol in an attempt to gain a competitive advantage (Pluim *et al.*, 2011). The main action of inhaled salbutamol is to act as a bronchodilator to reverse the bronchoconstriction of airway smooth muscle. These results in the asthmatic airway becoming dilated leading to a reduced airway resistance and improvements in V_E, increased oxygen uptake and exercise performance in asthmatics (Haverkamp *et al.*, 2007). Accordingly, a number of studies have examined the impact of acute and chronic inhalation of short-acting β_2-agonists on exercise performance. The vast majority of these studies have focussed on endurance performance (Pluim *et al.*, 2011).

Furthermore, previous studies that have focused on inhaled salbutamol have generally focused on non-specific performance trials such as running time to exhaustion or physiological markers of performance (Kindermann *et al.*, 2007). These previous studies have demonstrated no improvement following acute inhalation of up to 800 µg of salbutamol on running time to exhaustion, VO_{2max}, peak power and total work during a 30s Wingate test (Pluim *et al.*, 2011). In supportting of these findings and agreement with the findings in study 1 and 2, a number of previous studies have reported no ergogenic effect of acute inhalation of short-acting β_2-agonists on overall performance *i.e.* 20 km cycling performance and running to exhaustion (Pluim *et al.*, 2011).

Study 1 and 2 of this thesis are one of the first to examine the impact of inhaled salbutamol up to the World Anti-Doping Authority (WADA) recommended upper limit dose in a 24 hour period of 1600 µg versus 800 µg and placebo on time-trial endurance running performance

and simulated association football performance in male and female athletes. Furthermore, these studies are the first to examine the pharmacokinetics of inhaled salbutamol at a dose of 1600 µg and 800 µg following endurance and simulated association football performance in male and female athletes in temperate (18°C; 40% RH) and hot (30°C; 40% RH) environments.

The FIFA (Fédération Internationale de Football Association) has stated that the fundamental aims of doping controls and anti-doping policies to prevent any doping behavious, the policies are (FIFA Anti-Doping Regulations, 2016):

- to uphold and preserve the ethics of sport;

- to safeguard the physical health and mental integrity of the player;

- to ensure that all competitors have an equal chance.

There are many individual cases and programs were suspended by UEFA (The European football union) or FIFA after testing positive in doping test accoss 9 different countries in Europe (including Albania, Argentina, Australia, East Germany, England, France, Italy and Spain) in the recent decades (UEFA, 2007; BBC News, 2000; A-League, 2008; Focus Online, 1994; Hamburger Abendblatt, 2007; BBC Sport, 2003; The Daily Telegraph, 2008; ABC Sport, 2006). FIFA applies the minimum two-year ban for first-time offenders, however, there are exceptions. When a player accused of doping can prove the substance was not intended to enhance performance (*i.e.* inhalation of salbutamol for asthma or EIB), FIFA can reduce the sanction to a warning in a first offense, a two-year ban for a second offense and lifetime ban in case of repetition (The Associated Press, 2006). Therefore, in order to

avoid misjudgement, it is important for footballers to understand the maximum dosage of salbutomal to inhale at each time/day when it is used for necessary treatment.

To date, there are no published studies examining the impact of inhaled short acting β_2-agonists on team game performance. Given the global importance of team games such as football (soccer) it is imperative that the performance impact of inhaled short acting β_2-agonists be examined to support their inclusion in the WADA prohibited substance list. Accordingly, Study 2 investigated the ergogenic impact of salbutamol up to the WADA upper limit in associated football (soccer).

The results from Study 2 add to the current body of knowledge by focusing on an intermittent team sport, association football (soccer). No significant improvement in performance following the inhalation of up to 1600 µg of salbutamol was found in study 1, which suggests that the current WADA list of banned substances, which allows athletes to inhale up to 1600 µg in a 24 hour period is sufficient given the findings from this and previous studies. It is clear that salbutamol in a single bolus up to the WADA permitted daily limit of 1600 µg has no ergogenic effect on endurance performance. Further to our data, a recent study by (Elers *et al*., 2012) reported that inhaling an acute dose of up to 4000 µg of salbutamol resulted in no improvement in cycling time to exhaustion or oxygen uptake kinetics. According to Study 2 however, there is a possible improvement in performance following the inlalation of up to 1600 µg of salbutamol in non-asthmatic male and female association football (soccer) plers, it shows ergogenic effect on sprint, repeated sprint performance. Further studies with larger amount of participants are required, to corroborate whether inhalation of up to 1600 µg is sufficient to avoid pharmaceutical induced team performance enhancement according to

current WADA guidelines. Accordingly, from a performance perspective the current WADA upper limit of 1600 μg per day appears appropriate for endurance and team (multi-sprint) events given the absence of improvement in performance in non-asthmatic athletes.

There exists some ambiguity in terms of the therapeutic use of inhaled salbutamol. Whilst the recommended salbutamol maximal dosing regimen is 100 to 400 μg up to four times daily, it is typically prescribed *pro re nata* (PRN; when required) which may lead to confusion. Individuals encouraged to administer salbutamol PRN may dose up to the upper daily limit acutely (1600 μg) or over and above the maximal recommended daily dose either intentionally or inadvertently, however, in both instances individuals intent to dope for performance enhancement purposes may be nil. Such circumstances may lead to the current urinary threshold being unintentionally breached and thus bring about an adverse analytical finding (AAF). Clearly individuals administering inhaled salbutamol up to, and above the 1600 μg dose indicates uncontrolled asthma. Desensitisation or tolerances are experienced by those regularly administering inhaled salbutamol, which not only increases the risk of unsuccessful treatment in an emergency but also increases the likelihood of further overdosing in an attempt to control EIB.

The current urinary threshold imposed by WADA is intended to enable differentiation between the use of oral and inhaled salbutamol and also approved therapeutic use and misuse. Oral use has been linked with performance enhancement since it typically represents doses in the region of 10 times that of inhaled use, which is absorbed directly into the systemic circulation. Care is warranted in the continued promulgation of this assumed hypothesis as there has been limited research to examine this association. A small number of animal studies

have reported increased protein synthesis and muscle hypertrophy following the administration of oral β_2-agonists, particularly long acting β_2-agonists such as Clenbuterol (Beermann, 2002). From an endurance performance perspective in humans, only Collomp *et al.* (2000) have demonstrated enhanced performance whereby short-term oral administration of salbutamol (12 mg·day^{-1} for 3 weeks) improved time to exhaustion during sub-maximal cycling exercise. (Caruso *et al.*, 1995) and (Martineau *et al.*, 1992) have demonstrated an increase in muscle strength following prolonged oral administration of salbutamol (16 mg/day) The paucity of research examining the impact of oral salbutamol indicates the need for future research to examine further the claim that oral administration has a positive impact on sports performance.

The maximal recommended dosing regimen for salbutamol acts as an upper limit for those requiring immediate relief of asthma symptoms. Whilst there is no ergogenic impact on acute, single bolus inhalation of this upper limit, individuals who regularly use high doses of salbutamol or exceed these recommendations clearly demonstrate poor management of their condition. There lies the problem from an anti-doping regulatory perspective when the evidence to support any ergogenic properties is not present and the likelihood of poor control of asthma and EIB are high leading to a potential anti-doping violation in the absence of performance enhancement. From a sports medicine perspective improved care may constitute better diagnosis, management and education amongst athletes.

In addition to the absence of published data examining team games the factors effecting the presentation of a doping violation following the administration of therapeutic doses of short acting β_2-agonists remains unclear. The impact of prior exercise (competition), sporting

discipline (body composition), gender and race on the pharmacokinetics of short acting β_2-agonists has received little attention. The impact of environmental factors, in particular those environments leading to perturbations in hydration status, have been previously cited by athletes as a defence of a positive doping test. Exercise-induced sweat loss concentrates the urine, thus leading to a higher urine concentration of β_2-agonists (Lipworth, 1996; Dickinson *et al*., 2014; Hostrup *et al*., 2014). Sporer *et al*. (2008) observed that the urine concentrations of salbutamol were close to exceeding the urinary threshold in exercising subjects after inhalation of 800 μg. It is likely that the urinary threshold and decision limit for salbutamol would have been exceeded in that study, if subjects had inhaled the maximal allowed dose of 1600 μg salbutamol. Real-life doping control is mostly conducted immediately after a competitive event or a training session. Athletes using β_2-agonists, usually inhale them a prophylaxis prior to competition or training (McKenzie & Fitch, 2011; Price *et al*., 2014). While athletes typically inhale repetitive doses of salbutamol below the current therapeutic threshold of 1600 μg, the 2015 WADA anti-doping regulations for salbutamol allow inhalation of 1600 μg as a single dose. Therefore, pharmacokinetic data of the maximal allowed dose of 1600 μg salbutamol conducted during exercise in hydrated and dehydrated state in controlled settings are important to evaluate the WADA urinary threshold and decision limit for salbutamol on the list of prohibited substances. To date, not much data is available examining the impact of hydration status on the pharmacokinetics of inhaled short acting β_2-agonists and the resultant effect on urine concentration. Studies in this area will better define the upper normal limit following therapeutic administration of inhaled short acting β_2-agonist across different populations of athletes together with the impact of hydration status on urine concentrations.

There has been some concern expressed recently regarding the potential impact of dehydration on the urine concentration of salbutamol with claims that athletes inhaling up to the WADA limit may present with an AAF leading to sanctions. Findings from study three of this thesis demonstrates that following the inhalation of 1600 µg of salbutamol and in the presence of dehydration up to 5% body mass, it is possible to present with a urine salbutamol concentration above the current WADA threshold of 1000 ng·mL^{-1} (WADA, 2012). Furthermore, study three also demonstrated that following such a dosing regimen may also result in a urine salbutamol concentration in excess of 1200 ng·mL^{-1}. This concentration is significant since it represents a level referred to by WADA as the 'Decision Limit' above which a sample would be classified as an adverse analytical finding (AAF) and warrant further investigation (WADA, 2012). Of note, whilst the findings from study 3 do not suggest any differences according to gender or ethnic origin, there are large inter-individual variations between at the various salbutamol doses and %BM loss. This large inter-subject variation confirms the likelihood that individuals inhaling acute, high doses of salbutamol and exercising in conditions that may results in body mass loss, may present with an AAF. Study three is the first to investigate the impact of up to 1600 µg of inhaled salbutamol concomitant to a 2% and 5% body mass (BM) loss through dehydration on urine salbutamol concentration in a multi-ethnic population.

Following performance trials and in the absence of forced dehydration, study 3 demonstrated the possibility of a urinary salbutamol concentration above the current threshold following therapeutic use. However, the findings from study 1 and 2 would not warrant any sample to be reported as an AAF. This finding was true for a hot (30°C, 40% RH) as well as a temperate environment (20°C, 40% RH). Nevertheless, the inter-individual variation was high in both temperate and hot environments and combined with the low subject numbers caution is

advised and future studies should aim to examine the impact of high dose Salbutamol (1600 µg) administration on urine concentration following endurance and team game performance to establish the likelihood of the Decision Limit being breached.

Caffeine, taken prior to exercise, has been shown to be an effective prophylaxis for EIB (Inman, 1996), and has been examined by many previous studies. Vanhaitsma (2010) comparative study on effects of Caffeine and β_2-agonists for asthmatic athletes, showed that in athletes with asthma and EIB, moderate (6 mL/kg body mass) to high doses (9 mL/kg body mass) of caffeine provide a significant protective effect against EIB, and that a high dose of caffeine is equally as effective as salbutamol in attenuating the bronchoconstrictor response to exercise. Whilst the action of theobromine on the central nervous system (CNS) is generally considered weak or non-existent, a small number of studies have reported that theobromine primarily acts a diuretic and a bronchial smooth muscle relaxant, and it as another methylxanthine with potential bronchodilator properties, has been under researched. Theobromine is an active ingredient of bronchodilator drugs that are used in the treatment of acute and chronic asthma, and persistent cough (Irwin, 1997). The IOC does not consider theobromine an illicit substance in humans, though their administration to racing animals is prohibited. A large evidence base exists for the performance enhancing effects from caffeine. In contrast, little is known of the ergogenic effect of theobromine.

Caffeine has been shown to enhance several different modes of exercise performance including endurance (Graham & Spriet, 1995; Ivy *et al*., 1979; Hogervorst *et al*., 2008; Graham *et al*., 1998; McLellan & Bell, 2004; Pasman *et al*., 1995), high-intensity team sport activity (Collomp *et al*., 1992; Woolf *et al*., 2008; Glaister *et al*., 2008; Bruce *et al*., 2000;

Stuart *et al*., 2005; Schneiker *et al*., 2006), and strength-power performance (Woolf *et al*., 2008; Beck *et al*., 2006). A study conducted by Lieberman *et al*. (2002) showed the fact that caffeine continued to enhance performance in terms of repeated acquisition (assessment of motor learning and short-term memory) and Profile of Mood states fatigue eight hours following consumption. These results are in agreement with Bell & McLellan (2002), where aerobic capacity was assessed 1, 3, and 6 hours following caffeine consumption (6 mg/kg). Caffeine had a positive effect on performance for participants classified as users ($\geq$ 300 mg/d) and nonusers ($\leq$ 50 mg/d); however, nonusers had a treatment effect at 6 hours post-consumption, which was not the case for users – this group only had a significant increase in performance at 1 and 3 hours post-consumption. Taken together, results of these studies (Lieberman *et al.*, 2002; Bell & McLellan, 2002) provide some indication, as well as application for the general consumer and athlete. Specifically, while caffeine is said to have a half-life of 2.5-10 hour (Magkos & Kavouras, 2005), it is possible performance-enhancing effects may extend beyond that time point as individual response and habituation among consumers varies greatly. Recently, it was also suggested that caffeine can positively affect both cognitive and endurance performance (Hogervorst *et al.*, 2008). Overall, the previous studies examining the effects of caffeine on anaerobic exercise is equivocal, with some studies reporting a benefit (Collomp *et al.*, 1992; Woolf *et al.*, 2008; Glaister *et al.*, 2008; Bruce *et al.*, 2000; Doherty *et al.*, 2004; Wiles *et al.*, 2006) and others suggesting that caffeine provides no significant advantage (Greer *et al*., 1998; Collomp *et al.*, 1991). As with all sports nutrition research, results can vary depending on the protocol used, and in particular, the training status of the athlete as well as intensity and duration of exercise. According to the previous studies (Crowe *et al*, 2006; Hulston and Jeukendrup, 2008; Jackman *et al*., 1996; Kovacs *et al*., 1998; Graham *et al*., 2000), that a low dose of caffeine (3 mg/kg) was adequate for enhancing performance, but did not lead to increased levels of

epinephrine or subsequent effect of free fatty acid mobilization; whereas a high dose of caffeine (6 mg/kg) had no significant effect on increasing plasma FFA levels or glycerol concentrations, nor did it substantially enhance rates of whole-body fat oxidation during endurance exercise even though performance was significantly improved with the caffeine + glucose solution.

Theobromine is useful in asthma and in other respiratory tract problems such as cough for which no definitive drug has been developed, and it is commonly used by athletes during sports; it is a caffeine derivative and metabolite found primarily in chocolate, caffeine and theobromine are closely related alkaloid; and both of them are existing broadly in energy drinks and food stuffs, therefore, this study investigated whether they have ergogenic effect on athletes endurance performance in order to assit WADA with more accurate information on their prohibited list. Theobromine has often been described as a stimulant, with one-fifth the potency of caffeine on adenosine receptors (Svenningsson *et al.*, 1993). However, theobromine had no effect on alertness at any time, although it did decrease blood pressure. Thus it is possible that theobromine affects peripheral physiology, but lacks the strong CNS-activating properties of caffeine. Furthermore, there were no interactions of caffeine and theobromine on mood or blood pressure. Theobromine has been used as a bronchodilator in combination with caffeine; however, there is no objective information in the literature regarding the relative bronchodilator effects of the two drugs in *vivo*.

Study four explored the impact of a methylxanthine proposed as a bronchodilator, theobromine, compared with caffeine, a methylxanthine with known bronchodilator efficacy, and placebo on 3 km time-trial running performance in non-asthmatic recreational athletes.

The findings of this study suggest that caffeine and theobromine at high dose of 6 mg/kg did not result in an improved 3 km time-trial performance, which is similar to the most of the findings from previous studies. It has been shown that caffeine supplementation at a low dose of 3 mg/kg can significatnly enhance both endurance and high-intensity performance in trained athletes. Consequently, the International Olympic Committee mandates an allowable limit of 12 μg of caffeine per mL of urine (Graham, 1983; Spriet, 1995). A caffeine dose in the range of 9 to 13 mg/kg approximately one hour prior to performance will reach the maximum allowable urinary concentration for competition (Graham, 2001). Caffeine consumption and urinary concentration is dependent on factors such as gender and body weight (Ellender & Linder, 2005). Therefore, consuming 6-8 cups of brewed coffee that contain approximately 100 mg per cup would result in the maximum allowable urinary concentration (Spriet, 1995; Ellender & Linder, 2005). According to The National Collegiate Athletic Association, urinary concentrations after competition that exceed 15 μg/mL are considered to be illegal (The National Collegiate Athletic Association, 2009). In addition, the World Anti-Doping Agency does not deem caffeine to be a banned substance (World Anti-Doping Agency, 2009), but has instead included it as part of the monitoring program (World Anti-Doping Agency, 2009), which serves to establish patterns of misuse in athletic competition.

Although there is no improvement on endurance performance with consumption of caffeine or theobromine according to this study, theobromine appeared to enhance performance during the later stages of the time-trial to a similar magnitude as caffeine (6 mg/kg BM), with the ingestion of caffeine resulting in a more rapid enhancement of running performance than theobromine. The present study suggests theobromine abuse may provide an ergogenic effect,

which propses a field of future research to examine whether an upper limit of use for theobromine should be proposed on WADA list.

7.2 Conclusions

Prevention and management of exercise-induced bronchoconstriction (EIB) is a key issue in athletes. Inhaled corticosteroids are the most effective drug for long-term control of asthma and EIB (Carlsen *et al*., 2008), whereas inhald β_2-agonists taken before exercise provide immediate protection only (Carlsen *et al*., 2008). There are numerous negative findings in respect to severity, control, ad recovery from EIB when β_2-agonists are used daily. Recovery from EIB after a standard dose of β_2-agonists might, in some cases, be slower and need additional therapy when having EIB (Anderson, 1998). The present thesis has demonstrated that there is no improvement in endurance performance following the inhalation of up to 1600 μg of salbutamol in non-asthmatic athletes in temperate or hot environments. Furthermore, there is no improvement in association football (soccer) performance following the inhalation of up to 1600 μg of salbutamol in non-asthmatic male and female players. This would suggest that the current WADA guidelines, which allows athletes to inhale up to 1600 μg in a 24-hour period is sufficient to avoid pharmaceutical induced performance enhancement. However, such high doses not only suggest poor management of asthma they also mean that an athlete may be at risk of contravening the WADA current urinary threshold. If urine concentration of salbutamol exceeds 1000 ng·mL^{-1} in a doping test it is considered an adverse analytical finding, *i.e.* doping, unless the athlete proves through a controlled pharmacokinetic study performed in a WADA accredited laboratory, that the abnormal result was a consequence of use of therapeutic doses, *i.e.*, maximum 1600 μg over 24 hours, of inhaled salbutamol.

Furthermore, 2% body mass loss and greater concomitant with acute inhalation of 1600 µg of salbutamol in a single dose may result in a urine concentration above the current WADA upper limit and decision limit leading to a positive test finding. Hydration status *per se* is a critical factor relation to doping control. The results of this study suggest that WADA consider the role of normalizing drug concentrations to urine specific gravity in an attempt to negate the impact of hydration status on doping control tests. The present finding relate to urine concentration was independent of gender or ethnic origin. Data from this thesis will assist WADA in the implementation of regulations on the use of inhaled short acting β_2-agonist and assist in the resolution of contested doping violations.

The results of this thesis suggested that the consumption of theobromine (6 mg/kg) enhances the performance of 3 km time-trial at later stages to a similar magnitude as caffeine (6 mg/kg), even though the ingestion of caffeine resulting in a more rapid enhancement of running performance than theobromine. This indicates that abuse of theobromine may provide enhancement of athletic performance, which proposes a field of future research to examine whether theobromine should be considered part of the WADA prohibited list.

7.3 Limitations

One of the limitations to this thesis is the variability in actual dose inhaled. Whilst the use of a chamber was aimed to reduce this limitation in study 1, it remains possible that some participants with low urine concentration inhaled lower doses of salbutamol. This could have an impact on examining the relationship between urine concentration and inhaled salbutamol.

In addition, future work should investigate whether there is a relationship between body weight and the urinary concentration of salbutamol. A lighter athlete may be at a greater risk of breaching the threshold when administering high doses compared to a heavier athlete. Such findings would have implications to the care athletes receive in the future.

Funding issues resulted in relatively small participant numbers in the present thesis. Whilst participant numbers are commensurate with the majority of previous studies, small numbers means the extrapolation of results to the wider athletic population should be viewed with caution. An increase in the number of participants may provide more robust findings. Both female and male participants were examined in study 2 of this thesis and whilst no significant gender differences were observed there may have been value in including gender comparisons in all studies.

The same situation is related to the issue of ethnicity in the present thesis, which may also have an impact on the findings. Limitations to recruitment location and region issues resulting in small pools of potential participants. Whilst the results from the present thesis suggest an absence of an inter-race difference care is warranted in extrapolating from the small participant numbers in the wider community. Furthermore, this thesis only examined Afro-Caribbean Blacks, South East Asians and European Caucasians in study 3. Future studies should examine a broader cross-section of race to ensure an accurate understanding of the impact of race on pharmacokinetics. Further study is required in a larger number of participants including a range of gender and ethnicity to ensure an avoidance of Type II error.

Furthermore, the participants were not elite endurance athletes. Elite athletes could not be included in this study as they would have been at risk of a doping violation. Therefore we cannot claim our results directly represent the effects of inhaled salbutamol or the consumption of caffeine/theobromine in elite endurance athletes.

7.4 Future directions

Future studies should aim to examine higher dosages of salbutamol to discover if a lowest influential dose exists, as well as repetitive inhalation of salbutamol with interval for the maximal dosage within a day to find out the influence on urine concentration. Furthermore, a larger number of the participants including a range of gender and ethnicity would improve our understanding of the role of salbutamol on endurance and team game performance. Furthermore, the impact of acute short-acting β_2 -agonist administration on strength performance and cognitive function remains an area for further investigation.

Future work should investigate whether there is a relationship between body weight and the urinary concentration of salbutamol. A lighter athlete may be at a greater risk of breaching the threshold when administering high doses compared to a heavier athlete. Such findings would have implications for the care of athletes and anti-doping. Furthermore, few studies have examined the impact of long-term use of short-acting β_2- agonists on performance and anti-doping.

Future research should expand on the findings of study 4 in a larger number of participants and establish whether a limit on theobromine use and upper urinary threshold should be proposed on WADA prohibition list, it also needs to focus on the effect of combination of caffeine and theobeomine on endurance performance, to find out the inter-relations between caffeine and theobromine.

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