Intra-oral Spray Technology

Research and scientific studies

BetterYou™

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Foreword

An increasing problem is our body's growing inability to absorb the nutrients from the food we eat and from traditional oral supplementation. Our modern diet, increased stress levels and an aging population means that our digestive efficiency is worsening with malabsorption issues rising dramatically.

A solution to these malabsorption issues is the ability to bypass the digestive system, delivering active nutrients directly into the bloodstream. Developed in conjunction with Dr. Charles Heard, a pharmacy research professor at Cardiff university, BetterYou has pioneered intra-oral spray technology. A highly effective delivery mechanism that utilises the rich-vein network of the oral cavity to deliver essential nutrients directly into the body.

This collection of research presents a growing body of evidence supporting the efficacy of intra-oral spray technology. The research, undertaken by universities and scientists from the UK and abroad, indicates that the method of delivering nutrients intra-orally, via a spray mechanism, not only offers an alternative to tablets or capsules, but provides a superior alternative. With greater absorption achieved through the buccal membrane in the mouth, than through digestive absorption.

BetterYou considers research and education to be the lifeblood of its business and continues to commit growing resources into this area. As research continues to be undertaken this document will be updated regularly to incorporate the latest published works.

Andrew Thomas,
Founder and Managing Director of ***BetterYou***

An Overview on: Sublingual Route for Systemic Drug Delivery

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Abstract

Oral mucosal drug delivery is an alternative and promising method of systemic drug delivery which offers several advantages. Sublingual literally meaning "under the tongue", administrating substance via mouth in such a way that the substance is rapidly absorbed via blood vessels under tongue. Sublingual route offers advantages such as bypasses hepatic first pass metabolic process which gives better bioavailability, rapid onset of action, patient compliance, self-medicated. Dysphagia (difficulty in swallowing) is common among all ages of people and more in pediatric, geriatric, psychiatric patients. In terms of permeability, sublingual area of oral cavity is more permeable than buccal area which in turn is more permeable than palatal area. Different techniques are used to formulate the sublingual dosage forms. Sublingual drug administration is applied in field of cardiovascular drugs, steroids, enzymes and some barbiturates. This review highlights advantages, disadvantages, different sublingual formulation such as tablets and films, evaluation.

Introduction

Drugs have been applied to the mucosa for topical application for many years. However, recently there has been interest in exploiting the oral cavity as a portal for delivering drugs to the systemic circulation. Notwithstanding the relatively poor permeability characteristics of the epithelium, a number are offered by this route of administration. Foremost among these are the avoidance of first-pass metabolism, ease of access to the delivery site, and the opportunity of sustained drug delivery predominantly via the buccal tissues.

Oral mucosal drug delivery is an alternative method of systemic drug delivery that offers several advantages over both injectable and enteral methods. Because the oral mucosa is highly vascularised, drugs that are absorbed through the oral mucosa directly enter the systemic circulation, by passing the gastrointestinal tract and first-pass metabolism in the liver. For some drugs, this results in rapid onset of action via a more comfortable and convenient delivery route than the intravenous route. Not all drugs, however, can be administered through the oral mucosa because of the characteristics of the oral mucosa and the physicochemical properties of the drug[1].

The oral route of administration is considered as the most widely accepted route. The unique environment of the oral cavity offers its potential as a site for drug delivery. Because rich blood supply and direct access to systemic circulation, the oral mucosal route is suitable for drugs, which are susceptible to acid hydrolysis in the stomach or which are extensively metabolized in the liver. The continuous secretion of saliva results in rapid removal of released drug and this may desire that the oral cavity be restricted to the delivery of drugs, which have a short systemic circulation. The mucin film, which exists on the surface of the oral mucosa may provide an opportunity to retain a drug delivery system in contact with the mucosa for prolonged periods if it is designed to be mucoadhesive. Such system ensures a close contact with absorbing membrane, thus optimizing the drug concentration gradient across the biological membrane and reducing the differential pathway. The oral mucosa may be a potential site for controlled or sustained drug delivery. Oral route is most preferred route by medical practitioners and manufacturer due to highest acceptability of patients. About 60% of all dosage forms available are the oral solid dosage form. The lower bioavailability, long onset time and dysphagia patients turned the manufacturer to the parenterals and liquid orals. But the liquid orals (syrup, suspension, emulsion etc) have the problem of accurate dosing mainly and parenterals are painful drug delivery, so most patient incompliance[2]. The target sites for local drug delivery in the oral cavity include the following: Buccal, Sublingual, Periodontal region, Tongue, Gum. Other desirable targeting sites adjacent to oral cavity include pharynx, larynx, adenoids and tonsils. Within the oral cavity, delivery of drugs via the membranes of the oral cavity is classified into three categories:

i. **Sublingual delivery**
   which is systemic delivery of drugs through the mucosal membranes lining the floor of the mouth to the systemic circulation;

ii. **Buccal delivery**
   which is drug administration through the mucosal membranes lining the cheeks and the area between the gums and upper and lower lips to the systemic circulation.

iii. **Local delivery**
   which is drug delivery to periodontal, gingival, delivery for the local treatment of ulcers, bacterial and fungal infections and periodontal disease. Sublingual administration of the drug means placement of the drug under the tongue and drug reaches directly into the blood stream through ventral surface of the tongue and floor of the mouth. The drug solutes are rapidly absorbed into the reticulated vein which lies underneath the oral mucosa, and transported through the facial veins, internal jugular vein, and braciocephalic vein and then drained in to systemic circulation.[3]

The sublingual route usually produces a faster onset of action than orally ingested tablets and the portion absorbed through the sublingual blood vessels bypasses the hepatic first-pass metabolic processes[4-6]. The main mechanism for the absorption of the drug in to oral mucosa is via passive diffusion into the lipoidal membrane.

The absorption of the drug through the sublingual route is 3 to 10 times greater than oral route and is only surpassed by hypodermic injection. For these formulations, the small volume of saliva is usually sufficient to result in tablet disintegration in the oral cavity. Sublingual absorption is mostly rapid in action, but also short acting in duration. Nitroglycerine, for example, is an effective antianginal drug but is extensively metabolised when taken orally (>90%). It is rapidly absorbed through the sublingual mucosa, and its peak plasma level is reached within 1-2min. Because of its short biological half life (3-5min.), however the blood concentration of nitroglycerine declines rapidly to a level below the therapeutic concentration within 10-15min. In terms of permeability, the sublingual area of the oral cavity is more permeable than the buccal (cheek) area, which in turn is more permeable than the palatal (roof of the mouth) area. The differences in permeability are generally based on the relative thickness, the blood supply, and degree of keratinisation of these membranes. In addition to the differences in the permeability of the various mucous membranes, the extent of drug delivery is also affected by the physicochemical properties of the drug to be delivered[7].

Sublingual products have been developed for numerous indications ranging from migraines (for which rapid onset of action is important) to mental illness (for which patient compliance is important for treating chronic indications such as depression and schizophrenia.)[8]

Advantages

- Ease of administration to patients who refuse to swallow a tablet, such as pediatric, geriatric patients and psychiatric patients.
- Convenience in administration of drug and accurate dosing as compared to liquid formulations.
- Water is not required for swallowing the dosage form, which is convenient feature for patients who are traveling and do not have immediate access to water.
- Good mouth feels property helps to change the basic view of medication as “bitter pill”, particularly for pediatric patients.
- Fast dissolution of medicament and absorption which will leads to rapid, onset of action.
- Some drugs are absorbed from the mouth pharynx and esophagus as the saliva passes down into the stomach, in such cases bioavailability of drugs is increased.
- It provides advantages of liquid formulations in the form of solid dosage form.
- Pregastric absorption can result in improved bioavailability and as a result of reduced dosage, improved clinical performance through a reduction of unwanted effects.

Disadvantages

- Since sublingual administration of drugs interferes with eating, drinking, and talking, this route is generally considered unsuitable for prolonged administration.
- Although this site is not well suited to sustained-delivery systems.
- Sublingual medication cannot be used when a patient is uncooperative or unconscious.
- The patient should not smoke while taking sublingual medication, because smoking causes vasoconstriction of the blood vessels. This will decrease the absorption of the medication.

Sublingual glands

Salivary glands which are present in the floor of the mouth underneath the tongue. They are also known as sublingual glands. They produce mucin in turn produces saliva. The interior area of the mouth remains lubricated due to production of the saliva by the glands, which is necessary for chewing and food swallowing. The fluid which is produced by the glands gets mixed with the food, so the food gets easily chewed. Due to low secretion of the saliva it can create problem in swallowing the food and potential for food lodge in the throat increases. The absorption is transfer of the drug from its site of administration into systemic circulation, so it can be said that absorption is directly proportional layer thickness. The absorption of the drug follows in this way Sublingual > Buccal > Gingival > Palatal. Due to high permeability and rich blood supply, the sublingual route can produce rapid onset of action so the drug with short delivery period can be delivered and dose regimen is frequent. The drug gets diluted in the saliva and from there the drug is adsorbed across the oral cavity.

For example: Glyceryl nitrate-a potent coronary vasodilator which is used for rapid symptomatic relief of angina. After administration its gets pharmacologically active after 1-2minutes. Oral spray was found to provide rapid relief of symptom with first class metabolism. The extent of first class metabolism when compared to the sublingual spray decreased to 48% with sublingual tablets and 28% with the oral dose. Nitrate, which appears in the plasma concentration, can be maintained for 24 hours when administrated sublingually[9].

The Mechanism of Sublingual Absorption

The absorption potential of the buccal mucosa is influenced by the lipid solubility and therefore the permeability of the solution (osmosis), the ionization (pH), and the molecular weight of the substances. For example, absorption of some drugs via the buccal mucosa is shown to increase when carrier pH is lowering (more acidic) and decrease with a lowering of pH (more alkaline). The cells of the oral epithelium and epidermis are also capable of absorbing by endocytosis (the uptake of particles by a cell as if by hollowly wrapping itself around it. These engulfed particles are usually too large to diffuse through its wall). It is unlikely that this mechanism is used across the entire stratified epithelium. It is also unlikely that active transport processes operate within the oral mucosa. However, it is believed that acidic stimulation and uptake into the circulatory system.

The mouth is lined with a mucous membrane which is covered with squamous epithelium and contains mucous glands. The buccal mucosa is similar to the sublingual mucosal tissue.

The salivary glands consist of lobules of cells which secrete saliva through the salivary ducts into the mouth. The three pairs of salivary glands are the Parotid, the Sub mandibular and the Sublingual which lies on the floor of the mouth. The more acid the taste the greater the stimulation of salivary output, serving also to avoid potential harm to acid sensitive tooth enamel by bathing the mouth in copious neutralizing fluid. With stimulation of salivary secretion oxygen is consumed and vasodilator substances are produced, and the glandular blood flow increases, due to increased glandular metabolism.

The sublingual artery travels forward to the sublingual gland, it supplies the gland and branches to the neighbouring muscles and to the mucous membranes of the mouth, tongue and gums. Two symmetrical branches travel behind the jaw bone under the tongue to meet and join at its tip. Another branches meets and anastomoses with the sub mental branches of the facial artery. The sublingual artery system stems from the lingual artery – the body's main blood supply to the tongue and the floor of the mouth – which arises from the external carotid artery. The proximity with the internal carotid artery allows fast access to its route supplying the greater part of the cerebral hemisphere.

Drugs for sublingual administration

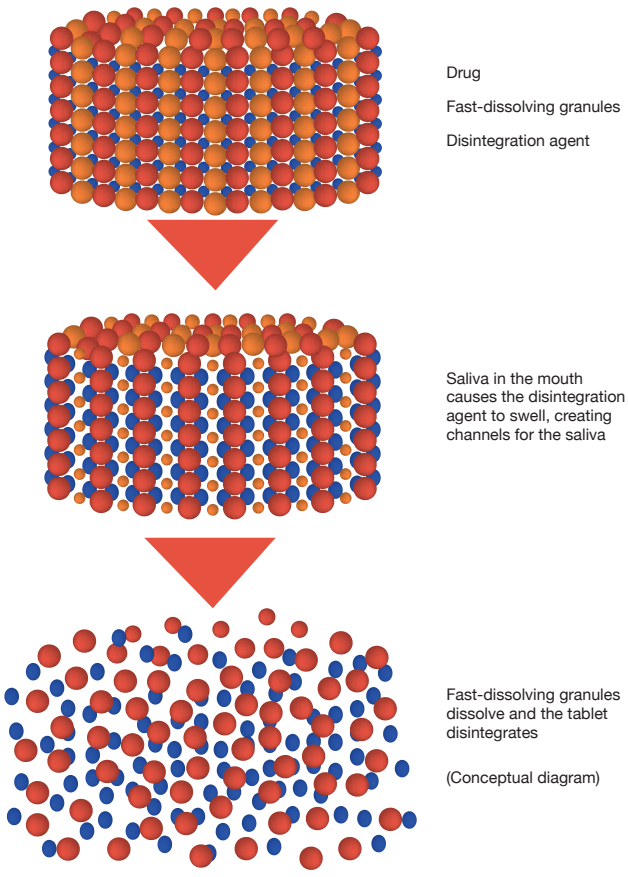
Sublingual drug administration is applied in the field of cardiovascular drugs, steroids, some barbiturates and enzymes. It has been a developing field in the administration of many vitamins and minerals which are found to be readily and thoroughly absorbed by this method. Sublingually absorbed nutrition, which avoids exposure to the gastric system and liver, means direct nutritional benefits, particularly important for sufferers of gastro-intestinal difficulties such as ulcers, hyperactive gut, coeliac disease, those with compromised digestion, the elderly and invalids – the nutritional benefit is independent of gastro-intestinal influences [10,11]. Examples of drugs administered by this route include antianginal like nitrites and nitrates, anti hypertensive like nifedipine, analgesics like morphine and bronchodilators like fenoterol. Certain steroids like estradiol and peptides like oxytocin can also be administered e.g. fentanyl citrate, apomorphine, prochlorperazine dimaleate {PRO}, and hydrazine HCl {HYD}.

Sublingual formulation Sublingual tablets

They are to be placed under the tongue and produce immediate systemic effect by enabling the drug absorbed directly through mucosal lining of the mouth beneath the tongue. The drug absorbed from stomach goes to mesenteric circulation which connects to stomach via portal vein. Thus absorption through oral cavity avoids first pass metabolism. The tablets are usually small and flat, compressed lightly to keep them soft. The tablet must dissolve quickly allowing the API to be absorbed quickly. It is designed to dissolve in small quantity of saliva. After the tablet is placed in the mouth below the tongue, the patient should avoid eating, drinking, smoking and possibly talking in order to keep the tablet in place. Swallowing of saliva should also be avoided since the saliva may contain dissolved drug. Bland excipients are used to avoid salivary stimulation. Various techniques can be used to formulate rapidly disintegrating or dissolving tablets.[12,13] Direct compression is one of these techniques which require incorporation of a superdisintegrant into the formulation, or the use of highly water-soluble excipients to achieve fast tablet disintegration. Direct compression does not require the use of water or heat during the formulation procedure and is the ideal method for moisture and heat-labile medications.

a. Fast disintegrating sublingual tablets (FDT)

FDT is defined as a solid dosage form that contains medicinal substances and disintegrates rapidly (within few seconds) without water when kept on the tongue. The drug is released, dissolved, or dispersed in the saliva, and then swallowed and absorbed across the GIT [14]. FDTs also are also called as Orodispersible tablet, mouth-dissolving, quick-dissolving, fast-melt, and freeze-dried wafers. Tablets that disintegrate or dissolve rapidly in the patient's mouth are convenient for young children, the elderly and patients with swallowing difficulties and in situations where potable liquids are not available. Direct compression is one of the techniques which require the incorporation of a superdisintegrant into the formulation, or the use of highly water soluble excipients to achieve fast tablet disintegration. Compared to conventional dosage form the drug dissolution, its absorption as well as onset of clinical action and its bioavailability may be significantly greater [15-17]. Though chewable tablets are available in the market, they are not same as the new FDTs. Patients for whom chewing is difficult or painful can use these FDTs. It can be used easily in infants and in children who have lost their primary teeth and who do not have full use of their permanent teeth [18].



Recent market studies indicate that more than half of the patients prefers FDTs than other conventional dosage forms [19] and most patients would ask their doctors for FDTs (70%), purchase FDTs (70%), or prefer FDTs than regular tablets or liquids (>80%)[20]. The US Food and Drug Administration Center for Drug Evaluation and Research (CDER) defines, in the, Orange Book, an FDT as "a solid dosage form containing medicinal substances, which disintegrates rapidly in saliva, usually within a few seconds, when placed upon the tongue"[21]. The implication of these dosage forms is emphasized by the term "Orodispersible Tablet", by the European Pharmacopoeia which defines it as a tablet that can be placed in oral cavity where it disperses rapidly before swallowing[22]. FDTs has been developed for numerous indications ranging from migraines (in which quick onset of action is necessary) to mental illness (in which patient compliance is necessary for treating chronic indications such as mental depression and schizophrenia)[23].

b. Bioadhesive sublingual tablets

The new sublingual tablet concept presented is based on interactive mixtures consisting of a water soluble carrier covered with fine drug particles and a bioadhesive component. With this approach, it is possible to maintain rapid dissolution in combination with bioadhesive retention of the drug in the oral cavity. Bioadhesion is usually defined as the bond formed between two biological surfaces or between a biological and a synthetic surface. Problem associated with sublingual tablet formulation is that there is always a risk that the patient will swallow part of the dose before the active substance has been released and absorbed locally into systemic circulation. This could result an unwanted prolongation of the pharmacological effect. Addition of a bioadhesive component is a well-known method of increasing the possibility of a more site-specific release. However, this concept is normally applied to non-disintegrating tablets or disc to achieve extended release of the active substance and, consequently, such a system will not be suitable for a fast acting formulation. Therefore, it would be of interest to study a disintegrating tablet which releases the drug quickly, but which also has bioadhesive properties which could prevent the drug from being swallowed.

Bioadhesion mechanisms

The mucus layer is often involved in the adhesion of a bioadhesive polymer and is present as either a gel layer adhering to the mucosal surface or a solution or suspension of various substances. The mucus layer mainly consists of mucin glycoprotein, inorganic salts, proteins, lipids and water with the composition varying depending on its source. The electronic theory involves an electronic transfer between the two materials causing a double layer of electric charge, which results in attraction forces. The adsorption theory involves adhesion between the mucosa and the adhesive material by van der waals interaction, hydrogen bonds and related forces. The wetting theory involves interfacial tensions between the two materials. Penetration of the polymer chains into the mucus network and vice versa, causing a mechanical bond, is referred to as the diffusion theory. The importance of water content and movement of water into the adhesive material from the mucosa, i.e. dehydration of the mucosa, has also been suggested as a mechanical for adhesion.

Measurement of bio-adhesive strength

Bio-adhesion strength of the tablets was measured on a modified physical balance. The method used bovine cheek pouch as the method mucosal and IPB ph 6.6 as the moistening fluid. The surface of the mucosal membrane was first blotted with a filter paper and then moistened with 25/L 1 of IPB pH 6.6. the weight in grams is required to detach the tablets from the mucosal surface gave the measure of bio-adhesive strength.

c. Lipid matrix sublingual tablets

Such tablets are formulated using advances in sublingual and liposomal technology to create a dosage form that offers a faster and more complete absorption than traditional oral routes of administration. The lipid matrix sublingual tablet is a bioavailable, quick, convenient and consistent dosage form for many neutraceuticals that are often taken orally. For e.g. Glutathione MB12(methylcobalamin) melatonin.

d. Sublingual vitamin tablet

Vitamin D i.e. cholecalciferol is a natural precursor of calcium regulating hormone calcitriol. Vitamin D is thus used in hypocalcaemia/hyperparathyroidism. Because of its incomplete absorption from GI tract, local intestinal degradation and hepatic metabolism, it is given sublingually.

2. Thin film drug delivery

Thin film drug delivery is a process of delivering drugs of the systemic circulation via thin film that dissolves when in contact with liquid, often referred to a dissolving films or strips and dissolve within 1min when placed in the mouth without drinking or chewing.

Such dissolving film or strip are typically designed for oral administration, with the user placing the strip on or under the tongue or along the inside of the cheek. Thin film's ability to dissolve rapidly without the need for water provides an alternative to patients with swallowing disorders and to patients suffering from nausea, such as those patients receiving chemotherapy.

The first developed fast-dissolving dosage form consisted in tablet form, and the rapid disintegrating properties were obtained through a special process or formulation modifications[24]. More recently, fast-dissolving films are gaining interest as an alternative to fast-dissolving tablets to definitely eliminate patients' fear of chocking and overcome patent impediments. Fast-dissolving films are generally constituted of plasticized hydrocolloids. Problems are caused by foaming during the film formation due to the heating of the material or solvent evaporation, the flaking during the slitting and the cracking in the cutting phase. The films should be stable to moisture, facilitate the handling, have to be flexible and exhibit a suitable tensile stress and do not stick to the packaging materials and fingers. Film can be prepared by five methods:

1. Solvent casting.
2. Semisolid casting.
3. Hot melt extrusion.
4. Solid dispersion extrusion.
5. Rolling.

1. Solvent casting method

Film is formulated using the solvent casting method, whereby the water-soluble ingredients are dissolved to form a clear viscous solution. The API and other agents are dissolved in smaller amounts of the solution and combined with the bulk. This mixture is then added to the aqueous viscous solution. The entrapped air is removed by vacuum. The resulting solution is cast as a film and allowed to dry, which is then cut into pieces of the desired size.[25]

2. Semisolid casting

Solution of water soluble film forming polymer is mixed with solution of acid insoluble polymer which forms homogenous viscous solution. The ratio should be 1:4. For e.g. cellulose acetate phthalate, cellulose acetate butyrate. It is then sonicated which is coated on non-treated casting film.

3. Hot melt extrusion

In present method the mass is prepared first under the control of temperature and steering speed. Afterwards, the film is coated and dried in a drying tunnel, once again the temperature, air circulation and line speed are controlled. Then follows a slitting and in the last step the films are punched, pouched and sealed.[26]

4. Solid dispersion extrusion

Solid dispersions are prepared by immiscible components and drug. Finally the solid dispersions are shaped in to films by means of dies.

5. Rolling

Solution or suspension drug is rolled on the carrier. The solvent is mainly water and mixture of water and alcohol. The film is dried on the rollers and gives desired shape and size[27].

Evaluation

Hardness and thickness

The test is done as per the standard methods. The hardness of three randomly selected tablets from each formulation is determined by placing each tablet diagonally between the two plungers of tablet hardness tester (with the nozzle) and applying pressure until the tablet broke down into two parts completely and the reading on the scale is noted down[28].The thickness of three randomly selected tablets from each formulation is determined in mm using a vernier caliper (Pico India). The average values is calculated[28].

Drug Content

Randomly ten tablets are selected from formulation, finely powdered and powder equivalent mg of drug is accurately weighed and transferred to 100ml volumetric flasks containing solution of desired pH. The flask is shaken to mix the contents thoroughly. The volume is made up to the mark with solution and filtered. One ml of the filtrate is suitably diluted and drug content is estimated using a double beam UV-visible spectrophotometer. This procedure is repeated thrice and the average value is calculated.

Wetting time (WT)

It is useful for quality control and provides supportive evaluation of these sublingual tablets. Unlike the disintegration test, the wetting test uses minimal water, which may be more representative of the quantity of moisture available sublingually. Using this test, the time required for moisture to penetrate the tablet completely is measured and possibly represents the time required to release drug in the presence of minute volumes of saliva The tablet was placed above absorbent paper fitted into a petri dish. After the paper is thoroughly wetted with distilled water, excess water is completely drained out of the dish. The time required for the water to diffuse from the wetted absorbent paper throughout the entire tablet is then recorded using a stopwatch[28].

Disintegration test

A relatively simple method with rigorous conditions is developed. Each individual tablet is dropped into 10ml glass test tube (1.5cm diameter) containing 2ml distilled water, and the time required for complete tablet disintegration is observed visually and recorded using a stopwatch. The visual inspection is enhanced by gently rotating the test tube at a 45° angle, without agitation, to distribute any tablet particles that might mask any remaining undisintegrated portion of the tablets. In the USP disintegration test for sublingual tablets, the disintegration apparatus for oral tablets is used without the covering plastic disks,[29] and 2 minutes is specified as the acceptable time limit for tablet disintegration[29].

Water absorption ratio

A piece of tissue paper folded twice is placed in a small Petri dish Containing 6ml of water. A tablet is put on the tissue paper and allowed to completely wet. The wetted tablet is then weighted. Water absorption ratio, R was determined using following equation[28].

R = 100 × Wa –Wb/Wa

where, Wa = Weight of tablet after water absorption
Wb = Weight of tablet before water absorption.

In vitro disintegrating test

Disintegration times for sublingual tablets is determined using USP tablet disintegration apparatus with desired medium. The volume of medium was 900ml and temp was 37± 2°C. The time in seconds taken for complete disintegration of the tablets with no palatable mass remaining in the apparatus is measured[28].

In vitro dissolution test

In-vitro release rate of sublingual tablets will be carried out using United State Pharmacopoeia (USP) XXIV dissolution testing apparatus (Paddle method). A aliquot sample of the solution is withdrawn from the dissolution apparatus. The samples are replaced with fresh dissolution medium of same quantity. The samples are filtered through Whatman filter paper No 40 and analysed in UV spectrophotometer. The percentage drug release is calculated using an equation obtained from the calibration curve[30].

Test for film

Tensile Strength

Tensile strength is the maximum stress applied to a point at which the film specimen breaks[31]. It is calculated by the applied load at rupture divided by the cross-sectional area of the film as given below:

$$\textbf{Tensile strength} = \frac{\textbf{Load at failure} \times 100}{\textbf{Film thickness} \times \textbf{film width}}$$

Percent Elongation

A film sample stretches when stress is applied and it is referred to as strain. Strain is basically the deformation of film divided by original dimension of the sample. Elongation of film increases as the plasticiser content increases.

Percent elongation

$$\frac{-L * 100}{Lo}$$

where,

L = Increase in length of film
Lo = Initial length of film.

Young's Modulus

Young's modulus or elastic modulus is the measure of stiffness of film. It is represented as the ratio of applied stress over strain in the region of elastic deformation as follows:

$$\textbf{Young's Modulus} = \frac{\textbf{Slope} * 100}{\textbf{Film thickness} * \textbf{Cross-head speed}}$$

Folding Endurance

Folding endurance is determined by drying process repeated folding of the film at the same place till the breaks. The number of times the film is folded without dry breaking is computed as the folding endurance value[32].

Superdisintegrant	Commercially available	Mechanism of action	Special comment
Cross linked Cellulose	Crosscarmellose® Ac-Di-Sol®, Nymce ZSX® Primellose®, Solutab®, Vivasol®, L-HPC	Swells 4-8 folds in <10 seconds. Swelling and wicking both	Swells in two dimensions. Direct compression or Granulation Starch free.
Cross linked PVP	Crosspovidone M® Kollidon® Polyplasdone®	Swells very little and returns to original size after compression but act by capillary action	Water insoluble and spongy in nature so get porous tablet
Crosslinked starch	Explotab® Primogel®	Swells 7-12 folds in <30 seconds.	Swells in three dimensions and high level serve as sustain release matrix
Crosslinked alginic Acid	Alginic acid NF	Rapid swelling in aqueous medium or wicking action	Promote disintegration in both dry or wet granulation.

Table 1.

Brand name	Category	Strength
Abstral Fentanyl Citrate	Opioid Analgesic	50, 100, 200, 300, 400, 600, 800µg
Subutex Buprenorphine	Opioid Analgesic	2 and 8mg
Avitan Lorazepam	Antianxiety	1, 2mg
Edular Zolpidem tartrate	Sedatives/ Hypnotics	5, 10mg
Isordil Isosorbide dinitrate	Vasodilators	2.5, 5 10mg
Suboxone Buprenorphine hydrochloride	Narcotic + Opioid antagonist	2/0.5, 8/2mg
Nitrostat Nitroglycerine	Antianginal	0.3mg, 0.4mg, or 0.6mg

Table 2. *Marketed Products of Sublingual Tablet*

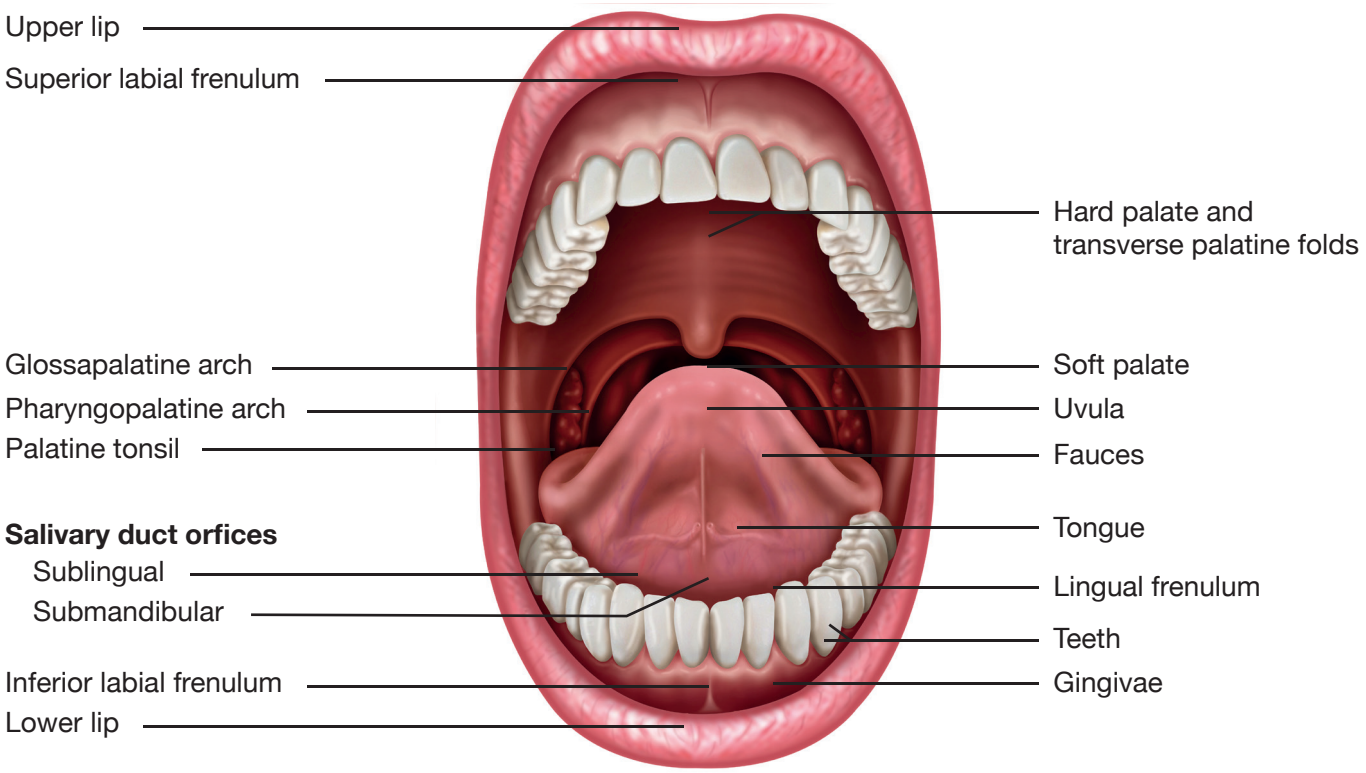


Figure 1 *Human mouth anatomy*

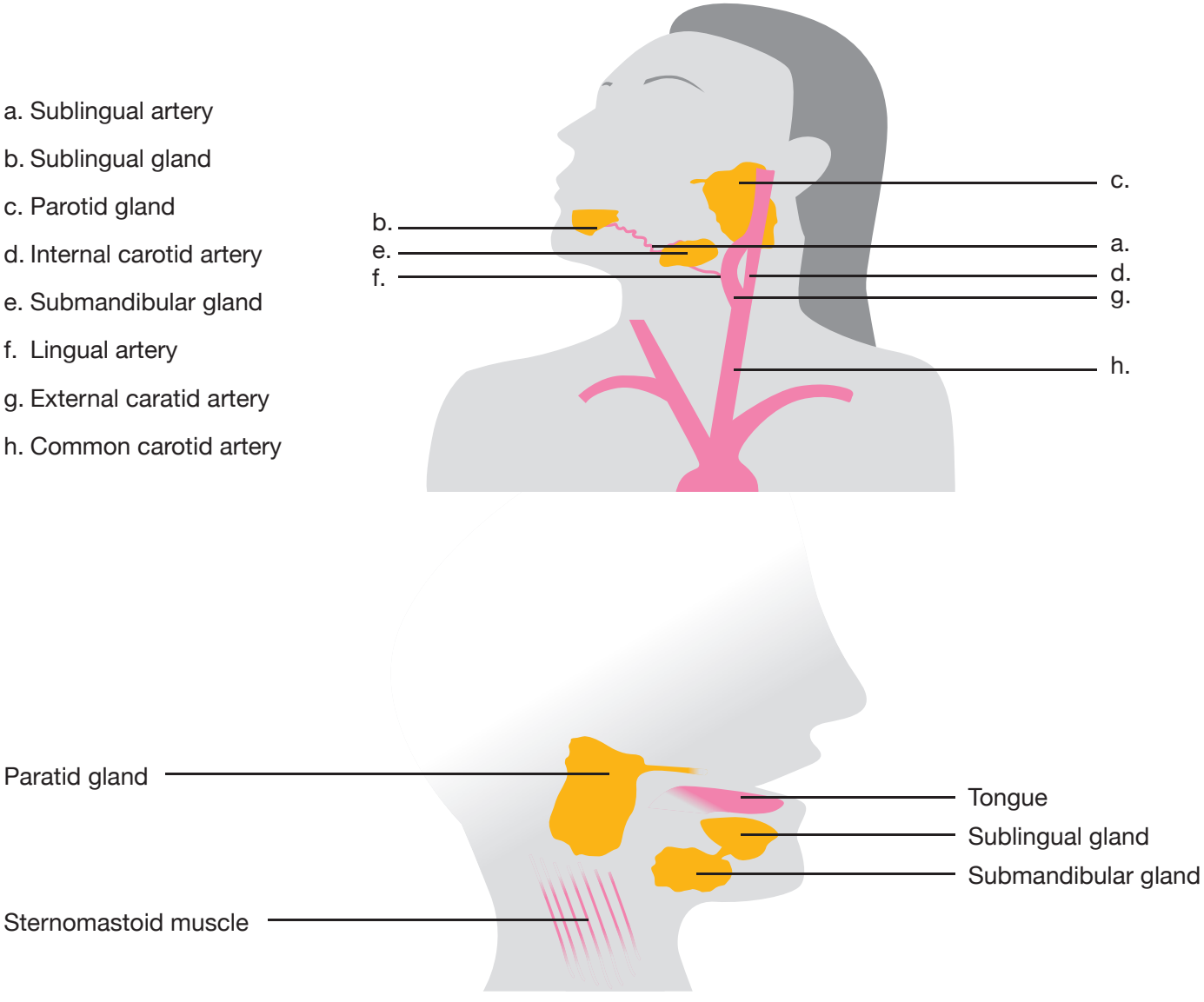


Figure 2: *Diagram of sublingual gland and sublingual artery*

Fig 3: *Thin Film Drug Delivery*

Thickness

The thickness of the polymer films was measured by using screw gauge. The thickness of each strip at six different areas was determined and standard deviation was calculated. [33]

In vitro disintegration time

In vitro disintegration time is determined visually in a glass dish of 25ml distilled water with swirling every 10sec. The disintegration time is the time when the film starts to break or disintegrates. The disintegration time of prepared films was measured in triplicate[34].

Uniformity of drug content

The film of area 1x1cm2 was cut and dissolved in 6.8 phosphate buffer solution and made up to 100mL in a volumetric flask. Then 1mL was withdrawn from the solution and diluted to 10mL. The absorbance of the solution was taken at 276nm and concentration was calculated. By correcting dilution factor, the drug content was calculated. The test was performed in triplicate[35].

In-vitro dissolution studies

Dissolution study was carried out in USP paddle type apparatus using 300mL of stimulated salivary fluid (pH 6.8) as a dissolution medium at 50rpm. Temperature of the dissolution medium was maintained at 37±0.5ºC. Samples of 5ml were withdrawn at every 4 minute interval, filtered (through 0.45µ) and replaced with 5ml of fresh dissolution medium. The samples were suitably diluted and estimated spectrophotometrically at 276nm by using ELICO-164 double beam UV-Visible spectrophotometer. The dissolution experiments were conducted in triplicate. Dissolution rate was studied for all designed formulations and dissolution parameters were calculated.

Conclusion

Sublingual drug delivery have been used for formulation of many drugs with view point of rapid drug release and quick onset of action. Sublingual products were developed to overcome the difficulty in swallowing conventional tablet, among pediatric, geriatric and psychiatric patients with dysphagia. The target population has expanded to those who want convenient dosing without water anywhere, anytime. The potential for such dosage forms is promising because strong market acceptance and patient demand. Peak blood levels of most products administered sublingually are achieved within 10-15 minutes, which is generally much faster than when those same drugs are ingested orally. Sublingual absorption is efficient. The percent of each dose absorbed is generally higher than that achieved by means of oral ingestion. Various types of sublingual dosage forms are available in market like tablets, films and sprays.

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Analysis of the oral delivery of vitamin D3 from BetterYou DLux spray formulations

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Abstract

This study tested the efficacy of the delivery of Vitamin D3 in vitro through the tissues of the buccal cavity from an oral spray.

"Vitamin D3 can be effectively delivered through the tissues of the buccal cavity from an oral spray"

1. Evaluation of the permeation of VD3 from commercially available preparations and compare that to laboratory made formulations through the buccal cavity – sublingual, buccal and soft palate membranes.
2. To estimate the total VD3 delivery to the system across these membranes.

Introduction

1.1 Overview

Sublingual drug delivery utilises the permeability of the mucosal membrane located on the ventral side of the tongue. The sublingual membrane is a preventative barrier for the permeation of many compounds into systemic circulation. The membrane therefore is a difficult route to utilise for the delivery of drugs. However in comparison to other delivery routes this pathway provides several advantages, as discussed later.

Vitamins are essential nutrients that humans require to sustain life. Each vitamin has a specific and vital role in the body and can be obtained from a variety of sources (food, drinks, sunlight and supplementation). There uptake and utilisation is intricate and relies upon a delicate balance of overall nutrition.

There are two main classes of vitamins:

1. Fats soluble vitamins; these can be obtained from fatty foods, they are stored in the liver and fatty tissues and therefore do not require a daily intake. The vitamins A, D (D1 and D2), E and K are included in this category.
2. Water soluble vitamins; these vitamins, not stored in the body, require daily intake. These are mainly acquired through the consumption of fruits, vegetables and grains. Included in this category are the B range of vitamins, vitamin C and folic acid.

Some vitamins although obtained from the diet can also be obtained through non-dietary sources e.g. Vitamins such as biotin and Vitamin K are naturally synthesised in the gut, Vitamin D3 (VD3) is mainly obtained via sunlight.

The progression of a westernised culture and poor diet regimes, has led to vitamin supplementation playing a vital role in maintaining the required levels nutrient uptake.

This research probed the efficacy of the sublingual VD3 supplementation from an oral spray via the in-vitro permeation of VD3 through the excised sublingual membranes; the efficacy of VD3 across the buccal and soft palate membranes is also included.

1.2 Vitamin D3 (VD 3)

Vitamin D is classed as a fat soluble vitamin that is naturally found in a few foods. There are two types- Vitamin D2 known as Ergocalciferol and Vitamin D3 known as Cholecalciferol. Both are produced from pre-vitamins.

VD3 is mainly synthesized in the skin through a photochemical reaction of ultra-violet rays from the sun. VD3, as absorbed naturally, is metabolised within the liver and kidneys to the active form Calcitriol (Institute of Medicine. National Academy Press, 2010.) as shown in Figure 1. In its metabolised form it plays an important role in the homeostatic control of calcium and phosphate; this is important in the development of bones, neuromuscular (Dhesi et al. 2004) and immune functionality and modulating skeletal cell proliferation.

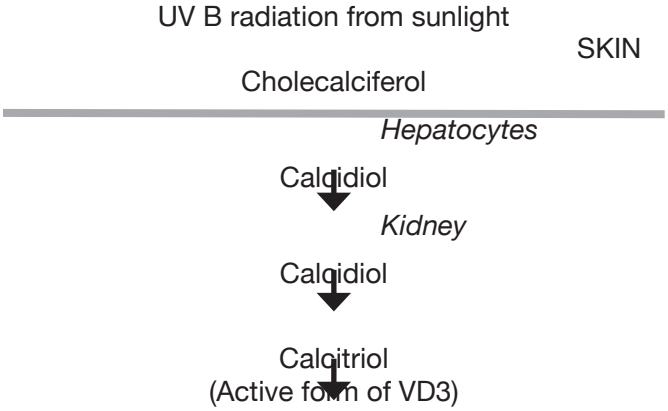

Figure 1: *Conversion pathway of the formation of the active form of VD 3.*

VD 3 supplementation is common in the elderly and pregnant. The recommended daily allowance varies between age groups, race and state of health.

1.3 Sublingual Drug Delivery

Sublingual drug delivery is defined as the permeation of a drug through the sublingual mucosal membranes, which cover the ventral side of the tongue and the soft palate; these membranes are part of the non-keratinised epithelia found in the oral cavity.

The total surface area of the oral cavity has been found to be approximately 214.7cm2 ± 12.9cm2. Of this surface area, 30% is found to be non-keratinised epithelia, involved in sublingual and buccal delivery. Non-keratinised epithelia line: the inner part of the cheeks (inside the mouth), the ventral part of the tongue and the soft palate (Collins and Dawes 1987).

An approximation of the non-keratinised membranes is:

(214 x 30) / 100 = 62.2 cm^2

60% of this surface area is represented by the sublingual membranes (the soft lower palate and the ventral side of the tongue) (Wilson 2005; Chen et al. 1999).

(62.2 x 60) / 100 = 37.32 cm^2

Of this, 13 cm2 makes up the ventral surface of the tongue (Ong and Heard 2009), with the rest making up the floor and soft palate. Therefore the rest of the surface area, 24.32 cm^2 makes up the floor and soft palate of the mouth.

Permeation is easily affected by substances such as alcohols and therefore permeability is classed as selective. This is a limiting factor in the selection of excipients for sublingual formulations.

The permeation of lipophilic compounds is greatly hindered by mucosal membranes, including the non-keratinised membranes, and therefore permeation enhancers are required. VD3 is a highly lipophilic

compound, and the excipients found in oral formulations are to aid permeation, solubility, taste and appearance. Formulations commonly known to be used in sublingual delivery are sprays, the most popular one being the Glyceryl Trinitrate spray (GTN) used to provide relief in angina. (BNF62).

As part of the oral cavity, these membranes are exposed to an abundant supply of saliva which is constantly secreted and continuously flushes the cavity. Continual movement of the tongue, speech and salivary secretions coupled with the swallowing reflex lead to a limited time period of application. Aspects of delivery such as particle size and the physico-chemical nature of a formulation (eg combinations of permeation enhancers (Sudhakar et al. 2006) and mucoadhesives etc) greatly affect the flux of a compound.

1.4 Buccal Delivery

Buccal delivery involves the membranes that line the inner cheek, inside the upper and lower lips in the oral cavity. It forms approximately 40% of the non-keratinised epithelia found in the oral cavity (Wilson 2005). This can be calculated from the overall surface area:

(40 x 62.2) / 100 = 24.88 cm²

The oral mucosal membrane, comprising of the buccal and sublingual membranes, varies in thickness and permeability. The buccal membrane is thicker, approximately 580μm (in comparison to the sublingual membrane which is approximately 190μm) and is generally less permeable (Squier and Wertz 1996) (Squier and Hall 1985b; Lesch et al. 1989).

This route of delivery is common for muco-adhesive formulations, enabling a longer application time. Similar to sublingual delivery, buccal delivery is affected by salivary secretion and mucus turnover. However, the increased application time in this area is due to the lower susceptibility of tongue movement.

1.5 Advantages of Sublingual and Buccal Delivery

Application of drugs onto the sublingual and buccal membranes have proven to be an easy alternative to those individuals who are incapable of ingesting formulations (i.e. patients who are nil-by-mouth, experiencing episodes of nausea and vomiting) or those that do not like or have difficulty taking tablets or liquid formulations (Narang et al. 2011). This route is non-invasive and is not as intimidating as injectable or rectal and vaginal routes.

The membranes are surrounded by a good vasculature which provides easy access into the systemic circulation bypassing the gastro-intestinal (system); this avoids any lag time of drug activation which is often experienced when dosing orally. The effects of drugs administered through theses membranes are therefore a lot more rapid and are not dependent on factors that commonly affect oral routes (stability of drugs in G.I fluid).

These areas are easily accessible for application and can be ideal for sustaining prolonged delivery. In case of any unwanted effects, the dosage form can be easily removed restricting delivery almost immediately.

Sublingual sprays offer a faster onset action in comparison to tablet which would require dissolution (Parker et al. 1986; Marmor 1990)

1.6 Hypothesis and Aims

This study tested the efficacy of the delivery of Vitamin D3 in vitro through the tissues of the buccal cavity from an oral spray.

“Vitamin D3 can be effectively delivered through the tissues of the buccal cavity from an oral spray”

1. Evaluation of the permeation of VD3 from commercially available preparations and compare that to laboratory made formulations through the buccal cavity – sublingual, buccal and soft palate membranes.
2. To estimate the total VD3 delivery to the system across these membranes.

2 Materials and Methods

2.1 Materials

Material/Chemical	Origin
Vitamin D3 (Cholecalciferol – Lot#:051M1682V)	Sigma-Aldrich Company (Poole, UK)
Menthol	
Cetrimide (Myrustyltrimethylammonium bromide, 99% Tetradecyltrimethylammoniumbromide, 99%)	Fisher Scientific UK Ltd. (Loughborough, UK)
Methanol	
Ethanol	
Phosphoric acid	
Vitamin D3 sublingual sprays: DLUX, Daily Vitamin D Oral Spray, 3000IU, 1000IU and 400IU	BetterYou Ltd. (Sheffield, UK)
High vacuum grease	Dow Corning (Michigan, USA)
Porcine tissues (tongues)	Local abattoir
Porcine heads – buccal and soft palate membranes	Local butchers

Table 1. *Materials used and their site of origin.*

2.2 Methods

In this study in-vitro analysis of sublingual delivery was carried out separately – ventral membrane of the tongue and the soft palate respectively. In reference to the main formulation concerned, an oral spray, it is hard to restrict delivery to only the sublingual membranes. Therefore we must account for delivery through other non-keratinised epithelia found in the cavity.

2.2.1 Preparation of Porcine Membranes

Porcine sublingual membranes were used to perform in-vitro studies. Human and porcine oral membranes are similar in structure (Squier 1991), composition (Heaney 1978) and permeability (Squier 1996). The sublingual area is comprised of 2 parts: the floor of the mouth and the ventral surface of the tongue. Previous studies conducted have shown that permeation via the ventral surface of the tongue is greater than through the floor of the mouth (Ong and Heard 2009). However, this does not rule out delivery through the soft palate and therefore must be accounted for. Buccal membranes were also used.

2.2.1.1 Sublingual membranes –Blunt dissection technique

Porcine tongues were collected from the local abattoir as soon as they were excised and transported immediately to the laboratory for membrane extraction.

The ventral surfaces of the porcine tongues were excised using blunt dissection. Separation of the membrane required careful scalpel dissection from the ventral surface before the membrane was cut into approximately 1cm^2 pieces ready to be used on Franz-diffusion cells (FDC) for permeation studies as shown in Figure 2. Each piece was microscopically examined to ensure its full intactness. The same technique was also used to extract membranes from the lower palate.

2.2.1.2 Buccal membranes –Heatseparation technique

The buccal membranes were cut and separated using heat separation. The porcine cheeks were excised from the inner cheek region of the porcine head and were placed in DI H_2O at 80°C for 60s. This allowed the membrane to be peeled away from the muscle using a forceps. This must be done carefully in order to extract large sections of the membrane for use on FDC's. Cells with a larger

diffusional area were used because these membranes are thicker and tougher. The membranes extracted were cut up into approximately 2.5cm^2 pieces, and microscopically examined before use.

2.3 Preparation of Donor and Receptor Phase solutions

2.3.1 Donor Phase Solutions

The donor phases consisted of 200µL of: Three commercial micro-emulsions VD3 supplement sprays (each at a different concentration) and a simple oil formulation. Water in the donor phase was used as a control.

The simple oil VD3 supplement was prepared using Cholecalciferol, olive oil and 1-methyl-2-pyrrolidinone. Two solutions were made with a ratio of 9:1 – Olive oil: 1-methyl-2-pyrrolidinone with the variant being the addition of 5% menthol in one of them. 1-methyl-2-pyrrolidinone was selected as it is a suitable solvent which0 acted as a mild penetration enhancer. Toxicology studies have shown that it is relatively safe over a range of concentrations and its metabolism does not lead to the formation of toxic compounds (Paulsson et al. 1997).

2.3.1.1 In-house Preparation of 9:1 – Olive oil: 1-methyl-2-pyrrolidinone donor phase

1µg of Cholecalciferol was weighed into a 1.5mL mini-centrifuge tube. 0.9 mL of olive and 0.1mL of 1-methyl-2-pyrrolidinone was measured using separate pipettes and added to the Cholecalciferol. The contents were mixed using a vortex mixer and sonication was used to ensure complete dissolution of VD3.

2.3.1.2 In-house Preparation of 9:1 – Olive oil: 1-methyl-2-pyrrolidinone + 5% menthol donor phase

1µg of Cholecalciferol was weighed into 1.5mL mini-centrifuge tube. 0.9mL of olive oil and 0.1ml of 1-methyl-2-pyrrolidinone was measured using separate pipettes and added to the Cholecalciferol. 75µg of menthol was weighed out and added to the oil and Cholecalciferol mixture. Mixing was carried out as previously mentioned in section 2.3.1.1.200µL of each preparation was used on the respective cells during the experiments.

2.3.2 Receptor Phase Solution

Cetrimide, at a concentration of 30µgmL-1 was used for the receptor phase. 12g of cetrimide was weighed out and dissolved in 400mL of de-ionised water. The solution was stirred using a magnetic stirrer until all the cetrimide had dissolved. This was added to each FDC together with a magnetic stirrer before application of the donor phases.

Cetrimide has no detrimental effect on the tissue or effect permeation. It acts as a sink for the compounds that permeate the membrane.

2.4 In Vitro Permeation Studies

The permeability of each type of membrane to VD3 was determined using all-glass FDC's. Two sizes of cells were used: Small size cells with a receptor volume of 2.4mL and a diffusion area of 0.1cm^2, large size cells with a receptor volume of 3.9mL and a diffusional area of 1.1cm^2. The cell flanges for both the cells were greased with high performance vacuum grease prior to the mounting of the membranes.

Prepared membranes were then mounted in between the receptor and donor compartments covering the diffusional area. They were positioned with the mucosal surface facing the donor compartment, with metal clamps holding the membrane in place between the cell top (donor compartments) and cell body (receptor compartment) together.

The receptor compartment was filled to the calibration mark with Cetrimide before adding magnetic stirrers and the sampling arm capped. The complete cells were placed in a water bath set at 37°C for 15 minutes to allow for equilibration before the addition of .200µL of donor phase (either the commercial sprays or simple oil VD3 supplements). The donor phase solutions varied from different concentrations of theoral spray and two simple laboratory mixed VD3 oil formulations of the same concentration, this is shown in Table 2 overleaf. Receptor phases were drawn after two hour time intervals over 12h from the sampling ports and replaced with fresh Cetrimide 0.03%. 1mL of the samples drawn, were then placed into HPLC vials for testing.

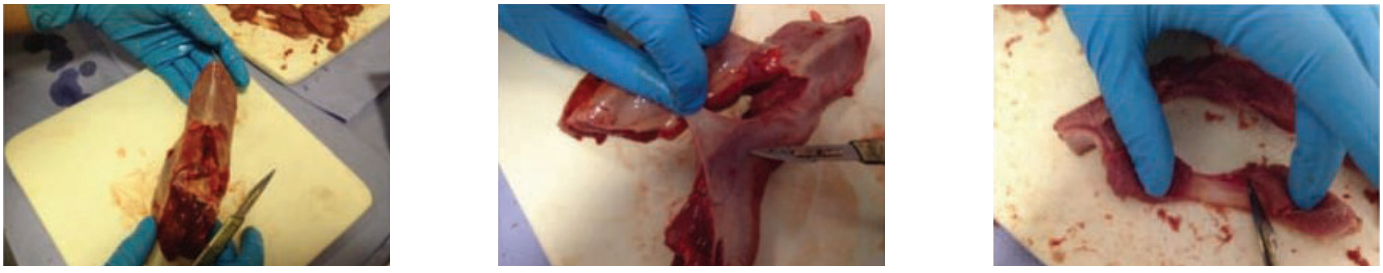

Figure 2: *Ventral side of the tongue (left), sublingual membrane (middle), display of the blunt dissection technique (right).of the active form of VD*

Preparation	VD3 Concentration (μgmL^{-1})	Solvents	Other
DLux400	71.43	Oil (sunflower lecithin) water emulsion	Xylitol, acacia gum, citric acid, preservative (potassium sorbate), peppermint oil.
DLux1000	178.57		
DLux3000	535.71		
9:1 (Olive oil: 1-methyl-2-pyrrolidinone)	1000	Olive oil	1-Methyl-2-Pyrrolidinone
9:1 + 5% menthol	1000	Olive oil	1-Methyl-2-Pyrrolidinone, 5% Menthol

Table 2. *Constituents of the formulations used in the donor phases.*

2.5 HPLC Analysis

Reverse phase HPLC was used to determine the amount of VD3 that permeated the membrane over the 12h time period. An Agilent 1100 fitted with Gemini NX C18 column was used; the UV detector was set at 254nm. The HPLC method used for the quantification of VD3 was developed in-house. A mobile phase of 70:30 – Methanol: Ethanol with 1% phosphoric acid was used; this aided elution of VD3 from the receptor phase.
VD3 has a retention time of 4.47 minutes (shown in Figure 3.). The LOD for VD3 was 0.25µgmL-1.

2.6 Data Processing and Statistical Analysis.

For each sample and each tissue cumulative amounts of VD3 permeated per unit area were plotted against time over 12h. Flux values were calculated using the linear portions of these graphs.

Statistical analysis was completed using InStat 3 for Macintosh GraphPad Software, Inc. (Hercules, CA, USA). A one way ANOVA with post t-test was used to investigate differences between the data sets of the various tissues. To be considered a significant p-value of <0.05 must be achieved. (Squier 1996).

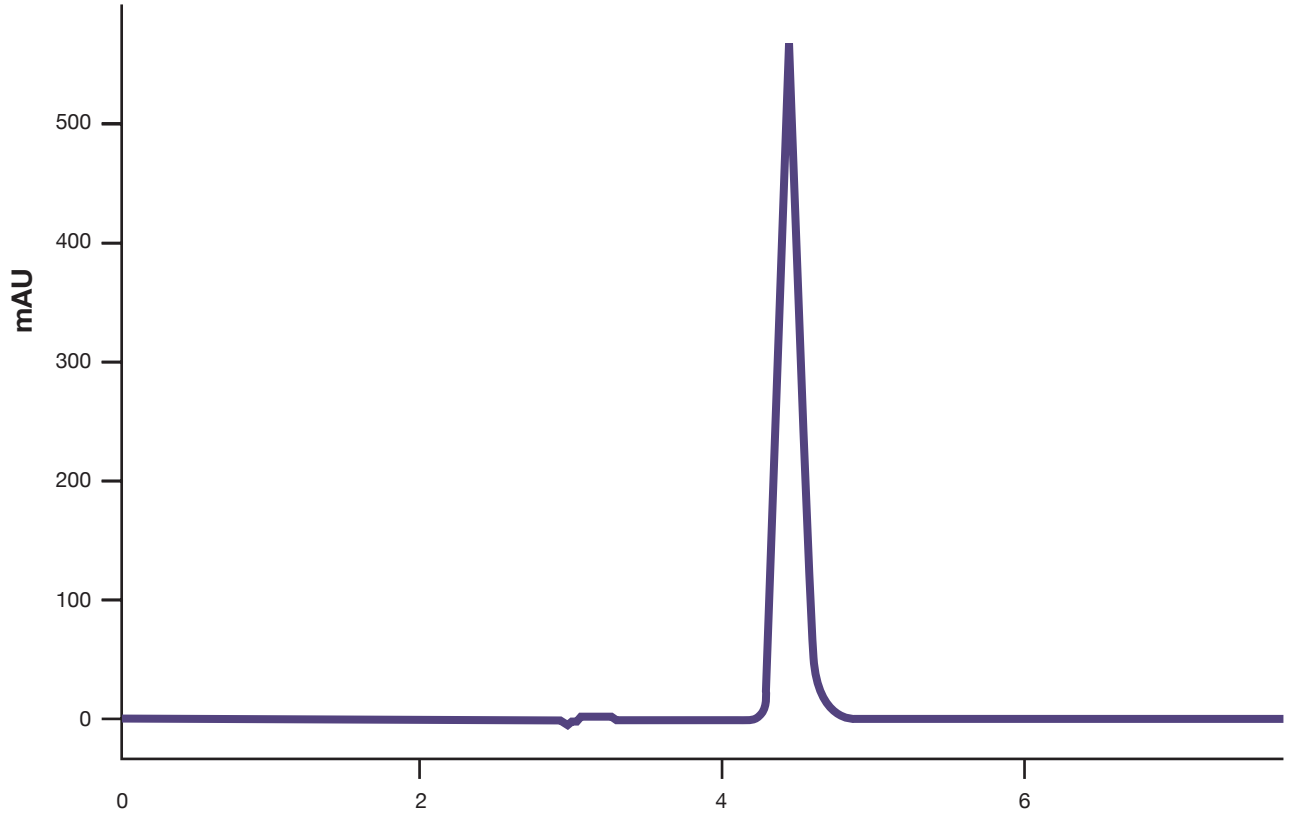

Figure 3: *HPLC of VD3 in ethanol - showing a retention time of approx. 4.5minutes*

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3 Results

3.1 Sublingual permeation

Each preparation was tested on porcine sublingual membranes – this included the ventral part of the tongue and the soft palate. The volume of donor phase applied each time was the same.

3.1.1 Ventral part of the tongue

The permeation profiles of VD3 across the tongue membranes for three commercial products was carried out in order to determine whether increasing the concentration of applied VD3 would result in increased permeation. Figure 4 graphically displays the permeation profiles of the three commercial sprays.

The DLux400 shows to have delivered the highest amount of VD3 over the 12h achieving a total mass of 0.0423mgcm2. The DLux1000 and the DLux3000 delivered significantly less 0.0350mgcm^2 and 0.0279mgcm^2 respectively. All three formulations show linear permeation.

An increase in applied VD3 concentration does not increase its permeation. Therefore delivery for the commercial spray is rate limiting.

Further investigations were carried out to test the efficacy of delivery of the commercial sprays (micro-emulsion) in comparison to the in-house (simple oil) formulations containing VD3. This is shown in figure 5.

Figure 7 displays the linear delivery of the commercial preparations as opposed to the delivery of the simple oil preparations which displays non-linear delivery. The DLux400 has the highest delivery of VD3 over 12h with a total mass of 0.0423mgcm-2 whilst the other two commercial sprays show lower delivery. The simple oil formulations showed lower overall delivery. However, we can see that the initial delivery of the oil formulation with menthol was similar to that of the DLux400 up until the 4h time point, achieving a total delivery of 0.0390mgcm-2 over 12h. The preparation without the menthol showed considerably less delivery. The DLux3000 shows a lower overall delivery in comparison to the simple oil formulations and overall worst delivery of the three sprays over 12h time period.

The average flux values of the three preparations have been calculated and shown in Table 3.

The DLux3000 shows the highest flux of 2.80 x 10-3mg cm-2 h-1. The difference between the flux values for the three preparations is not significantly different. This shows that a change in formulation concentration does not appreciably affect the flux values ($p > 0.05$).

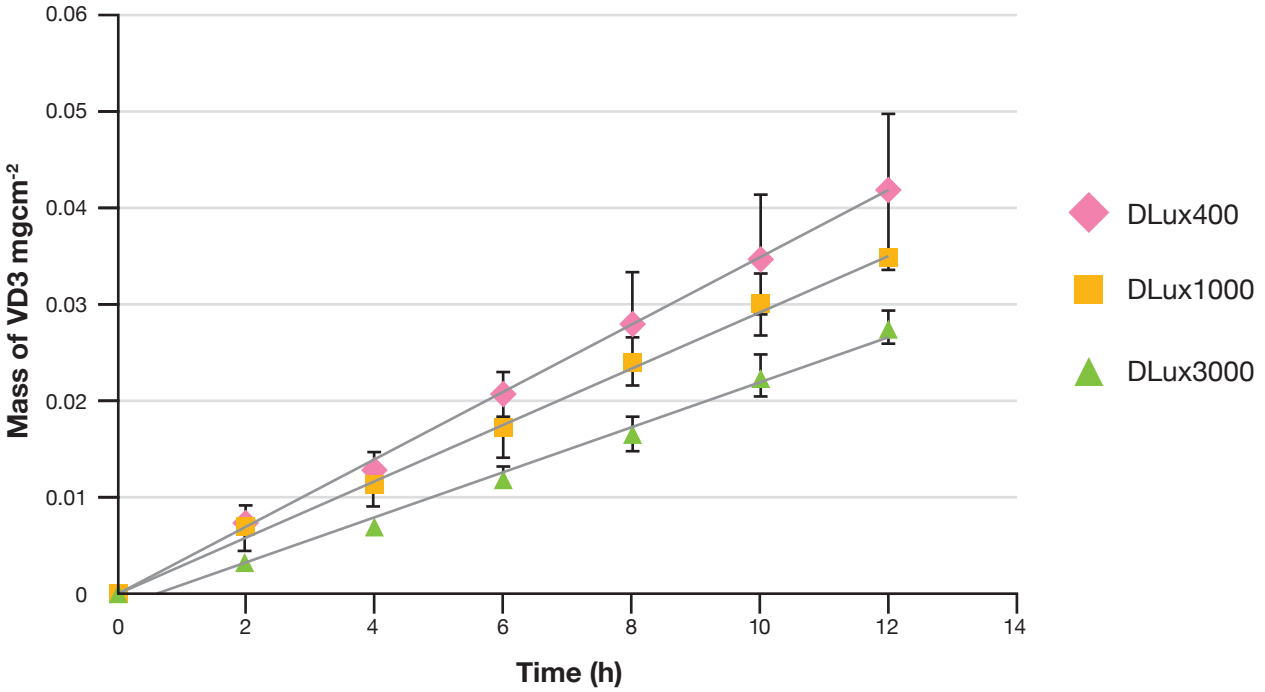

Figure 4: *Plot showing the cumulative delivery of VD3 across the ventral surface of the tongue for 3 commercial sprays.*

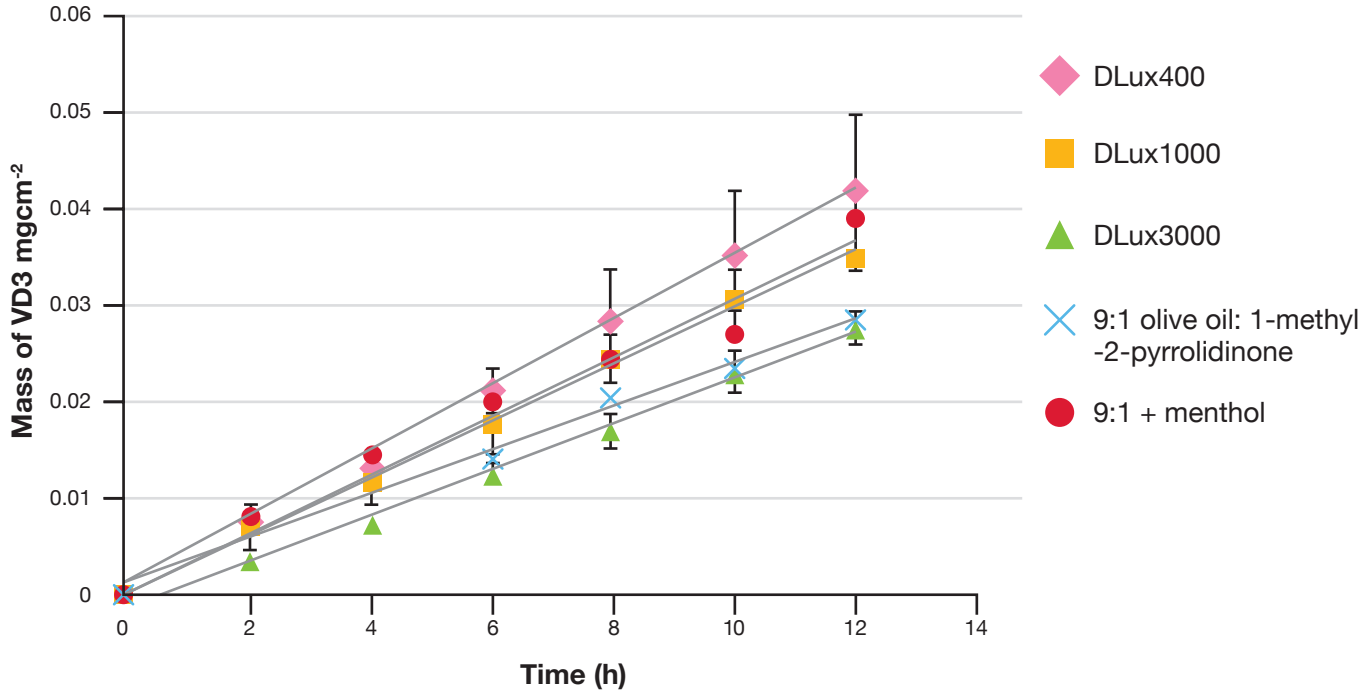

Figure 5: *Plot comparing the cumulative delivery of VD3 between commercial preparations and in-house made preparations across the ventral surface of the tongue.*

Preparation	Average J_{ss} ($x10^{-3}$mg cm^{-2} h^{-1})
DLux400	2.53
DLux1000	2.67
DLux3000	2.80

Table 3. *Average steady state flux of three commercial sprays across the ventral surface of the tongue.*

Preparation	Average J_{ss} ($x10^{-3}$mg cm^{-2} h^{-1})
DLux3000	0.00870
9:1 olive oil: 1-methyl-2-pyrrolidinone	0.00130
9:1 + menthol	0.00125

Table 4. *Average steady state flux of three preparations (1 commercial spray and 2 simple oil formulations with a menthol variant) across the porcine buccal membrane.*

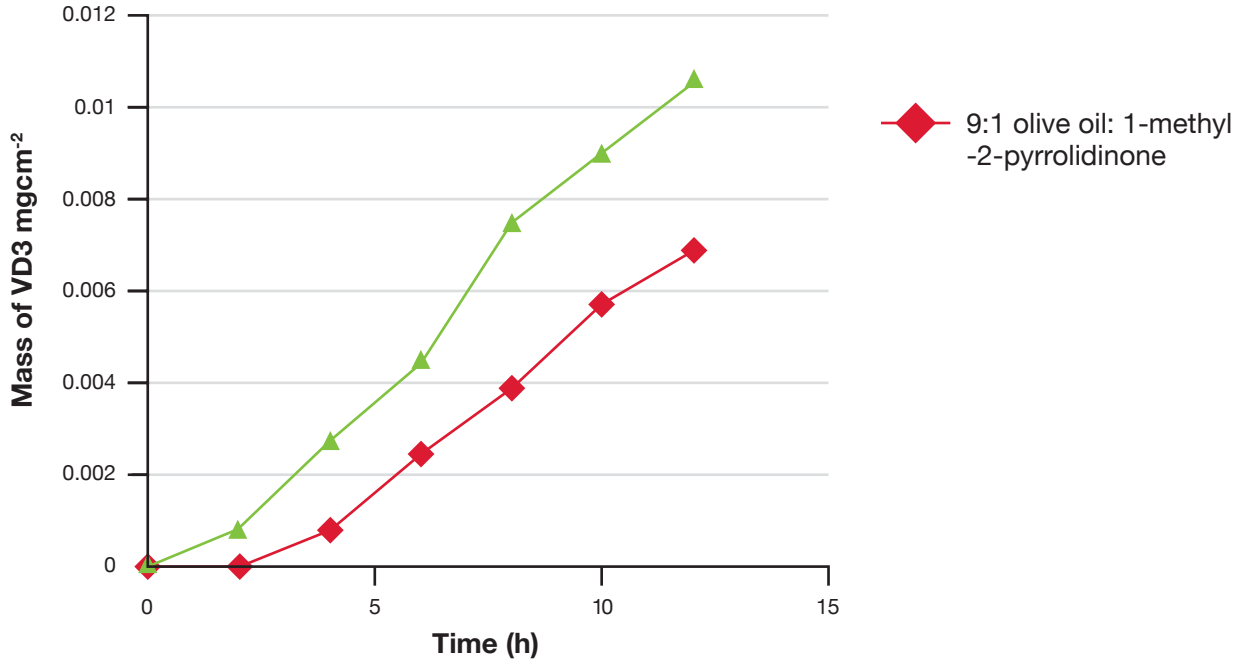

Figure 6: *Plot showing the cumulative mass of VD3 that has permeated the soft palate over a 12h time period.*

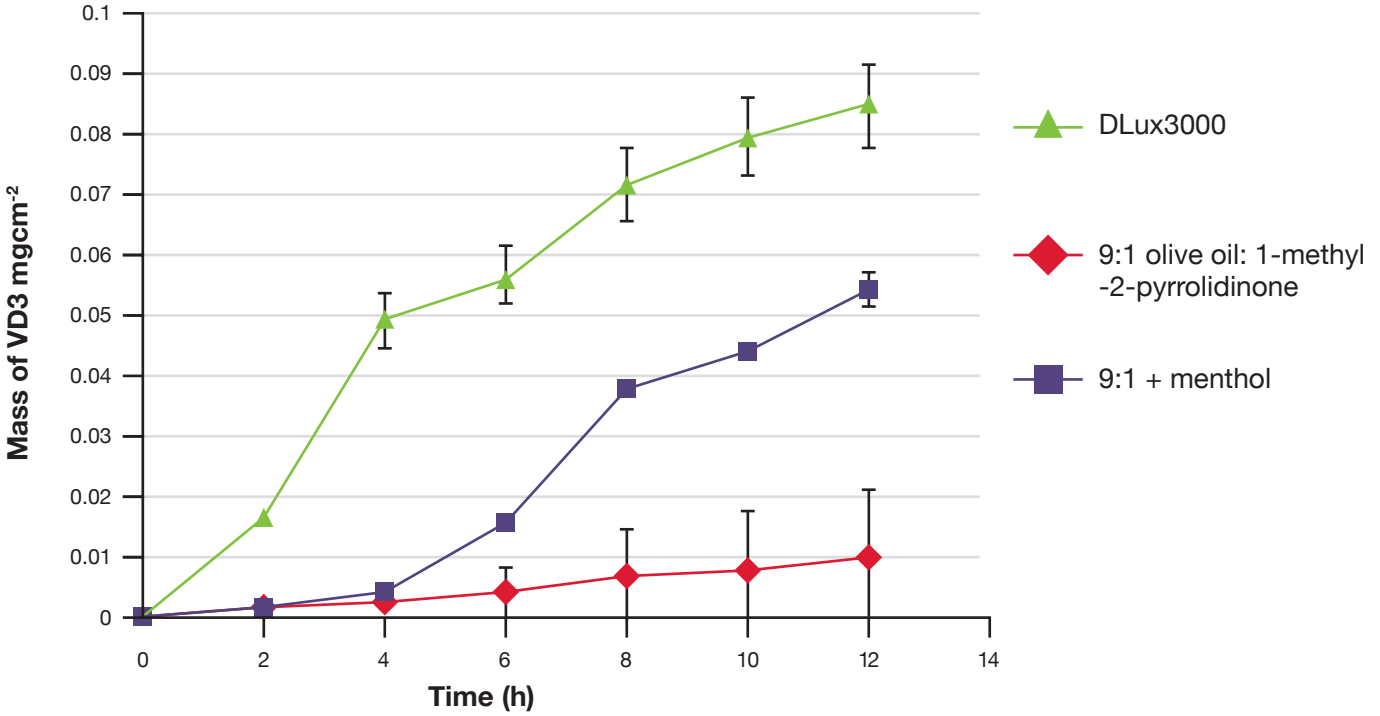

Figure 7: *Plot showing the cumulative delivery of VD3 across the buccal membranes over 12h.*

Membrane	Membrane area over which dose is distributed (cm^2)	VD3 permeated after 0.25h ($x10^{-4}$mg)	VD3 permeated after 0.25h (%)
Ventral surface	3.85	21.23	2.83
Lower soft palate	2.90	0.65	0.87
Buccal (membranes on inner cheeks)	1.80	36.45	4.86

Table 5. *Area that is covered during sublingual administration of spray and the percentage permeation of that dose.*

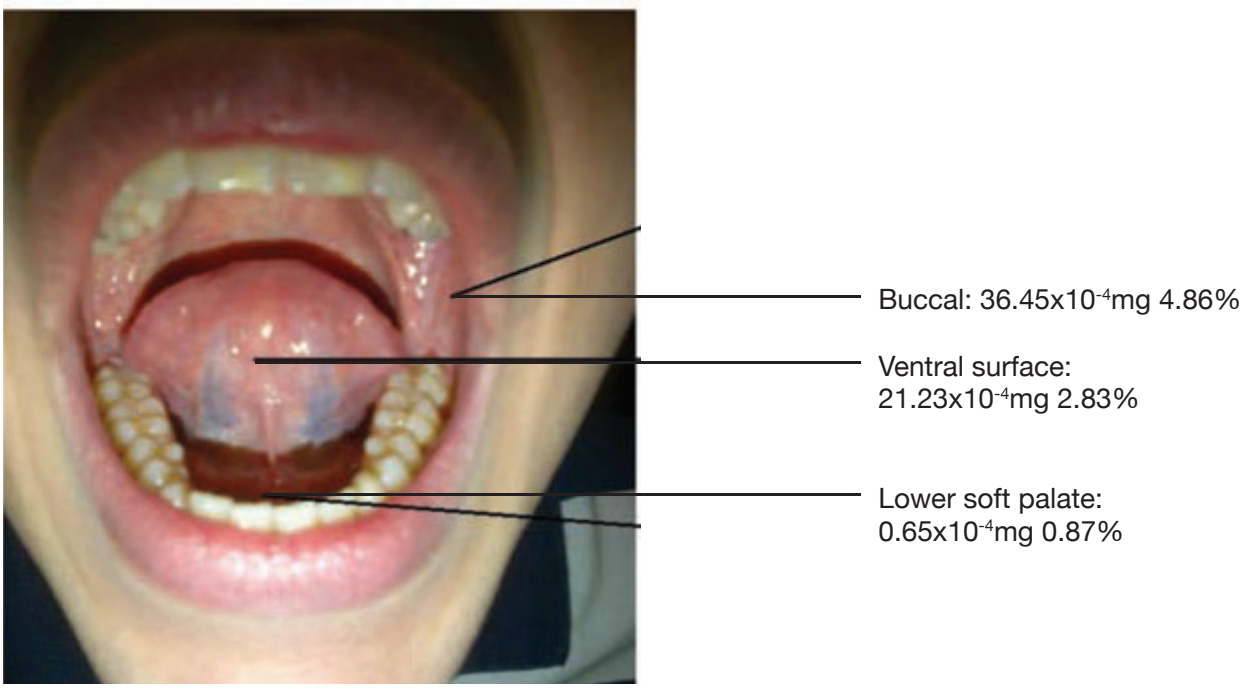

Figure 8: *Permeation values of VD3 when spray is directed to the ventral tongue surface.*

3.1.2 Soft Palate

The permeation of each formulation across the soft palate has been tested over 12h and data collected and represented graphically. This is shown in figure 6.

Better permeation is seen from the DLux3000, with a total mass of 0.0107mgcm-2 permeating after 12h. The graphs for each formulation show a lag phase followed by a linear part and then tail off. The simple oil formulation delivers approximately half the amount of VD3 delivered by the DLux3000. Delivery from both formulations is non-linear.

3.2 Buccal permeation

Buccal membranes include the membrane that lines the inner part of the lips and the inner cheeks.

VD3 is able to permeate the buccal membranes. This is shown in Figure 7.

Delivery of VD3 from all three preparations is non-linear. The DLux3000 has the best permeation profile, achieving maximum delivery of 0.0848mgcm-2 after 12h. The simple oil preparations show much lower delivery; however the preparation containing the menthol displays a similar delivery profile to that of the commercial spray, delivering a total mass of 0.0538mgcm-2 of VD . The 9:1 olive oil:1-methyl-2-pyrrolidinone delivers the least VD3 across the membrane with a delivery profile which looks a lot more linear in comparison to the other two preparations. It delivers almost ten times less the amount of VD3 (0.0087mgcm-2) after 12h in comparison to the DLux3000

The average flux values of the three preparations have been calculated and shown in Table 4.

The DLux3000 shows the highest flux across the buccal membrane with a value of 0.0087 x10-3mgcm-2 h-1 which is approximately eight times greater than the flux of the simple oil preparations.

3.3 Estimation of the VD3 delivered from oral spray

The spray plume of a single dose covers an area of 8.55cm2 , with the spray distance being approximately one inch from the application site. Permeation will differ depending on the position of the device when spraying which determines the membrane area that is exposed. It is also apparent that a proportion of the dose will target other membranes and/or be swallowed.

3.3.1 Spray aimed at the ventral tongue surface

Here, the dose is sprayed directly at the ventral surface of the tongue and an estimation of the dose distribution is shown in table 5.

With this type of application method a total of 8.56% (58.33x10-4mg) of the overall dose permeates the membrane with the rest of the dose being swallowed. The data in table 5 is displayed in figure 8.

3.3.2 Spray applied directly to one of the inner cheek lining (buccal)

Having determined that the buccal membrane is significantly more permeable than sublingual, it is worthwhile considering the outcome should the spray be directed solely at the inner cheek. This would involve facing the device at the buccal membrane; either, the right or left cheek, directly exposing only one buccal membrane. In this case the amount that would reach the other areas would be lower, especially as there would be greater retention between the cheek and gums, and where saliva is less likely to wash the dose away. An estimated dose distribution for this is shown in table 6.

This method shows a much higher permeation percentage in comparison to the sublingual technique. We already know that the commercial spray shows a higher degree of permeation through the buccal membrane. However, even with this dosing method the overall percentage permeation achieved is only 19.51% (146.33x10-4 mg), less than one fifth of the dose administered.

The data in table 6 is displayed in figure 9.

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The data in table 6 is displayed in figure 9.

Membrane	Membrane area over which dose is distributed (cm^2)	VD3 permeated after 0.25h ($x10^{-4}mg$)	VD3 permeated after 0.25h (%)
Ventral surface	~1	5.48	0.73
Lower soft palate	~1	2.33	0.31
Buccal (membranes on inner cheeks)	6.84	138.53	18.47

Table 6. *Area that is covered by buccal administration and the percentage permeation of that dose.*

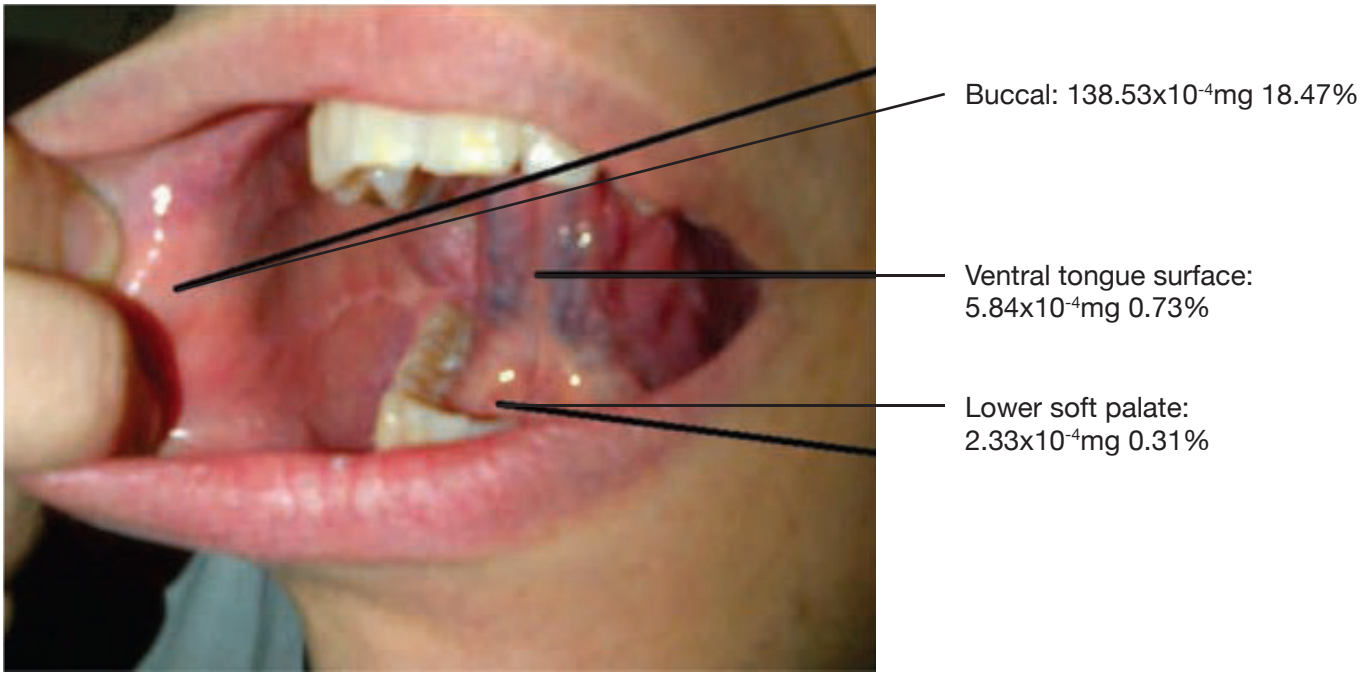

Figure 9: *Permeation values of VD 3 when spray is directed to the buccal region (one cheek).*

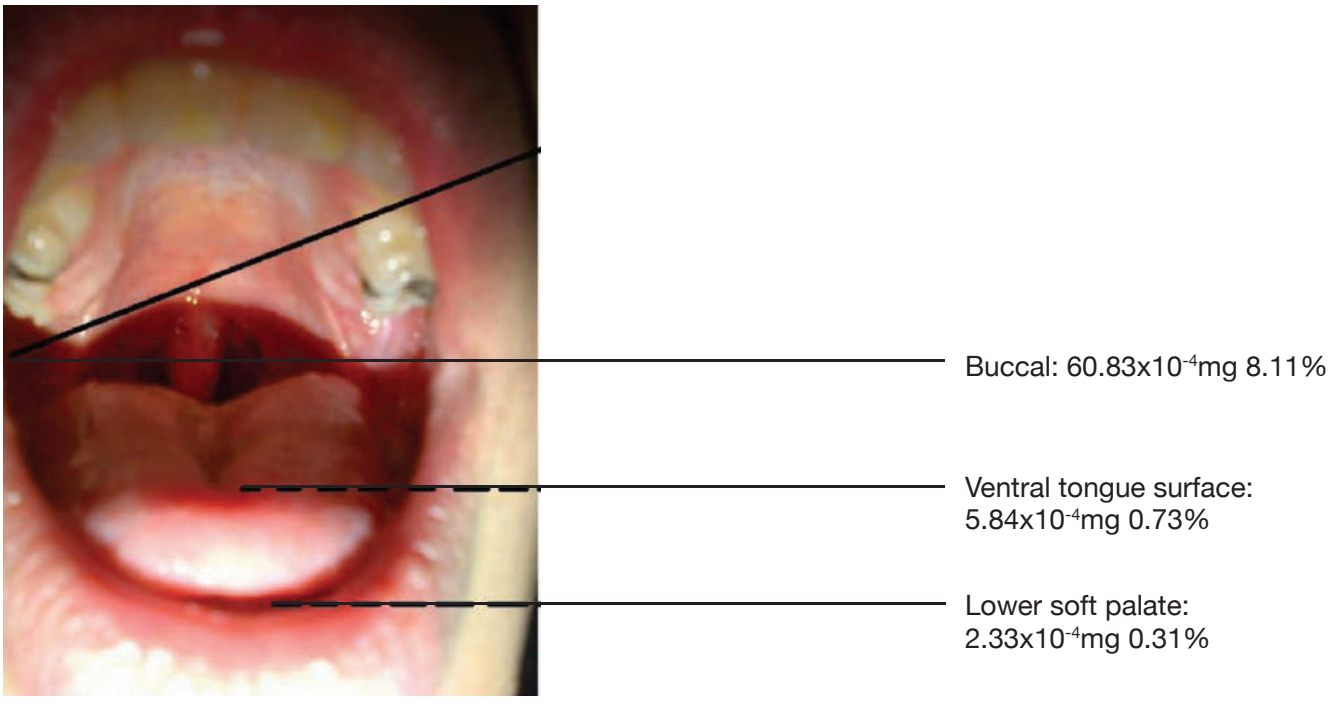

Figure 10: *Permeation values of VD 3 when the formulation is sprayed directly into the oral cavity.*

3.3.3 Spraying into the oral cavity (as alluded to by Instructions)

Spraying directly into the cavity would result in most of the dose being sprayed on the surface of the tongue or hard palate - in both these areas absorption is very poor due to their keratinised nature. We can assume that a small proportion of the sprayed dose will be deposited on the buccal membranes. Table 7 shows an estimate of the dose distribution areas.

Even by spraying the dose directly into the cavity we do achieve a small percentage of permeation, approximately 9.15% (68.63x10-4mg). Most of the dose, 90.85% (681.38x10-4mg) will follow the oral route. The tabulated data is shown in figure 10.

4 Discussion

4.1 Permeability of porcine oral membranes

Delivery through the membranes of the oral cavity occurs through passive diffusion (Kurosaki et al. 1998). The design of the formulation is a major important factor in achieving permeation; molecular weight, size, degree of lipophilicity and charge. Absorption into the systemic circulation occurs via the jugular vein; this is shown in figure 11.

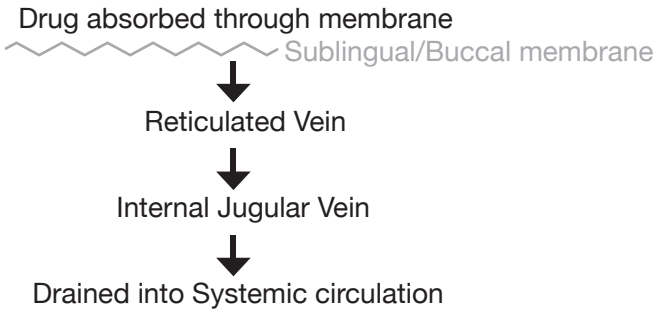

Figure 11: *Absorption pathway from the membrane into the systemic circulation.*

Drug characteristics are an important factor for permeation; VD3 has a molecular weight of 384.6 and a logP of 9.1 making it a good compound for sublingual and buccal permeation. Both membranes are lipophilic in nature, favouring the permeation of lipophilic compounds, which penetrate the membranes faster than hydrophilic compounds (Hiroshi et al. 1993). However the drug must be able to permeate the surface membrane and then the mucosal membrane; therefore a highly lipophilic drug such as VD3 needs to be dissolved in suitable solvent to achieve permeation (Loftsson et al. 2002).

4.1.1 Comparison of the oral membranes involved in the delivery of VD3.

Sublingual and buccal delivery are both forms of topical delivery, although permeation across each of the membranes varies. Studies on the different membranous regions of the oral cavity have shown this. Sublingual membranes have the highest permeability, of which the ventral surface of the tongue is more permeable than the lower soft palate, followed by the buccal membrane which is the least permeable (Lesch et al. 1989). The buccal membrane does not provide the same rapid absorption and bioavailability which is seen with the other two membranes (Singh et al. 2011). This however can be argued based on the variation seen in this study, where the buccal permeation is greater than the sublingual, as shown in Section 3.

The difference in permeability can be seen with reference to the flux values of the DLux3000 between the ventral membrane of the tongue and the buccal membrane. The flux across the ventral membrane of the tongue is approximately 321 times greater than that of the buccal membrane. This difference can be attributed to glucosylceramide, which is an important mucosal membrane constituent. Studies have shown that the greater the glucosyleceramide content the poorer the membrane permeability (Squier et al. 1991).
The amount of glucosylceramide present in the buccal mucosa is almost 3 times more than in the sublingual membranes. This allows us to understand the difference that is seen with the permeability results obtained (Wertz et al. 1986).

However when the entire membranous region is considered, the overall delivery of the commercial spray through the buccal membranes is significantly higher than through the sublingual. This can be seen in Figure 8 showing an increase in buccal delivery which is almost twice that of the sublingual. Looking at the 9:1 olive oil:1-methyl-2-pyrrolidinone preparation the results are reversed. The percentage permeation is higher in sublingual than in buccal, but not significantly. This may be attributed to the type of formulation and the variation in the selectivity of the membrane.

Comparisons of the sublingual membranes have shown that permeation across the ventral surface of the tongue is almost double to that of the lower soft palate. The DLux3000 and 9:1 olive oil:1-methyl-2-pyrrolidinone respectively have both shown increased permeation through the ventral surface of the tongue, with permeation being approximately 2.6 times and 2.9 times higher respectively. This can be attributed to the difference in the lipid composition of the epitheliums (Squier et al. 1986).

Several permeation studies, when specified as sublingual, do not consider drug permeation occurring in other parts of the oral cavity. Most drugs

delivered through membranes in the oral cavity will be exposed to an abundant supply of saliva, which will ultimately result in parts of the drug being moved to other membranous regions, hence the basis of this study. This study has shown the difference in permeability in the prominent regions of the oral cavity, proving that delivery can occur all over but to different extents. Whilst vascularity is important, it is not the limiting factor in this type of delivery.

4.2 Preparation analysis: spray (DLux400, DLux1000, DLux3000) and simple oil ((9:1) olive oil: 1-methyl-2-pyrrolidinone and 9:1 + menthol)

The commercial preparation is classed as an oral spray, with no specific guidance on use. It is difficult to control the area of application as the spray plume would vary depending on individual use. The excipients used in all three preparations remain constant ruling out variability in the delivery of VD 3. Oils have been used as solubilising agents due to the lipophilic nature of VD 3. The purpose of the other excipients is outlined below:

Xylitol – A sugar alcohol that is commonly used as a sweetener. It is safe and known to reduce the incidence of tooth decay (Lynch 2003) and has some permeation enhancing effects and as a solubilising agent (Nep et al. 2011).

Acacia gum – Used as a demulcent and suspending agent in several pharmaceutical preparations with some mild permeation enhancing effect, and is known to inhibit growth of periodontic bacteria.

Peppermint oil – It is famously known that peppermint and menthol oils are commonly used for taste and as permeation enhancers (Abdullah et al. 1996).

The lipophilic nature of VD3 makes choosing a solvent a lot harder. VD3 is freely soluble in ethanol and methanol, but, these are not suitable solvents for permeation studies as they can alter membrane viability. 1-methyl-2-pyrrolidinone is used as a solubilising agent for poor soluble drugs (Uch et al. 1999). With a low toxicity profile it is safe to use and has slight permeation enhancing effects. VD3 is freely soluble in olive oil and therefore the 1-methyl-2-pyrrolidinone is to aid permeation through the surface layer of the membranes, before permeating the lipid part. Olive oil is used in several traditional commercial VD3 supplements, “oral drops”.

Menthol is a permeation enhancer, making up 30-55% of peppermint oil (Gelal 2008). The minty smell helps mask the formulation smell. For the in-house simple oil preparation, menthol was the permeation enhancer of choice. By keeping the oil formulations simple we were able to test the effect of adding a penetration enhancer. Based on the results seen with the commercial preparations, the results obtained verified what we expected. The preparation containing the menthol shows a better permeation profile than the preparation without, as seen in figures 7. Tables 4 and 5 show that the percentage permeation of the simple oil formulation is better suited to sublingual membranes, showing a larger permeation percentage over the whole sublingual membranes.

The results shown in Figures 5 to 7 confirms once more that the commercial micro-emulsion mixture provided much higher permeation of VD 3 in comparison to the simple oil formulations. Surprisingly, a change in concentration did not appreciably increase flux values as seen in Table 3; showing that delivery is rate limiting. The percentage permeation values shown in Tables 5,6,7 and Figures 8,9,10 show that the commercial preparations are more suited to buccal delivery.

4.3 Comparisons of the two types of preparations

The commercial spray is a micro-emulsion preparation whilst the other is a simple oil preparation, with the main difference being the types of excipients used. Both contain oils and permeation enhancers. We either expected similar permeation patterns or expected the commercial preparation to have a poorer permeation profile. This was because the commercial spray has a lot more excipients. On investigation, the commercial preparations showed better permeation profiles through all the membranes. These can be seen in figures 8, 9 and 10. This can be attributed to the micro-emulsion formulation, possibly in addition to the presence of excipients.

5 Conclusion

The current study has confirmed that VD3 permeates the major membranes in the oral cavity from an applied spray dose. However, there was considerable variation in the permeability of VD3 across individual membrane types.

Predicted percentage permeation values have shown that the inner cheek/buccal membrane provided significantly greater VD3 permeation from the commercial spray compared to the other membranes. Comparing the different types of preparations this work has shown that the micro-emulsion commercial preparations have a higher degree of permeation in comparison to the simple oil preparations.

Lack of specificity regarding the Instructions for use of the oral spray has the potential to lead to differing VD3 permeation obtained by users administering in different manners. In particular, when the spray is

targeted towards the inner cheek ~20% of the dose is absorbed, whereas when directed towards the sublingual region ~9% will be absorbed – approx. the same as spraying into the mouth without the tongue raised. The balance of the sprayed doses will presumably be swallowed.

VD3 can sufficiently be delivered as an oral spray, with an overall absorption potential within the mouth of ~37% and buccal permeation delivering the highest individual absorption of ~20%, easily reaching the RDA, this does not include the levels still delivered after swallowing. It can be reasonably argued that a rapid and more constant delivery is achieved through this method of application.

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In vitro analysis of sublingual vitamin B12 permeation

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Abstract

Oral mucosal drug delivery is an alternative and promising method of systemic drug delivery which offers several advantages. Sublingual literally meaning is “under the tongue”, administrating substance via mouth in such a way that the substance is rapidly absorbed via blood vessels under tongue. Sublingual route offers advantages such as bypasses hepatic first pass metabolic process which gives better bioavailability,rapid onset of action, patient compliance, self-medicated. Dysphagia (difficulty in swallowing) is common among in all ages of people and more in pediatric, geriatric, psychiatric patients. In terms of permeability, sublingual area of oral cavity is more permeable than buccal area which is in turn is more permeable than palatal area. Different techniques are used to formulate the sublingual dosage forms. Sublingual drug administration is applied in field of cardiovascular drugs, steroids, enzymes and some barbiturates. This review highlights advantages, disadvantages, different sublingual formulation such as tablets and films, evaluation.

1. Introduction

1.1 Overview

Sublingual drug delivery utilises the permeability of the mucosal membrane located on the ventral side of the tongue. The sublingual membrane is a preventative barrier for the permeation of many compounds into systemic circulation. The membrane therefore can be a difficult route to utilise for the delivery of drugs. However in comparison to other delivery routes this pathway provides several advantages, as discussed later.

Vitamins are essential nutrients that humans require to sustain life. Each vitamin has a specific and vital role in the body and can be obtained from a variety of sources (food, drinks, sunlight and supplementation). Their uptake and utilisation is intricate and relies upon a delicate balance of overall nutrition.

There are two main classes of vitamins:

1. Fat soluble vitamins; these can be obtained from fatty foods, they are stored in the liver and fatty tissues and therefore do not require a daily intake. The vitamins A, D (D1 and D2), E and K are included in this category.
2. Water soluble vitamins; these vitamins, not stored in the body, require daily intake. These are mainly acquired through the consumption of fruits, vegetables and grains. Included in this category are the B range of vitamins, vitamin C and folic acid.

Some vitamins although obtained from the diet can also be obtained through non-dietary sources e.g. Vitamins such as biotin and Vitamin K are naturally synthesised in the gut, Vitamin D3 (VD3) is mainly obtained via sunlight.

The progression of a westernised culture and poor diet regimes, has led to vitamin supplementation playing a vital role in maintaining the required levels nutrient uptake.

This research probed the efficacy of the sublingual VB12 supplementation from an oral spray via the in-vitro permeation of VB12 through the excised sublingual membranes.

1.2 Vitamin B12 (VB12)

Vitamin B12 is one of the water soluble B vitamins which is bound to protein within food. It is generally obtained through dietary means from animal products such as meat, fish, milk and eggs. It is involved in the body's ability to maintain normal neurological and psychological functions, particularly those aspects of the brain and nerve functions which determine concentration, learning, memory and reasoning. VB12 plays an important role in the contribution to normal homocysteine levels, ensuring healthy red blood cell formation and has been proven to be linked to the reduction of tiredness and fatigue.

Adequate VB12 levels can be achieved through a balanced diet but certain people may have difficulty achieving these levels. VB12 is a particularly large molecule and relies upon the presence of a chemical called intrinsic factor, a glycoprotein secreted by the stomach's parietal cells, in order for it to be absorbed from food. The resulting complex undergoes absorption within the distal ileum by receptor-mediated endocytosis.

Vegans and vegetarians are typically vulnerable unless they take supplementary measures. Its role in supporting healthy cell division and folic acid metabolism also raises the importance of adequate levels within women who are pregnant or breastfeeding. Finally people who have had part of their gut surgically removed, who have bowel problems such as coeliac disease, Crohn's or ulcerative colitis will also be considered for supplementary activity.

1.3 Sublingual Drug Delivery

Sublingual drug delivery is defined as the permeation of a drug through the sublingual mucosal membranes, which cover the ventral side of the tongue and the soft palate; these membranes are part of the non-keratinised epithelia found in the oral cavity. The total surface area of the oral cavity has been found to be approximately 214.7cm2 ± 12.9cm2. Of this surface area, 30% is found to be nonkeratinised epithelia, involved in sublingual and buccal delivery. Non-keratinised epithelia line: the inner part of the cheeks (inside the mouth), the ventral part of the tongue and the soft palate (Collins and Dawes 1987). An approximation of the non-keratinised membranes is:

(214 x 30) / 100 = 62.2cm^2

60% of this surface area is represented by the sublingual membranes (the soft lower palate and the ventral side of the tongue) (Wilson 2005; Chen et al. 1999).

(62.2 x 60) / 100 = 37.32cm^2

Of this, 13cm2 makes up the ventral surface of the tongue (Ong and Heard 2009), with the rest making up the floor and soft palate. Therefore the rest of the surface area, 24.32cm2 makes up the floor and soft palate of the mouth.

Permeation is easily affected by substances such as alcohols and therefore permeability is classed as selective. This is a limiting factor in the selection of excipients for sublingual formulations.

As part of the oral cavity, these membranes are exposed to an abundant supply of saliva which is constantly secreted and continuously flushes the cavity. Continual movement of the tongue, speech and salivary secretions coupled with the swallowing reflex lead to a limited time period of application. Aspects of delivery such as particle size and the physico-chemical nature of a formulation

(eg combinations of permeation enhancers (Sudhakar et al. 2006) and mucoadhesives etc) greatly affect the flux of a compound.

1.4 Buccal Delivery

Buccal delivery involves the membranes that line the inner cheek, inside the upper and lower lips in the oral cavity. It forms approximately 40% of the non-keratinised epithelia found in the oral cavity (Wilson 2005). This can be calculated from the overall surface area:

(40 x 62.2) / 100 = 24.88cm^2

The oral mucosal membrane, comprising of the buccal and sublingual membranes, varies in thickness and permeability. The buccal membrane is thicker, approximately 580μm (in comparison to the sublingual membrane which is approximately 190μm) and is generally less permeable (Squier and Wertz 1996) (Squier and Hall 1985b; Lesch et al. 1989).

This route of delivery is common for muco-adhesive formulations, enabling a longer application time. Similar to sublingual delivery, buccal delivery is affected by salivary secretion and mucus turnover. However, the increased application time in this area is due to the lower susceptibility of tongue movement.

1.5 Advantages of Sublingual and Buccal Delivery

Application of drugs onto the sublingual and buccal membranes have proven to be an easy alternative to those individuals who are incapable of ingesting formulations (i.e. patients who are nil-by-mouth, experiencing episodes of nausea and vomiting) or those that do not like or have difficulty taking tablets or liquid formulations (Narang et al. 2011). This route is non-invasive and is not as intimidating as injectable or rectal and vaginal routes.

The membranes are surrounded by a good vasculature which provides easy access into the systemic circulation bypassing the gastro-intestinal (system); this avoids any lag time of drug activation which is often experienced when dosing orally. The effects of drugs administered through theses membranes are therefore a lot more rapid and are not dependent on factors that commonly affect oral routes (stability of drugs in G.I fluid). These areas are easily accessible for application and can be ideal for sustaining prolonged delivery. In case of any unwanted effects, the dosage form can be easily removed restricting delivery almost immediately.

Sublingual sprays offer a faster onset action in comparison to tablet which would require dissolution (Parker et al. 1986; Marmor 1990)

2. Materials and Methods

2.1 Materials

Material/Chemical	Origin
Vitamin B12 (labelled 1713 02783A)	Supplied by Cultech
Methanol Tri-flouroacetic acid	Fisher Scientific UK Ltd. (Loughborough, UK)
Vitamin B12 Boost Oral Spray	Better You Ltd. (Sheffield, UK)
Porcine tissues (tongues)	Local abattoir

Figure 1.

2.2 Preparation of Porcine Membranes

Porcine sublingual membranes were used to perform in-vitro studies. Human and porcine oral membranes are similar in structure composition and permeability, and therefore an appropriate model for human sublingual membranes.

Porcine tongues were collected from the local abattoir as soon as they were excised and transported immediately to the laboratory for membrane extraction.

The ventral surfaces of the porcine tongues were excised using blunt dissection. Separation of the membrane required careful scalpel dissection from the ventral surface before the membrane was cut into approximately 1cm2 and 2 cm2 pieces ready to be used on Franz-diffusion cells (FDC) for permeation studies as shown in Figure 2. Each piece was microscopically examined to ensure its full intactness. (Collins and Dawes 1987).

2.3 In Vitro Permeation Studies

The permeability of the membrane by the vitamin B12 spray was determined using all-glass FDC's. Two sizes of cells were used: Small size cells with a receptor volume of 2.4mL and a diffusion area of 0.1cm^2, large size cells with a receptor volume of 3.9mL and a diffusional area of 1.1cm^2. The cell flanges for both the cells were greased with high performance vacuum grease prior to the mounting of the membranes.

Prepared membranes were then mounted in between the receptor and donor compartments covering the diffusional area. They were positioned with the mucosal surface facing the donor compartment, with metal clamps holding the membrane in place between the cell top (donor compartments) and cell body (receptor compartment) together. De-ionised water was used as the receptor phase and added to each FDC together with a magnetic stirrer before application of the donor phases. The complete cells were placed in a water bath set at 37°C for 15 minutes to allow for equilibration before the addition of 200µL of donor phase to the small cells to represent maximal delivery or a single spray to the large cells to represent in-situ use.

The receptor phases were drawn at 0.5, 1, 2, 3, 6, 12 and 24 hours for the maximal delivery test. The in-situ analysis receptor phases were drawn at 10, 15, 20, 25, 30 and 60 minutes.

2.4 HPLC Analysis

Reverse phase HPLC was used to determine the amount of vitamin B12 that permeated the membrane over the time periods. An Agilent 1200 fitted with Gemini NX C18 column was used; the UV detector was set at 278nm. The HPLC method used for the quantification of vitamin B12 was developed in-house. The mobile phase used a gradient elution timetable represented in figure 3. Vitamin B12 has a retention time of 6.87 minutes. The LOD for vitamin B12 was 1.75ngmL-1. 2.5 Data Processing and Statistical Analysis. For each sample and each tissue cumulative amounts of vitamin B12 permeated per unit area were plotted against time. Flux values were calculated using the linear portions of these graphs.

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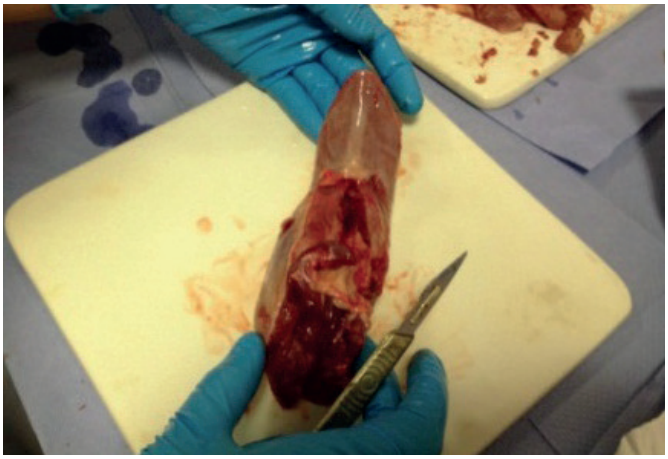
For each sample and each tissue cumulative amounts of vitamin B12 permeated per unit area were plotted against time. Flux values were calculated using the linear portions of these graphs.



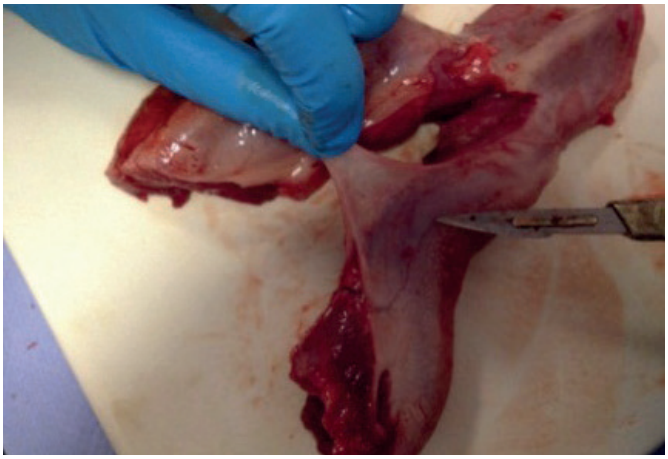


Figure 2: *Ventral side of the tongue (left), sublingual membrane excision (right)*

Time (mins)	% MeOH	% H_20 (0.1%TFA)
0	20	80
20	20	80
25	80	20

Figure 3.

3. Results

Figure 4 shows the cumulative permeation of vitamin B12 across sublingual membranes from an INFINITE dose of Boost oral spray as nanomoles/cm^2.

Figure 5 shows the cumulative permeation of vitamin B12 across sublingual membranes from an INFINITE dose of Boost oral spray as micrograms/cm2.

Figure 6 shows the cumulative permeation of vitamin B12 across sublingual membranes from a FINITE (SINGLE SPRAY) dose of Boost oral spray as nanomoles/cm2.

Figure 7 shows the cumulative permeation of vitamin B12 across sublingual membranes from a FINITE (SINGLE SPRAY) dose of Boost oral spray as micrograms/cm2.

Figure 8 shows the cumulative percentage permeation of vitamin B12 across sublingual membranes from a FINITE (SINGLE SPRAY CONTAINING 300 MICROGRAMS of vitamin B12) dose of Boost oral spray as micrograms/cm2.

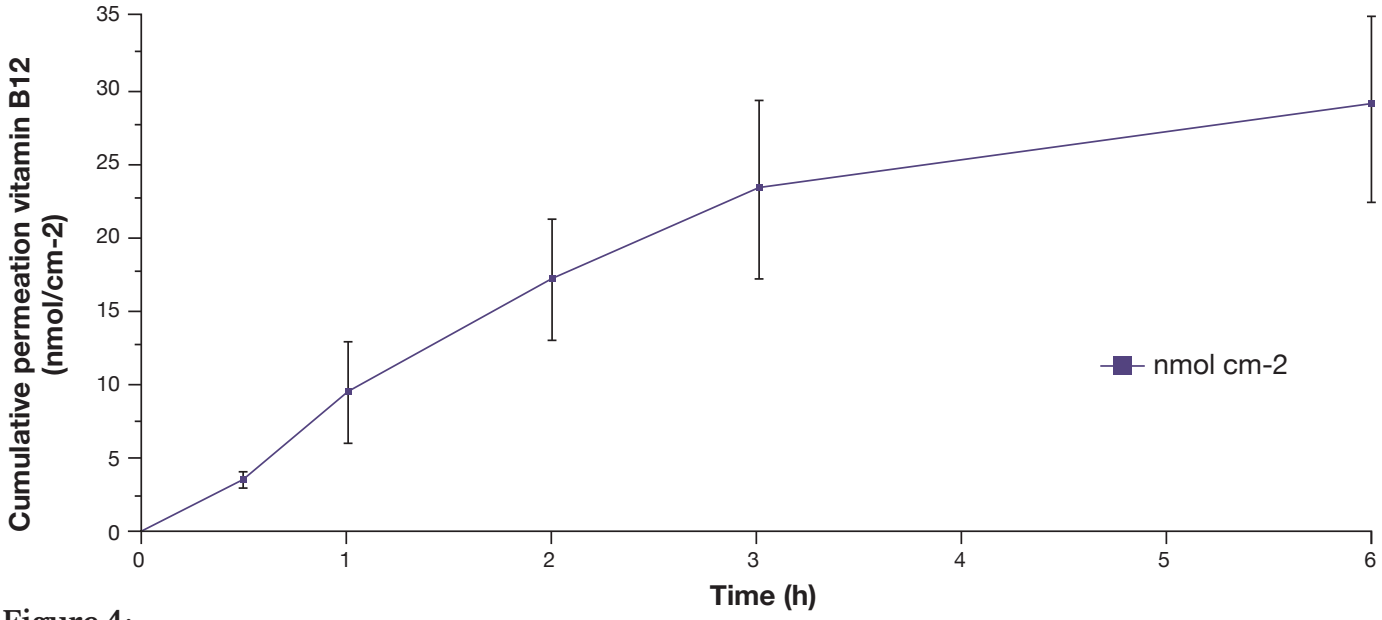


Figure 4:

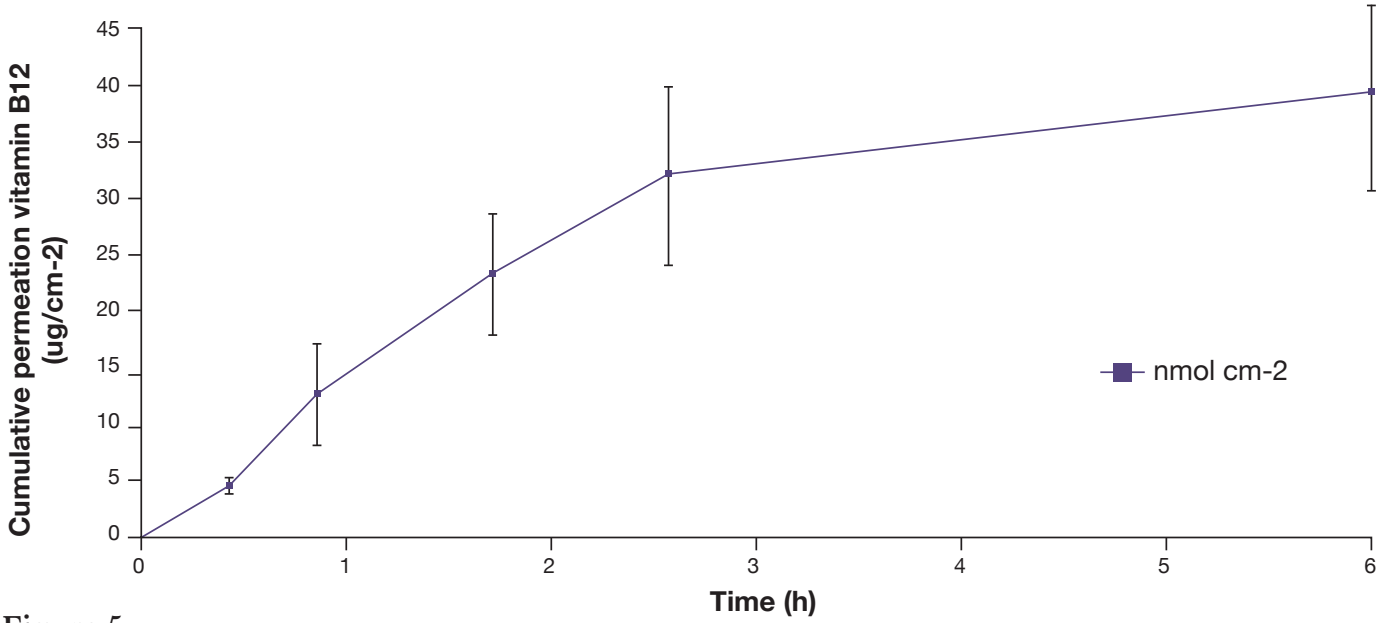


Figure 5:

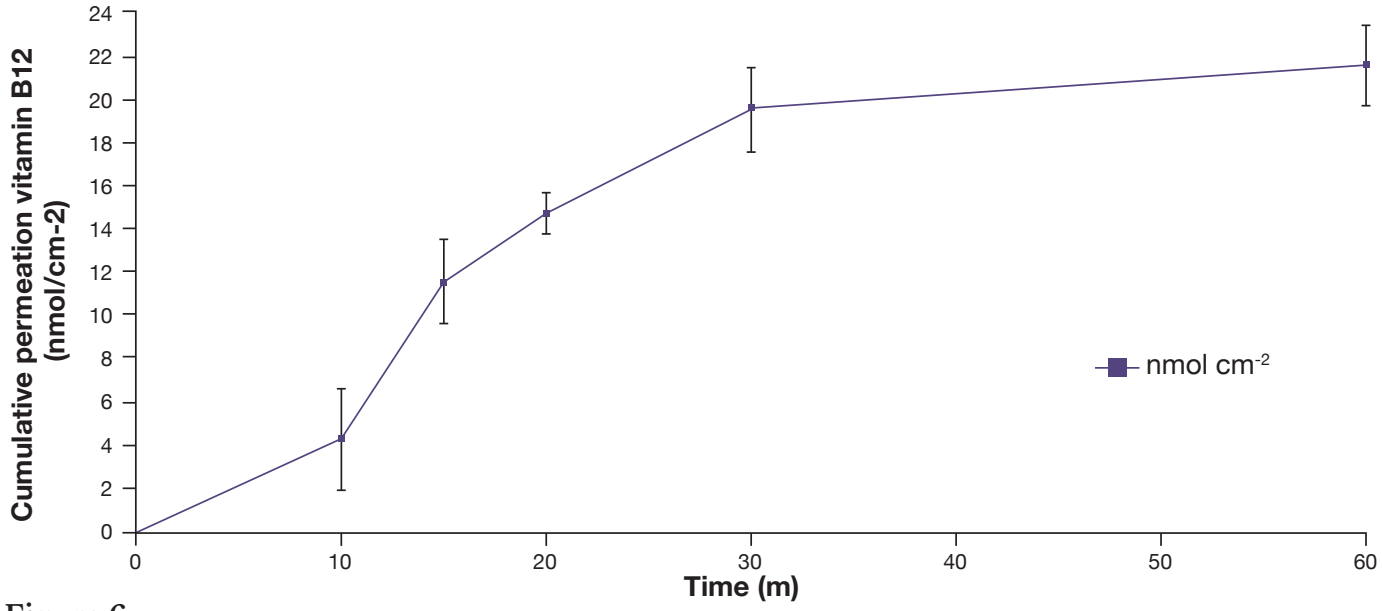


Figure 6:

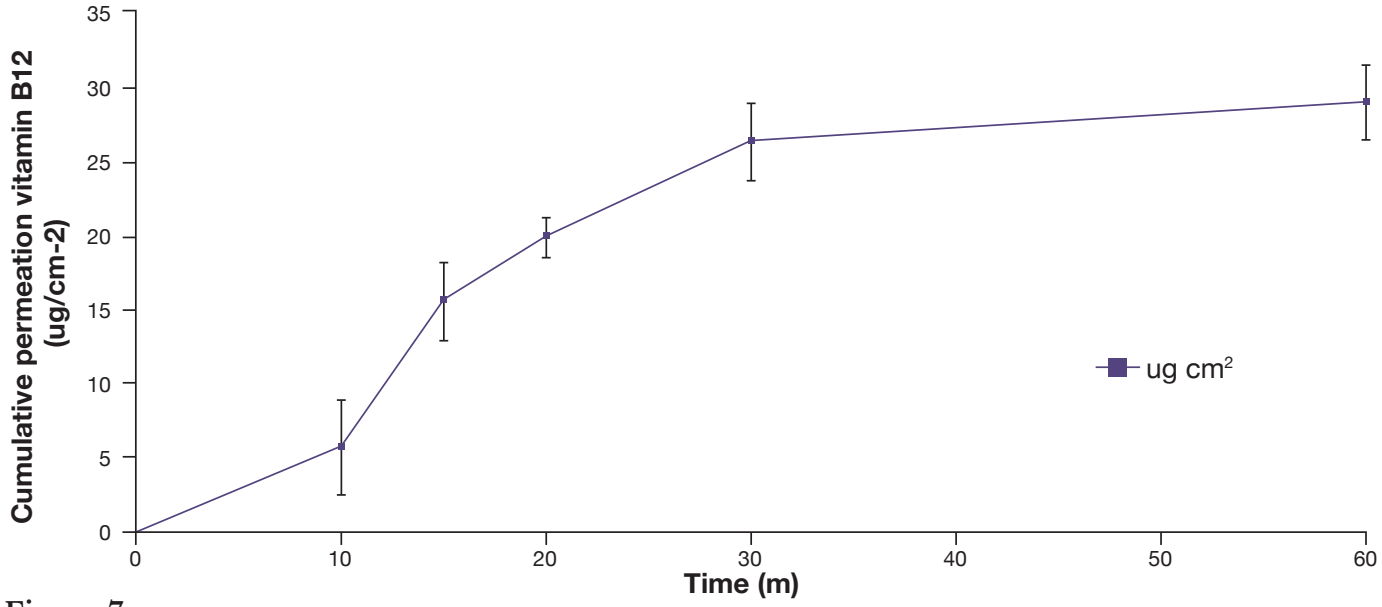


Figure 7:

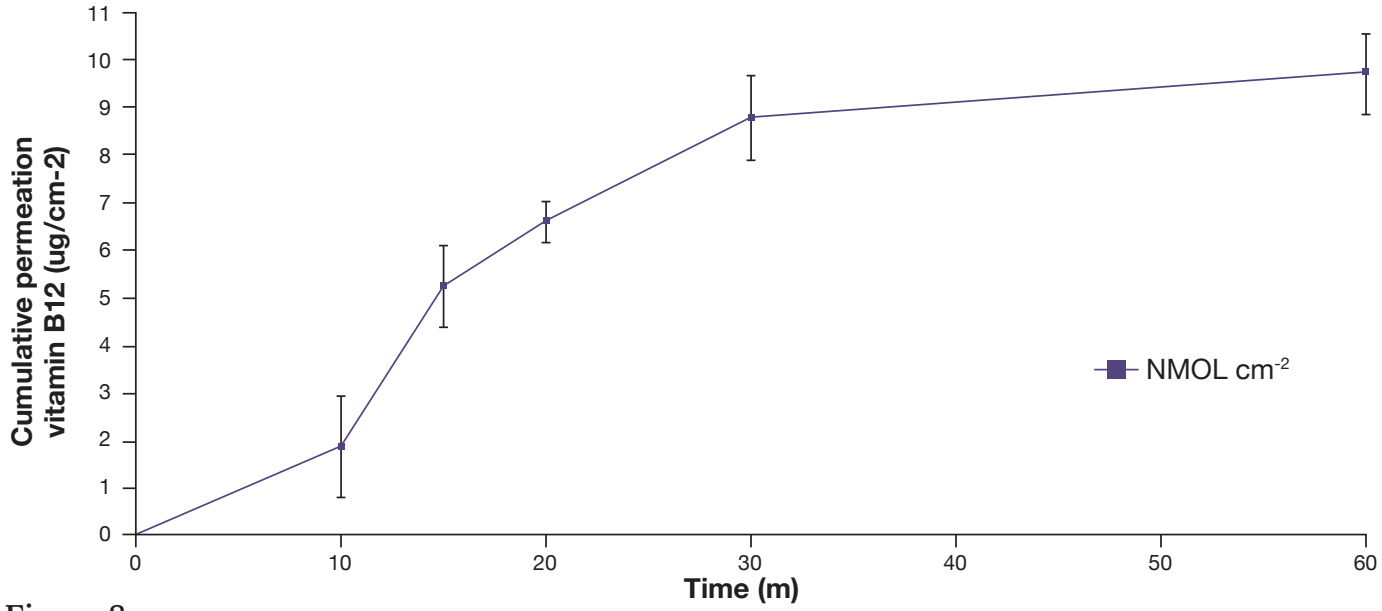


Figure 8:

4. Conclusions

1. ‘Infinite’ refers to the maximal amount that can permeate the membrane; ‘finite’ refers to the application of a single spray as per in-use conditions.
2. Vitamin B12 readily permeated excised sublingual membranes. This result is unexpected, given it is a large water-soluble molecule, with a molecular weight of 1355.37. The micro emulsion formula and spray delivery mechanism may explain, in part, this exceptional result.
3. The dietary reference intake for an adult ranges from 2 to 3 µg per day – this figure is readily attained from a SINGLE spray application.
4. Based on the in vitro data contained herein, the B12 Boost spray product appears to be an effective supplement for the rapid attainment of recommended levels of vitamin B12.
5. In practical terms the findings indicate that at least 30mcg of vitamin B12 will enter the bloodstream one hour after a single spray application.

A randomised two way cross over study for comparison of absorption of vitamin D3 buccal spray and soft gelatin capsule formulation in healthy subjects and in patients with intestinal malabsorption

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Abstract

An open label, randomised, two-periods, two-way cross over study to investigate the comparative uptake of vitamin D via a buccal spray with a capsule containing to same dosage. The study further investigated the relative serum concentration increase of subjects who were considered healthy compared to those with pre-diagnosed intestinal malabsorption issues.

Introduction

Vitamin D is essential for active intestinal calcium absorption and plays a central role in maintaining calcium homeostasis and skeletal integrity. It is derived mainly from cutaneous synthesis in the presence of ultraviolet sunlight while dietary intake constitutes a minor fraction [1]. Vitamin D deficiency is a common problem through the world [2, 3] and is assessed by low serum concentration of the major circulating metabolite 25-hydroxyvitamin D (25(OH)D) [4, 5]. The prevention of vitamin D deficiency and insufficiency remains a priority of international health services [6–8]. Vitamin D deficiency has been proposed to contribute to the development of intestinal bowel diseases like Crohn's disease, steatorrhea and ulcerative colitis [9]. Conversely, people who have such illnesses have a reduced absorption of vitamin D3 through the intestine [10, 11]. In addition, osteomalacia occurs in patients with a wide variety of disorders affecting the stomach and small bowel, especially when associated with steatorrhea. The pathogenesis of this osteomalacia has in part been explained by malabsorption in vitamin D [9]. Earlier reports show that orally administered tritiated vitamin D3 was malabsorbed in patients with celiac disease, biliary obstruction or pancreatic disease [12]. The pathogenesis of vitamin D deficiency in these patients remains unclear but it is thought to result primarily from fat soluble vitamin malabsorption due to the presence of intestinal disease conditions.

Despite vitamin D malabsorption in patients with gastrointestinal or liver disease, the vitamin status of these patients is often neglected. Although vitamin D supplements are often prescribed, adequate absorption of these formulations has not been documented [10]. Vitamin D is known to be liposoluble, and its relative bioavailability could result in unfavorable conditions when administered in solid form (capsule), since the process of its release is a factor limitingthe rate of absorption, bearing in mind that bioavailability is related not only to the pharmaceutically active molecules, but also, to the formulation and excipients used.

Vitamin D3 taken by oral route (peroral delivery) is absorbed in the intestine, where the lining of the digestive tract is aqueous in nature. Therefore vitamin D3, a fatsoluble molecule, in order to be absorbed, must be made water soluble in the intestine. This is accomplished in two steps: emulsification of vitamin D3 in the intestinal lumen, through the action of bile salts, forming small droplets which are dispersed and incorporated into micelles-complex aggregates formed by the interaction of free fatty acids, monoglycerides, and bile salts. Micelles are sufficiently water-soluble to access the intestinal brush border where upon the vitamin D3 content is released and then absorbed [13].

When sprayed inside the mouth, the fine micro sized droplets of vitamin D3 are believed to be quickly and completely absorbed through the buccal mucosa into the numerous capillaries and veins lying close to the tissue surface [13]. Considering the possibility of reduced vitamin D absorption in healthy subjects and even in patients with malabsorption syndrome, a buccal spray formulation was developed. Therefore, the objective of the present work was to compare the absorption of vitamin D3 through the oral route (soft gelatin capsule form) and buccal spray in healthy subjects and patients with intestinal malabsorption syndrome.

Methods

Study design and patients

An open label, randomized, two-periods, two-way cross over study was conducted, first in healthy subjects and then in patients with malabsorption syndrome, with a similar study design except the presence of disease status in patients with malabsorption syndrome. After approval from the Spandan-IEC ethics committee (registration no: ECR/67/Indt/GJ/2013), the informed consent of study participants were taken and the formulation was administered for 30 days in period I where half of the subjects and patients (collectively participants) received capsule formulation and half of the participants received buccal spray formulation. After completion of treatment in period I, all participants were given 30 days wash out before initiating period II where treatment has changed in cross over fashion, i.e. the participants who received capsule formulation in period I have received buccal spray in period II and vice versa. The clinical study was registered in a centralized clinical trial registry of India (CTRI) before initiating the enrollment of the first patient in the study (CTRI/2013/06/003770).

The inclusion criteria were as follows: subjects of either sex between 18 and 65 years of age, with a Body Mass Index (BMI) between 18.0 and 30.0 kg/m2, and the ability to comply with study procedures in the opinion of the investigators.For healthy subjects: no history of liver, kidney or cardiovascular disease, or of any other medical conditions or medications likely to affect vitamin D3 absorption or metabolism. For patients with malabsorption syndrome: confirmed diagnosis of any one of the following malabsorption disease conditions like ulcerative colitis, Crohn's disease or steatorrhea. Patient with history of above diseases, who are on therapy, were selected for the screening. In these patients, malabsorption syndrome was diagnosed by clinical symptoms like abdominal pain, vomiting, diarrhea, and subcutaneous fat loss together with blood tests like haematology and biochemistry. Stool examination was also performed for all patients to confirm rectal bleeding, presence of occult blood, infectious organisms, or fat. Finally, colonoscopy was performed in all patients to objectively assess the extent of inflammation to

confirm the diagnosis. The exclusion criteria were as follows: systemic inflammatory or malignant disease; hepatic or renal failure; uncontrolled hypo- or hyperthyroidism, or the use of drugs that are known to affect bone metabolism such as bisphosphonates, glucocorticoids and anti-convulsants. A pregnant or desired to be pregnant woman during study period was also excluded.

Data collected at baseline

Each participant completed a self-administered questionnaire before enrollment. During completion of the questionnaire, they had the possibility to ask for assistance (i.e. clarification of question or any other issue) from one of the project leaders. The questionnaire included questions about usual intake of vitamin D containing foods, clothing and sun exposure habits, as well as height & weight, date of birth, and education. Body mass index was derived as wt/ht2 (kg/m2). Blood pressure was measured through sphygmomanometer and vitals (heart rate, body temperature) were also recorded for safety purposes.

Collection and analyses of blood samples

Fasting blood samples were collected to measure baseline 25(OH)D levels in all the participants at day 0 (Screening visit), day 30 (completion of period I), day 60 (end of wash out and initiation of period II) and day 90 (completion of period II). Blood samples were centrifuged (15 min; 2000g at 4°C) within 30min of blood collection and separated serum samples were immediately frozen. Serum samples were stored at -20°C until analyzed. Serum 25(OH)D levels were measured by Electrochemiluminescence (ECLIA) assay method. This assay was carried out through quantitative determinations of total 25-hydroxyvitamin D in serum samples using a standard kit available from Roche diagnostics GmbH, Germany. All analyses were done in a central independent clinical analysis laboratory (APL Institute of Clinical Laboratory & Research Pvt. Ltd., Ahmedabad, India). The kit has a limit of detection of 3ng/mL and has a linearity of 0.0 to 60.0ng/mL. The intra-assay and inter-assay co-efficient of variation were 4% and 6%, respectively. Elecsys eimmunoassay analyzers were used for this assay.

Safety parameters were evaluated including hematology analyses (complete blood counts), biochemical analyses (serum creatinine, total bilirubin, urea, SGOT, SGPT, alkaline phosphates, calcium) and urine was collected for urine routine and microbiological analyses at screening visit (day 0) and at the end of period II visit (day 90).

Intervention randomization and compliance

Randomization and group allocation: The participants were enrolled at two different hospital sites in India; one physician's site where all healthy subjects were recruited and a gastroenterologist's site where all patients with intestinal malabsorption were recruited. Out of the forty-eight participants who met the eligibility criteria, forty (twenty healthy and twenty patients) had agreed to participate and were found eligible, signed a written consent form, and completed a self administered questionnaire concerning usual diet and sun exposure. Subsequently, a venous blood sample was drawn, and a participant received a sealed, non-transparent envelope with the allocated treatment intervention. The randomization procedure was performed beforehand by a statistician by block randomization with blocks of two to one ratio, where the first two participants were randomly given each treatment and a third participant served as a control and didn't receive any treatment. The participants were allocated to the interventions as they visited the clinic i.e. the first subject was allocated to buccal spray group, second to soft gelatin capsule group and third to the control group. This was done in order to distribute the participants equally in the two interventions (Group I and Group III) and half of the participants served as a control (Group II and Group IV).

Twenty healthy subjects enrolled for the study were recruited at a physician's site. They were randomized and enrolled into group I and II: fourteen subjects were enrolled for vitamin D3 treatment and labeled as group I (healthy subjects), while every third subject (total six subjects) were not given any treatment (labeled as group II) and acted as the control for group I.

The twenty patients with confirmed diagnosis of malabsorption syndromes (nine with ulcerative colitis, four with Crohn's disease and seven with steatorrhea) were recruited at a gastroenterologist's hospital site. Fourteen subjects were enrolled for vitamin D3 treatment and labeled as group III (patients with malabsorption syndrome), while every third subject (total six subjects) were not given any treatment (labeled as group IV) and acted as the control for group III. All patients continued to take their treatment for ulcerative colitis, Crohn's disease and steatorrhea as prescribed by gastroenterologists.

Treatment allocation was assigned to each participant according to their number of sequence of attendance at the blood sampling. Treatment allocation was concealed in envelopes numbered in ascending order that they met. Study personnel involved in recruitment and data collection were blinded to the participant's treatment allocation. All participants were instructed to maintain their routine lifestyle including diet habits and sun exposure during the entire study period, to minimize interference with the daily routine.

Intervention

The buccal spray and soft gelatin capsules containing vitamin D3 were supplied by Pharma Base, India (a subsidiary of Pharma Base SA, Switzerland). The soft gelatin capsule formulation was purchased from the Indian market. The analysis of both the formulations was done in triplicate according to the method described in European Pharmacopeia at an independent analytical laboratory (Oasis Testing House, Ahmedabad, India). The soft gelatin capsule formulation had a label content of 1000 IU per capsule and the buccal spray formulation had a label content of 500 IU/spray shot.

All participants in group I & III were randomized to receive either the vitamin D3 buccal spray (2 sprays each of 500 IU) or soft gelatin capsule containing vitamin D3 (1000 IU) for 30 days. Of the fourteen subjects in each group, half received buccal spray and half received soft gelatin capsule. This was considered as period I of the study. After the completion of the 30-day treatment, all participants were given a 30-day washout. The next treatment in period II was changed in a crossover fashion; those participants who had received the buccal spray formulation (vitamin D3) in period I received the soft gelatin capsule formulation in period II and vice versa. The treatment in period II continued in group I and group III participants for the next 30 days. The detailed study flow chart is described in Fig. 1.

Compliance

All participants were instructed to take the two buccal sprays and one soft gelatin capsule every day irrespective of the period of the study. Formulation for 7 days was handed over to each participants under group I & III at baseline, along with the compliance form. The participants were instructed to mark the intake and time of intake of number of spray shots or capsule of each day of the study period, as well as to note any extraordinary event that occurred during the period (forgetting to take a spray shot or capsule). If forgetting to take spray shot or capsule, the participants were instructed to take a double dose on the following day, in order to take altogether 60 spray shots or 30 capsules during the study periods. The participants were recommended to take spray shots or capsules after the main meal of the day. However, participants were not instructed to standardize meals or time between meals. All participants were also instructed not to change their daily routine, meal habits, as well as their sun exposure. This was done to minimize interference with daily routine and thus maximizing compliance with taking study medication. All participants in group I and III were instructed to visit the site every week to check compliance with the intake of study medication. Participants in control groups (Group II and Group IV) were instructed to visit their respective clinic after 30 days for their blood sample collection.

All participants who received treatment were also instructed to note any adverse events during the entire 90 days of the study which included period I, washout period and period II in a separately given adverse event recording form.

Stastical Analysis

Sample size

The sample size of this crossover study was based on the changes in 25(OH)D levels where goal was to detect: 1) percent increase between pre-dose and post-dose levels within treatments, and 2) to have significant difference of percent change in 25(OH)D levels between the treatments. To achieve type-I error rate of less than 5% (2 tailed), a sample size of 12 subjects in buccal spray group (healthy or patients) was sufficient to provide a statistical power of 80% to detect a clinically significant mean difference of 5% in 25(OH)D levels.

Demographic and baseline characteristic

A descriptive statistics was applied for demographics; age (years), weight (kg), body mass index (kg/m2); and were presented as mean ± standard deviation.

Efficacy analysis

Statistical analyses were performed using GraphPad Prism 5, version 5.03 (GraphPad Software, Inc., CA, USA). Differences of mean and percentage change from baseline of 25(OH)D levels between two formulation groups, soft gelatin capsules and buccal spray, were evaluated using two tailed Paired t-test with 95% confidence interval separately for both healthy subjects and patients with intestinal malabsorption. For comparison between control group and either of the treatments, unpaired t-test with 95% confidence interval was used.

Safety analysis

The number and proportion of subjects with changes in laboratory value (change from baseline to end of study visit for complete blood count, serum creatinine, total bilirubin, urea, SGOT, SGPT, alkaline phosphates and calcium) was summarized and the difference was analyzed by chi –square test from normal to abnormal.

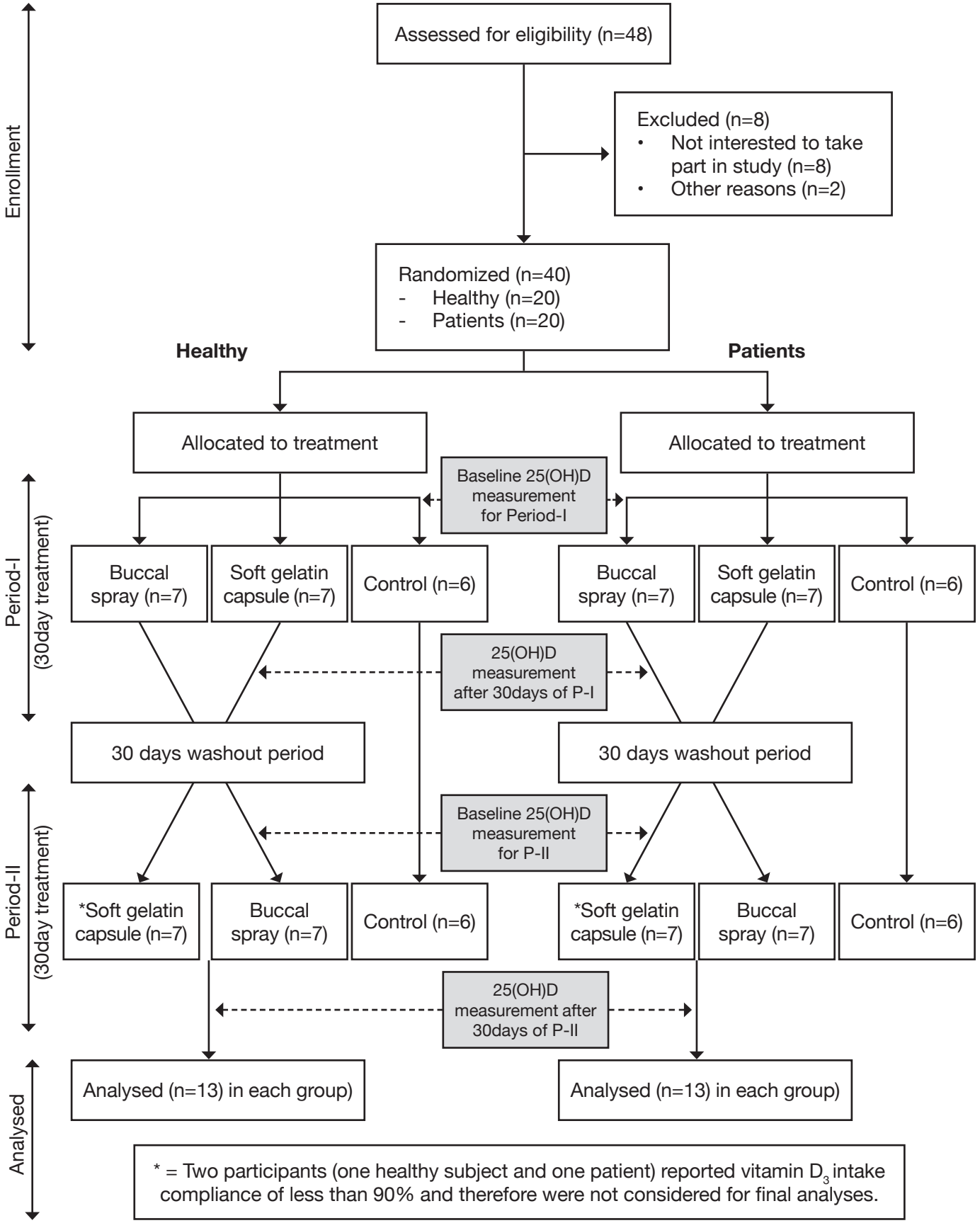

Figure 1: *Study flow chart.*

Results

Baseline characteristics:

In total, thirty eight individuals, thirteen healthy individuals with six control and thirteen patients with malabsorption syndrome with six control, fulfilled the eligibility criteria and completed the study (Fig. 1). Two participants (one healthy subject and one patient) reported vitamin D3 intake compliance of less than 90% and therefore were not considered for final analyses.

In healthy subjects, at baseline, 85% (n = 12) of the subjects had serum 25(OH)D concentration between 10 and 30ng/ml, and one individual each (7.14%) had serum 25(OH)D concentration >30ng/ml and <10ng/ml each in the oral soft gelatin capsule group. While in the buccalspray group, at baseline all subjects had serum 25(OH)D concentration between 10 and 30ngml. Similarly, in patients with malabsorption disease who received oral soft gelatin capsules, at baseline 8 (57.14%) patients had serum 25(OH)D concentration below 10 ng/ml, 5 (35.71%) patients had serum 25(OH)D concentration between 10 and 20 ng/ml, and one individual (7.14%) had serum 25(OH)D concentration above 20 ng/ml. However, in the buccal spray group, at baseline 9 (64.28%) patients had serum 25(OH) D concentration below 10 ng/ml and 5 (35.71%) patients had serum 25(OH)D concentration between 10 and 20ng/ml. There were no striking differences in baseline characteristics between healthy individuals or patients with malabsorption disease with their corresponding control groups (Table 1).

Parameters	Healthy Subjects Group I	Healthy Subjects Control Group II	Patients with malabsorption syndrome Group III	Patients with malabsorption syndrome Control Group IV
N	14	6	14	6
Sex	Male = 7, Female = 7	Male = 3, Female = 3	Male = 7, Female = 7	Male = 3, Female = 3
Age (Yrs)	36.21±9.97	34.00±6.42	39.93±11.65	44.17±5.56
(Range)	(25–60)	(25–42)	(26–63)	(38–53)
Height (cms)	159.86±13.43	161.33±14.12	162.29±8.54	164.33±8.55
BMI	23.39±3.88	21.40±2.39	21.48±2.82	23.64±3.02
All values are expressed in Mean ± SD; N-number subjects in each group				

Table 1. *Demographic Data for Healthy Subjects and Patients with malabsorption syndrome*

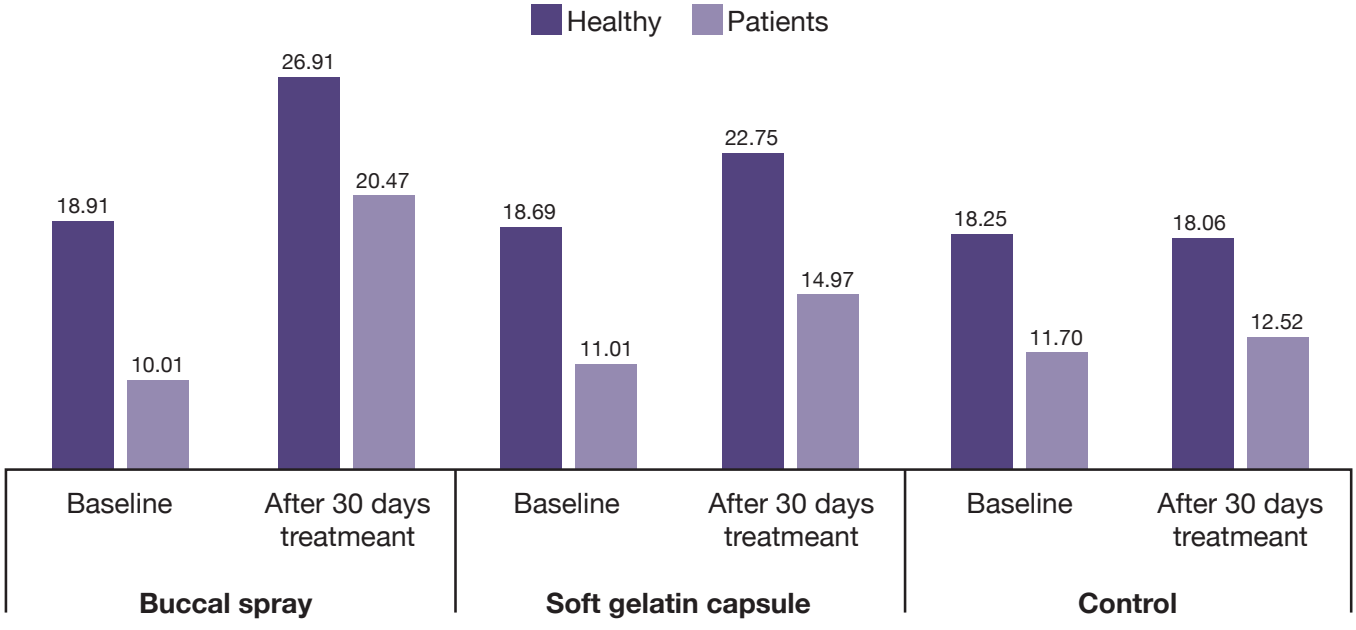

Figure 2: *Mean 25(OH)D level in study subjects*

Effect of intervention

The control groups in each subject population were compared with their corresponding treatment group. The control groups for healthy subjects as well as patients with malabsorption syndrome showed no change in 25(OH)D concentration over a period of 30 days. The mean baseline levels of 25(OH)D in healthy subjects was 18.25ng/ml and in patients with malabsorption syndrome was 11.7ng/ml. These mean levels remained at 18.06ng/ml and 12.52ng/ml after 30 days in healthy subjects and patients with malabsorption syndrome respectively (Fig. 2), which was statistically non-significant. When control group in healthy subjects was compared with their corresponding treatment groups, it was found that the difference of mean between control group and buccal spray group was 7.47 (95% CI, 5.27, 9.67) which was significant ($p < 0.05$), and the same between control group and soft gelatin capsule group was 3.53 (95% CI, 1.79, 5.28) which was also statistically significant ($p < 0.05$). Similarly in patients, the difference of mean between control group and buccal spray group was 8.53 (95% CI, 2.74, 14.31) which was significant ($p < 0.05$), while the difference of mean between control group and soft gelatin capsule group was 2.03 (95% CI, −1.44, 5.50) which was statistically not significant. This shows that buccal spray was more effective to increase mean 25(OH)D levels as compared to oral soft gelatin capsule. Table 2 describes these data.

The efficacy of buccal spray and soft gelatin capsule to increase the 25(OH)D levels after 30 days of administration was evaluated and compared with each other. After 30 days of administration, overall mean serum 25(OH)D concentration in healthy subjects was 22.75 (sd 6.75) ng/ml as compared to baseline value of 18.69(sd5.88)ng/ml in the soft gelatin capsule group, with the mean increase of 4.06 (95% CI 3.41, 4.71) ng/ml (Table 3). On the other hand, in the buccal spray group, the mean serum 25(OH)D concentration was 26.9 (sd 5.72) ng/ml as compared to baseline value of 18.91 (sd 4.3) ng/ml, with the mean increase of 8.0 (95% CI 6.86, 9.13) ng/ml (Fig. 2). The difference in mean increase between both the groups was 3.95 (95% CI 3.19, 4.69) which was statistically significant ($p < 0.0001$).

When calculated from baseline, the mean percentage change in serum 25(OH)D concentration in healthy subjects after 30 days treatment with soft gelatin capsule was 21.72% (95% CI 16.42, 24.42), while the same in buccal spray group was 42.99% (95 % CI 37.19, 48.79) with a mean difference of 20.42 % (95% CI 16.42, 24.42) between two groups ($p < 0.0001$) (Table 3). A total of 11 (85%) subjects now had serum 25(OH)D concentration between 10 and 30 ng/ml, and two individuals (15.38%) now had serum 25(OH)D concentration >30 ng/ml in the soft gelatin capsule group. However, in the buccal spray group, 10 (75.88%) subjects had serum 25(OH)D concentration between 10 and 30 ng/ml and 3 (23.1%) subjects now had serum 25(OH)D concentration >30 ng/ml.

Similarly in patients with malabsorption syndrome, overall mean serum 25(OH)D concentration after 30 days of administration of soft gelatin capsule was 14.97 (sd 9.01) ng/ml as compared to aseline value of 11.01 (sd 6.43) ng/ml, with the mean increase of 3.96 (95% CI 2.37, 5.56) ng/ml. While in buccal spray group, mean serum 25(OH)D concentration was 20.47 (sd 7.89) ng/ml as compared to baseline value of 10.01 (sd 4.29) ng/ml, with the mean increase of 10.46(95 % CI 6.89, 14.03) ng/ml (Fig. 2). The difference in mean increase between both the groups was 6.50 (95 % CI 3.78, 9.22) which was statistically significant ($p < 0.0001$).

The mean percentage change in serum 25(OH)D concentration in patients with malabsorption syndrome after 30 days treatment with soft gelatin capsule was 36.02% (95 % CI 30.42, 41.62), while the same value in buccal spray group was 117.8% (95% CI 64.71, 170.8) with a mean difference of 81.75% (95% CI 29.80, 133.7) between the two treatments ($p < 0.005$). A total of four (31%) subjects now had serum 25(OH)D concentration below 10 ng/ml, eight (61%) subjects now had serum 25(OH)D concentration between 10 and 30 ng/ml, and one individual (7.7%) had serum 25(OH)D concentration above 30 ng/ml. However, in the buccal spray group, only 7.7% (1 subject) now had serum 25(OH)D concentration below 10 ng/ml, ten (76.9%) subjects now had serum 25(OH)D concentration between 10 and 30 ng/ml and two (15.4%) subjects now had serum 25(OH)D concentration more than 30 ng/ml (Table 3).

Statistical considerations with respect to period, sequence and power:

Statistical analyses were performed using SAS v9.2 (SAS Institute Inc, Cary, NC, USA) for the post-hoc evaluation of sequence and period effect. The statistical method adopted for this analysis was period + sequence + Subject (sequence) + treatment. It was observed that there is no significant difference in sequence effect in healthy subjects ($p = 0.5251$) and in patients with malabsorption syndrome ($p = 0.0532$). It was also revealed that there is no statistically significant period effect in healthy subjects ($p = 0.6920$) as well as in patients with malabsorption syndrome ($p = 0.0715$). The post-hoc power analyses with intra-subject variability were also derived for both the group of subjects. In healthy subjects, statistical power obtained was 99.42 with an 11.81% intra-subject variability. While, in patients with malabsorption syndrome it was 81.62 with an intra-subject variability of 21.86%.

Safety evaluation

There were no significant changes in any of the hematology and biochemistry parameters studied. There are also no notable changes in the vitals for any of the participants. No adverse event reported after administration of the buccal spray or soft gelatin capsule formulations of vitamin D3 during entire study period in healthy or patients with malabsorption disease and hence the product is considered safe.

Discussion

Supplemental fat-soluble vitamin D is usually made without determination of whether oral doses are adequately absorbed. The evidence of vitamin D malabsorption (Osteomalacia, rickets, hypocalcaemia, or reduced circulating concentration of 25(OH)D) persists despite routine vitamin D supplementation in cystic fibrosis [14], Crohn's disease [15], Intestinal resection [16–19], ulcerative colitis, liver disease [20–22] and other malabsorption syndrome [23].

Many factors are involved in the absorption of vitamin D, including gastric, pancreatic, and biliary secretions, micelle formation, and diffusion through the unstirred water layer, brush border membrane uptake, and transport out of the intestinal cell [24, 25]. As vitamin D is a relatively nonpolar sterol, it must be solubilized by incorporation into a bile salt micelle solution in order to be absorbed in the aqueous phase [26]. This process is severely inhibited if there is any interruption of normal pancreatic or biliary secretion. As fat-soluble vitamins are fairly sensitive to disturbances in lipid absorption, vitamin malabsorption may occur in conditions like steatorrhea, ulcerative colitis, and Crohn's disease. Serum concentrations of 25-hydroxyvitamin D are good indicators of long term vitamin D levels in the body but are insensitive to single doses of vitamin D and do not rise out of the normal range unless doses of vitamin D are chronically administered [27].

Considering the malabsorption in intestinal disease and a possible poor and complex absorption with oral formulations, a novel nanoemulsion formulation of buccal spray was developed where vitamin D3 is suspended in an aqueous base which can be easily absorbed through the mucosal layer of the mouth. We compared the serum concentration of vitamin D after 30 days administration with the soft gelatin capsule and the aqueous based buccal spray formulation. To the best of our knowledge, this is the first randomized two way cross over trial comparing the increase in 25(OH)D levels in healthy adults and patients with intestinal malabsorption receiving similar oral doses of two different formulations (per oral and buccal spray).

The analyses of baseline levels of study participants showed that vitamin D deficiency was prevalent in both healthy subjects and patients with intestinal malabsorption syndrome. However, patients were more vitamin D deficient as compared to healthy subjects. Four weeks administration of 1000 IU per day increased mean serum 25(OH) D in all treatment groups. In healthy subjects, soft gelatin capsule increased serum 25(OH) D level by 22.5% (Range 15.4 to 37.8%), while the buccal spray increased serum 25(OH)D level by 43% (range 29.2 to 68.7. Similarly, in patients with intestinal malabsorption, soft gelatin capsule increased serum 25(OH)D level by 36% (range 24.6 to 58.7%) and the buccal spray increased serum 25(OH)D by 117.8% (range 61.3 to 381.1%).

The result implies that the buccal spray formulation had a significantly higher mean increase in both the subject groups, healthy subjects and patients with intestinal malabsorption syndrome. Interestingly, the mean increase was much higher in the patients group as compared to the healthy subjects group. This may be because increase in serum 25(OH) D after supplementation is known to be inversely related to baseline 25(OH)D concentration [28].

In the present study, the mean baseline levels were almost half in patients with intestinal malabsorption as compared to the baseline levels found in healthy subjects. This indicates the presence of vitamin D3 deficiency in the patient group as compared to healthy subjects. The primary and the most important source of vitamin D is sunlight. Although excessive exposure to sunlight and vitamin D have been positively associated with non-melanoma skin cancer [29], ecological studies suggest that sunlight may protect against female breast, ovarian, prostate, and colon cancer [30]. Solar UV-B exposure and the amount of exposure to sun are related inversely with cancer mortality and survival in detailed epidemiological studies [31]. Some analytical studies suggest a protective association between circulating vitamin D in blood, which is largely derived from sunlight or dietary vitamin D, and colorectal cancer and prostate cancer [30]. However, looking at the overall baseline levels of all participants in the present study, it is also advisable to increase the moderate daily sun exposure and to improve clothing apart from vitamin D3 supplementation.

Conclusion

We conclude that the buccal spray formulation was able to increase mean serum vitamin D3 concentration significantly higher as compared to the soft gelatin capsule, in both healthy subjects (1.9 times) as well as in patients with intestinal malabsorption syndrome (2.6 times).

Availability of supporting data

The data set(s) supporting the results of this article is (are) included within the article.

Abbreviations

25(OH)D: 25-hydroxyvitamin D; CTRI: clinical trial registry of India; BMI: Body Mass Index; ECLIA:

Electrochemiluminescence; IU: International Unit.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MCS participated in design of the study, analyses and interpretation of data, performed the statistical analyses, and drafting revising, and finalizing the manuscript. AGM participated as investigator in the study, involved in healthy subject recruitment, their compliance and acquisition of the data. KDT participated as investigator in the study, involved in patient recruitment, their compliance and acquisition of the data. MSB participated as investigator in the study, involved in patient recruitment, their compliance and acquisition of the data. All authors have read and approved the final manuscript.

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BetterYou study

Athens University

Abstract

A study to investigate the comparative uptake of vitamin D via oral spray and tablet, both of the same strength and both commercially available in the Greek market.

After a period of 30 days those using the oral spray demonstrated a serum elevation 52% greater than those taking the tablet. In addition those subjects with low initial vitamin D levels exhibited, in most cases, higher percentages of elevation than those with pre-study high vitamin D levels.

Summary report

The results of the study are summarised in Table 1. A solid 95% of the subjects demonstrated an increase in vitamin D levels a daily intake (1000 units) of BetterYou oral spray. Only in one case (Subject 7) vitamin D levels were not elevated and a small decrease of 3.85% was observed. Figure 1 presents the % change in vitamin D levels in comparison with subjects' Body Mass Index.

*It has to be noted that the daily dosage of 1000 IU is not adequate in cases of significant vitamin D deficiency (<20ng/ml). Still, it was preferred not to provide subjects with large dosages (>=3.000 IU) in order to better determine the bioavailability of the product.

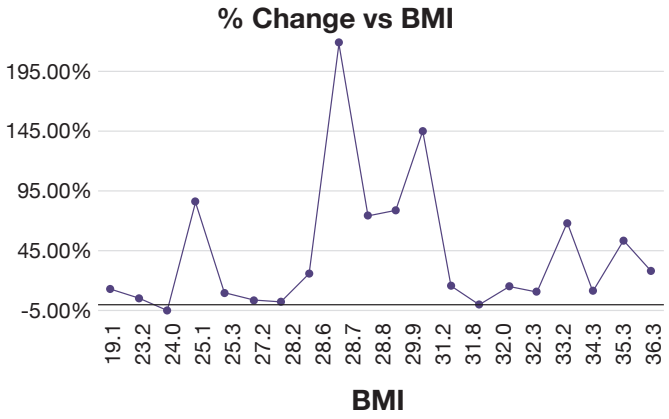

Figure 1: *% Change in vitamin D levels vs BMI*

Subject	Vitamin D before (ng/ml)	Vitamin D after (ng/ml)	% Change	Weight (kg)	Height (m)	Body Mass Index
1	24.10	24.40	1.24	95.10	1.73	31.8
2	10.26	32.60	217.74	84.60	1.72	28.6
3	13.90	21.20	52.52	95.90	1.64	35.3
4	18.50	30.90	67.03	72.70	1.48	33.2
5	12.50	14.40	15.20	84.00	1.62	32.0
6	19.00	24.20	27.37	71.00	1.58	28.4
7	26.00	25.00	-3.85	60.70	1.59	29.9
8	8.80	21.40	143.18	75.70	1.59	29.9
9	13.30	23.90	79.70	73.70	1.60	28.8
10	23.40	26.60	13,68	60.00	1.77	19.1
11	15.00	26.50	76.67	81.00	1.68	28.7
12	20.60	24.00	16.50	86.00	1.66	31.2
13	13.60	25.30	86.03	76.00	1.74	25.1
14	24.10	26.80	11.20	89.00	1.66	32.3
15	27.40	30.60	11.68	105.00	1.75	34.3
16	28.75	31.97	11.20	75.00	1.72	25.3
17	22.90	24.10	5.24	61.00	1.62	23.2
18	25.20	26.40	4.76	67.00	1.57	27.2
19	27.60	28.30	2.54	75.00	1.63	28.2
20	17.10	21.98	28.54	88.30	1.56	36.3
Average	**19.60**	**25.53**	**30.24**	**78.79**	**1.65**	**29.15**

Table 1. *Study results**

The average increase of vitamin D levels is 65.93 ng/ml, which represents an on average increase of 30.24%. The results compare good with those of the two competing products, as shown in table 2.

BetterYou – DLux1000 (1000 IU/daily spray)	**Solgar – Vitamin D3** (1000 IU – 1 tablet daily)
5.93ng/ml	3.88ng/ml
30.24%	21.27%

Table 2.

Specifically for BetterYou, 35% of the patients had an increase greater than 50%, and 40% an increase between 10 and 50%. It has to be noted that an extreme upper value was observed, that of Subject 2, which demonstrated an increase of vitamin D levels reaching 217.74%. We decided to include it in our calculations. Finally, as expected, subjects with low initial vitamin D levels exhibit in most cases higher percentages of elevation than those subjects with pre-study high vitamin D levels. This finding becomes more obvious if on elooks in the radiant diagram of Figure 2.

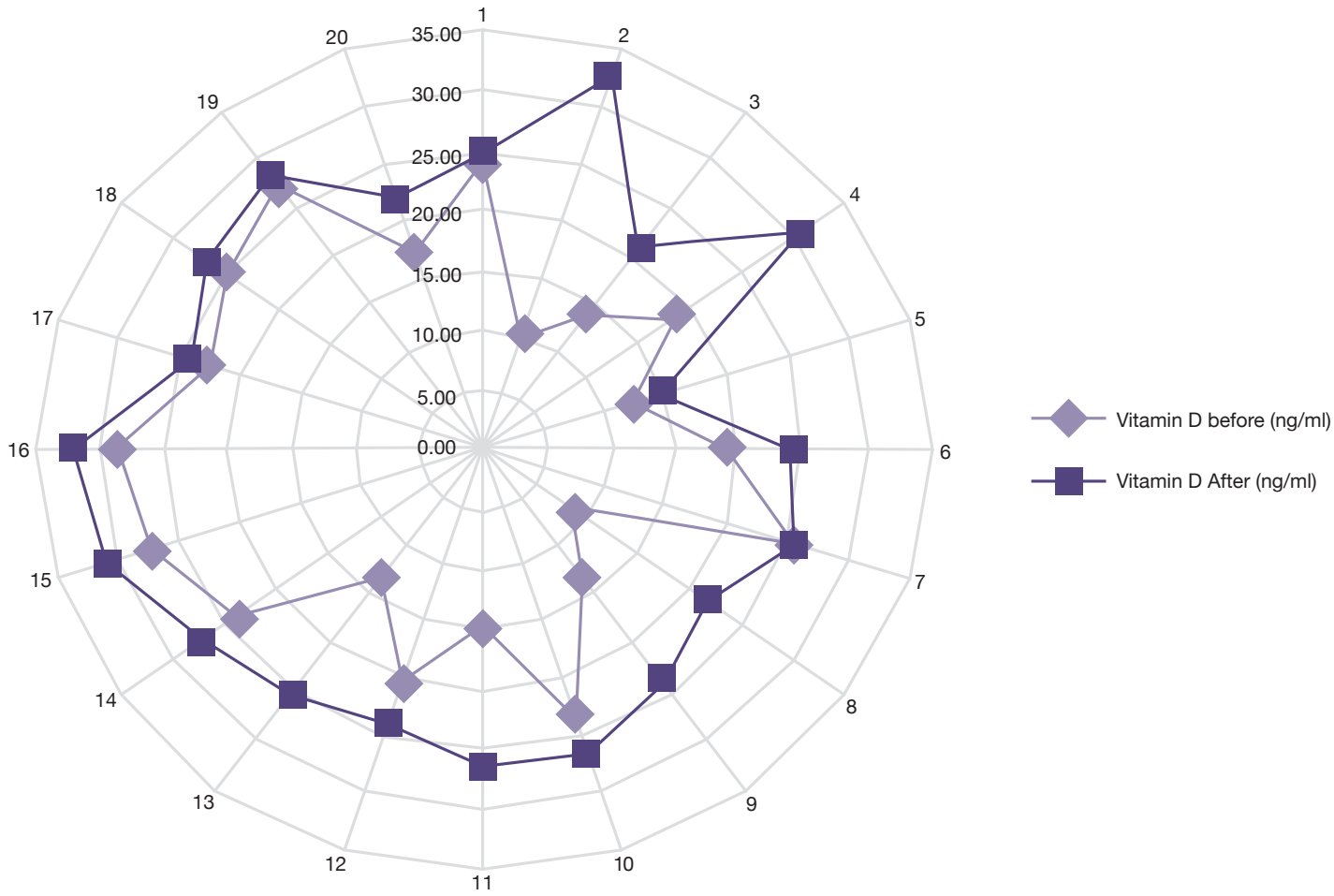

Figure 2: *Vitamin D levels 'Before & After' (BetterYou)*

Vitamin D and D-Light

Pharma Base S.A[2]

Date of study: 2013 (Unpublished)
Clinical trial registry number: CTRI/2013/06/003770

Abstract

A randomised, open-label, two-piece, two-treatment, two-way cross-over clinical study investigating absorption of vitamin D via an intra-oral spray compared to a capsule containing twice the vitamin D level.

Two cohorts were used, providing healthy and unhealthy subjects, where unhealthy subjects were those demonstrating a pre-trial vitamin D serum level of below 50nmol/L (a sub-optimal level).

Results demonstrated a dramatically higher uptake of vitamin D via the spray compared to the capsule over the 90 day trial. In addition there was a market difference recorded between the results of the healthy and unhealthy subjects, with spray elevating vitamin D levels 167% higher in healthy subjects and 227% higher in unhealthy subjects.

Absorption of vitamin D3

Vitamin D3 taken by oral route (pre-oral delivery) is absorbed in the intestine, where the lining of the digestive tract is aqueous in nature: therefore vitamin D3, which is a fat-soluble molecule, in order to be absorbed must be made water soluble in the intestine.

This is accomplished in two steps: emulsification of vitamin D3 in the intestinal lumen, through the action of bile salts, into small droplets and the dispersion and incorporation of these droplets into micelles, complex aggregates formed in the intestinal lumen by the interaction of free fatty acids, monoglycerides, and bile salts. Micelles are sufficiently water-soluble to access the intestinal brush border whereupon the vitamin D3 content is released and then absorbed.

Factors which influence the absorption of vitamin D3

The fed/fasting state and type of fat; the gastric, pancreatic and biliary secretions; the micelle formation; any illness resulting in malabsorption of intestinal fat, are all factors which may impair the absorption of vitamin D3.

The gastric, pancreatic and biliary secretions are necessary for the formation of fatty acids from the fats ingested with the diet.

The formation of micelles depends on bile salts formed in the liver and secreted by the gall bladder. The presence of fat in the duodenum stimulates the release of bile acids to facilitate lipid absorption.

Several studies have reported differences in the bioavailability of vitamin D3 oral supplements, which have been correlated to the fed/fasting state, and to the type of dietary fats. It has been demonstrated that taking vitamin D3 with the largest meal improves absorption and results in higher serum levels of 250HD3, and that the intakes of different dietary fats are associated with the increase in serum levels of 250HD3:

Mulligan et al: Taking vitamin D with the largest meal improves absorption and reults in higher serum levels of 25-Hydroxyvitamin D; Journal of Bone and Mineral Research, 2010.

Niramitmahapanya et al: Type of dietary fat is associated with the 25-Hydroxyvitamin D3 increment in response to vitamin D supplementation. J Clin Endocrinol Metab, 2011.

Fat malabsorption is associated with a variety of medical conditions including pancreatic enzyme deficiency, Crohn's disease, cystic fibrosis, celiac disease, surgical removal of part of the stomach or intestines, and some forms of hepatic failure. People which have such illnesses have a reduced absorption of vitamin D3 through the intestine:

Lo et al: Vitamin D absorption in healthy subjects and in patients with intestinal malabsorption syndromes. Am J Chin Nutr, 1985.

Javorsky et al: Vitamin D deficiency in gastrointestinal disease. Practical geastroenterology, 2006

The intraoral delivery

Intraoral delivery of drugs through the mucosal linings of the oral cavity offers distinct advantages over peroral delivery through the GI tract. The oral mucosae are extremely rich in blood vessels and lymphatic vessels that allow a faster and higher absorption of the drugs and their direct entering into the systemic circulation, via the internal jugular vein, bypassing the gastrointestinal tract, and therefore enhancing their bioavailability.

D-Light

D-Light is an emulsion of micro sized droplets of vitamin D3 in water, supplied in a non-pressurised container, equipped with a spray pump, a finger-actuated mechanism, for the metered dispencing of the product into the oral cavity and under the tongue.

When sprayed inside the mouth, the dine droplets of D-Light are quickly and completely absorbed through the buccal mucosae into the numerous capillaries and veins lying close to the tissue surface. In this way, the fine droplets of D-Light enter *directly* the bloodstream within seconds.

Each actuation of the spray pump delivers 200mcl of the liquid emulsion, which may contained 500 IU of vitamin D3.

Assesment of the bioavailability of vitamin D3 after intraoral and peroral delivery

A randomised, open-label, balance, two-piece, two-treatment, two way cross-over clinical study have been conducted for studying the absorption of D-Light (vitamin D3 oral spray 500 IU) in comparison with the reference product Uprise-D3 (vitamin D3 capsule 1000IU) in 14 healthy and 14 unhealthy subjects under fed conditions.

Clinical study, protocol no: ECTS 13/003; Clinical trial registry no. CTRI/2013/06/003770; duration 5 months.

The results of the study showing the percentage increase in vitamin D3 absorption, measured as plasma level of 250HD, after the administration of vitamin D3 spray and vitamin D3 capsules in a dose of 1000 IU daily for 30 days in healthy and unhealthy subjects are graphically presented in Fig. 1 (R: reference product, capsules; T: D-Light, spray).

The respective control groups in healthy and unhealthy subjects were not showing any significant difference in 250HD levels between baseline and over a period of 90 days.

The data show:

- In healthy subjects there is a 26.38% increase in 250HD levels after capsules administration as compared to 44.12% increase observed after administration with spray formulation. Therefore, there is a 167% higher absorption observed with spray formulation as compared to capsule formulation.
- In unhealthy subjects there is a 49.52% increase in 250HD levels after capsules administration as compared to 112,81% increase observed after administration with spray formulation. Therefore, there is a 227% higher absorption observed with spray formulation as compared to capsules formulation.

Conclusions

D-Light spray formulation has significantly higher absorption as compared to vitamin D3 oral capsule formulation in both healthy (1.67 times) as well as unhealthy subjects (2.28 times).

Advantages of D-Light intraoral delivery over tablets and capsules peroral delivery

Greater bioavailability: D-Light spray release in the oral cavity vitamin D3 in the form of micro sized droplets, which are absorbed by buccal mucosae.

Absorption independent from fasting and fed state, and from the composition and type of food: Vitamin D3 fron D-Light is absorbed through the oral cavity and not through the gastro-intestinal tract.

Bioavailability not affected by malabsorption and related illnesses: The absorption of D-Light through the oromucosal route overcomes the negative effect of these illnesses on the bioavailability of vitamin D3.

Compliance from people: Better compliance from people having difficulties in swallowing tablets and capsules, and from people which are not obliged to observe a defined timing for taking the product, or to modify their diet.

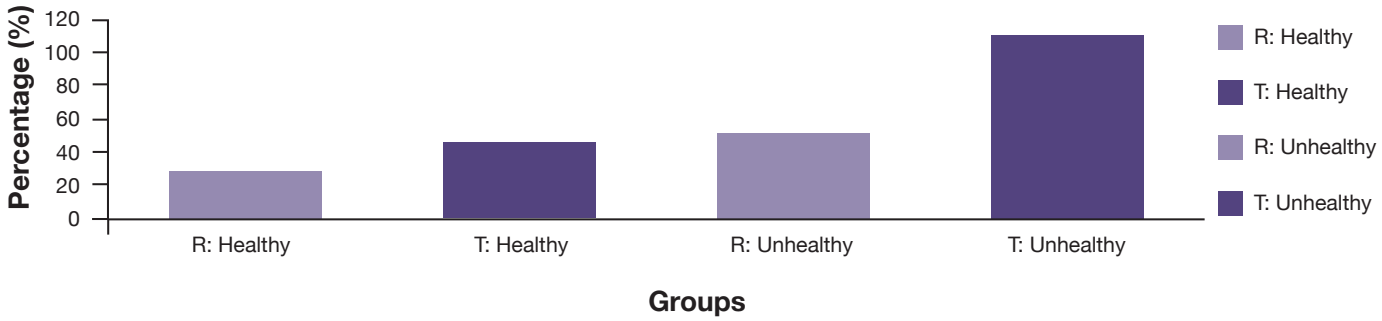

Figure 1: *Percentage increase in 250HD level after 30 days treatment*

Vitamin D associates with improved quality of life in participants with irritable bowel syndrome: outcomes from a pilot trial

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Date of publication **2015**

Abstract

Background: Vitamin D deficiency has been associated or implicated with the pathophysiology of the gastrointestinal conditions inflammatory bowel disease and colorectal cancer, as well as with depression. No trials or epidemiology studies to date have investigated a link with irritable bowel syndrome (IBS). A single case report has suggested a benefit in IBS of vitamin D supplementation. We hypothesised that IBS participants with vitamin D insufficiency would benefit from repletion in terms of their IBS symptoms. We undertook a pilot trial to provide data to support a power calculation and to justify a full trial.

Methods: This was a randomised, double blinded, three-arm parallel design trial of vitamin D, placebo or a combination of vitamin D and probiotics. Participants were further stratified according to whether they were vitamin D replete or insufficient. Vitamin D status was determined by blood test at baseline and exit; IBS symptoms were assessed by validated questionnaire; dietary intakes were assessed by food frequency questionnaire.

Results: A significant proportion of the IBS population were vitamin D deficient, such that the replete stratum could not be adequately recruited. There was a significant association in the baseline data between circulating vitamin D level and quality of life ("How much has IBS affected your life?"). Supplementation significantly improved vitamin D level versus placebo. IBS symptoms were not significantly improved in this pilot, although a power calculation was enabled from the intervention data.

What is already known about this subject?

- Irritable bowel syndrome (IBS) is a chronic functional gastrointestinal (GI) disorder that affects 10–15% of western populations, yet its pathogenesis and management are poorly characterised.
- Vitamin D supplementation has been associated with positive outcomes in other lower-GI conditions, including cancer prevention and colitis.
- Few studies to date have reported any relationship between vitamin D and IBS, although we recently reported a case study in this area.

What are the new findings?

- The IBS population exhibits low concentrations of serum 25OHD; these levels respond to supplementation.
- There is a significant positive association between quality of life and circulating 25OHD concentrations.
- We provide data on which to inform power calculations for a larger clinical trial of vitamin D supplementation in IBS patients.

How might it impact on clinical practice in the foreseeable future?

- The data provide evidence for widespread vitamin D insufficiency in people with IBS. There may be benefit to testing IBS patients' vitamin D status and providing supplementation. There is a positive association between vitamin D status and quality of life measures, supporting a potential interaction between vitamin D and mental health and well-being. Clinicians may consider trialling vitamin D with patients with IBS as part of a nutritional management strategy. The results suggest a larger, adequately powered trial may be justified.

Introduction

Irritable bowel syndrome (IBS) is a chronic disorder which profoundly affects quality of life with a prevalence of 10–15% in the industrialised world.1 IBS is a relapsing condition that has a large social impact and is associated with significant direct and indirect healthcare costs.[1]
It is a heterogeneous disorder that can be subtyped depending on the bowel habits of patients; IBS with constipation (IBS-C), IBS with diarrhoea (IBS-D) or IBS with mixed bowel habits (IBS-M).[2]

IBS pathogenesis is poorly understood and it is generally regarded as a multifactorial disorder involving host and environmental factors, including diet. Most hypotheses involve altered intraluminal milieu, immune activation, enteric neuromuscular dysfunction and brain–gut axis dysregulation. It is thought that the intestinal microbiota might play an important role as bacterial infection, antibiotic use and chronic low-grade inflammation are associated with IBS onset.[3] Several studies have implicated changes in the colon microbiota may be associated with IBS symptoms, including perturbation in bile acid metabolism and electrolyte absorption.[4] This theory is further supported by generally beneficial effects of bacteriotherapeutic interventions (vide infra).

Therapy for IBS is primarily targeted at treating the symptoms experienced, and include loperamide for diarrhoea, methylcellulose for constipation and smoothmuscle relaxants for abdominal pain.[2] Nonetheless, symptom treatment meets with limited success and may not be effective for long-term management of IBS.

Mounting data suggest that probiotics may be beneficial in the management of IBS. A recent systematic review of 35 trials investigating probiotic use in IBS showed that 25 of these studies reported a beneficial effect on the primary outcomes including global symptom severity.[5 6] The effect on secondary outcomes varied between studies with improvements seen in flatulence and abdominal bloating following treatment with VSL#3.[7 8] Other studies demonstrated a decrease in abdominal pain after 4 weeks of treatment with *Lactobacillus plantarum* 299V when compared to control.[9] Our previous work has demonstrated an improvement in global symptom severity in people with IBS following 8 weeks of supplementation with LAB4 probiotic.[10]

We recently reported a case study of an IBS patient taking high dose (3000 IU daily) vitamin D. The participant reported remission of IBS symptoms following supplementation, with a recurrence of the symptoms on supplementation cessation. Additionally, analysis of social media (blogs/forums) reports from 37 IBS patients found that 70% described improvements in their symptoms with supplementation and the majority of these individuals reported being vitamin D deficient before supplementation.[11] A role for vitamin D supplementation in gastrointestinal health is also supported by studies showing associations between vitamin D deficiency and inflammatory bowel disease (IBD).[12] A recent systematic review suggested there may be benefits of vitamin D supplementation in IBD.[13]

On the basis of our previous case report,[11] we hypothesised that patients with IBS who are vitamin D insufficient, would report improvement in symptoms following vitamin D supplementation. We further sought to test whether vitamin D supplementation in combination with a probiotic preparation would act synergistically.

There are no published randomised controlled trial of vitamin D supplementation in IBS and therefore no data on which to base a power calculation. A formal aim of this trial is to provide data to allow such calculations to be undertaken, enabling properly scaled clinical trials to be designed.

Materials and methods

Participants

Ethical approval for this study was granted by The University of Sheffield Research Ethics Committee (Ref: SMBRER278). Participants were recruited via poster advertisements at the University of Sheffield. All participants had a previous clinical diagnosis of IBS and met the Rome III criteria at baseline.[14] Participants who reported any antibiotic use in the past 4 weeks prior to recruitment, recent changes in IBS medication, pregnancy, current use of vitamins or probiotic supplements, history of gastrointestinal surgery, diabetes or current use of antidepressants or antipsychotics were excluded. The study population was stratified by vitamin D status hypothesising that deficient or insufficient individuals would respond if deficiency were causal in IBS.

Patient measures

Throughout the study participants were assessed using a number of outcomes. To assess vitamin D status participants provided a blood sample from which baseline and exit 25OHD was measured in serum. Participants were given a food frequency questionnaire (FFQ) from which dietary intake was derived using FETA opensource software. [i] Baseline IBS symptom questionnaires were completed. [15] The questionnaire assessed abdominal pain (pain severity and number of days with pain), bloating, bowel habits (minimum and maximum bowel movement per day and satisfaction with bowel habit) and quality of life over each 2-week period.

Study design

This was a 12-week double-blind, placebo-controlled, stratified study.

Participants attended the Clinical Research Facility, Royal Hallamshire Hospital, Sheffield for three visits (figure 1). At the first visit, baseline 25OHD was measured in serum, participants completed FFQ and baseline IBS symptom questionnaires were completed.[15]

The second visit took place approximately 2 weeks following visit 1. Participants were issued with a 12-week supply of supplement and were given IBS symptom questionnaires to be completed biweekly from the date of visit 2.

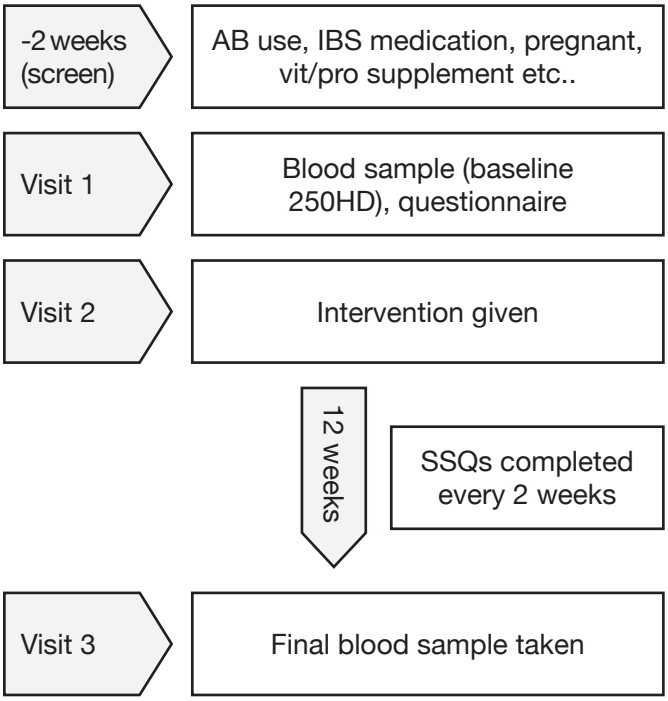


Figure 1: *Flow diagram of study protocol. IBS, irritable bowel syndrome; SSQ, Symptom Severity Questionnaires.*

At the final visit participants provided a second blood sample from which serum 25OHD was measured. Of the 51 patients issued with supplements 9 failed to return questionnaires for the full period (4 in the placebo group, 3 vitamin D alone and 2 combined intervention).

Sample size and randomisation

Based on our previous study[10] we aimed to recruit approximately 150 participants. By the end of the study 51 participants were recruited. Recruitment was between January 2014 and April 2014. Participants were stratified by baseline vitamin D statusii ('Vitamin D deficient' (25OHD <20 ng/mL) and 'Vitamin D replete' (25OHD >20 ng/mL)) and randomised. Participants were randomised to receive either double placebo (n=17), vitamin D supplementation and probiotic placebo (n=16) or probiotic and vitamin D supplementation (n=18). Participants were allocated in a 1:1:1 ratio to the three arms of the study according to a computer-generated random sequence using block randomisation with a block-size of six. The randomisation was performed by an independent statistician. Participants were enrolled and assigned sequentially to placebo or active products. The allocation sequence was not available to any member of the research team until databases had been completed and locked.

Intervention

The probiotic, vitamin D3 and corresponding placebos were provided by Cultech Ltd, Port Talbot, UK. Vitamin D3 and the corresponding placebo were provided as 15mL liquid sublingual sprays.

Both contained identical buffers with placebo lacking the active vitamin D3. Each spray of vitamin D3 gave a dose containing 3000 IU vitamin D3.

The probiotic preparation contained two strains of *Lactobacillus acidophilus*, CUL60 (NCIMB 30157), CUL21 (NCIMB 30156), *Bifidobacterium bifidum* CUL20 (NCIMB 30153) and *Bifidobacterium animalis* subsp. *lactis* CUL34 (NCIMB 30172) at a total of 2.5×10^{10} colony forming units (cfu) per capsule. Volunteers were instructed to ingest one capsule per day with water and one spray orally per day for 12 weeks. Compliance was assessed by counting the number of capsules remaining and weighing sprays at the end of the intervention—98% compliance was achieved.

Adverse events

There were no adverse events. One participant on the placebo arm reported feeling unwell after starting the trial with acute indigestion, heartburn and pain in the back which subsided after 2 weeks. The participant halted supplements for 4 weeks and then returned to the study under the initial time frame.

Biochemical assay

Serum 25OHD was measured using the Cobas e411 automated immunoassay from Roche Diagnostics (Germany). The inter assay coefficient of variation (CV)was 4.6%.

Statistical analysis

Number of days with pain were expressed as a percentage of each 14-day period. All other variables were assessed using a visual analogue scale and scored of 100. Individual scores were then combined to give a total symptom severity score of 500.[15] x^2 Test was used to assess the distribution of participants by vitamin D stratification. Associations between baseline 25OHD stratification (deficient: <12.5 ng/mL; insufficient: 12.5–20 ng/mL; replete 20–50 ng/mL; toxic: >50 ng/mL, no recordings were made at or near the toxic range) and baseline symptom scores, follow-up 25OHD and change in symptom score were determined by analysis of variance(ANOVA) with Bonferroni correction. Pearson's correlations for vitamin D intake and baseline serum 25OHD were undertaken. Change in symptom score over 7 time points were analysed by repeated measures ANOVA, missing data were not imputed and were assumed to be missing at random in the ANOVA model. Comparisons of deficient and replete symptom scores were assessed by independent t-tests. All tests were two sided with a significance value of <0.05. Analysis was carried out using SPSS V.22 (IBM, Armonk, New York, USA).

Results

Baseline vitamin D status shows a significant association with quality of life

Demographics of the population are shown in table 1. The three arms were similar in terms of number, gender, age, IBS subtype and serum 25OHD. Overall IBS symptom severity at baseline (week -2) was similar between groups (243±67 placebo, 244±92 vitamin D and 237±67 vitamin D with probiotic, p=0.241) and between IBS subtype (figure 2A) as was the baseline serum 25OHD (15±8.4, 16±8.0, 14±8.3 ng/mL, p=0.295).

The majority of participants had baseline 25OHD levels that are considered insufficient/severely deficient16 with an overall sample mean 25OHD of 15.3±7.9 ng/mL and 81.8% of IBS-C (n=7), 70% of IBS-D (n=9) and81.6% of IBS-M (n=24) with <20 ng/mL circulating 25OHD levels. This level of deficiency was similar to that seen in a BMI-matched cohort in Sheffield at this time of year (S.Bowles personal communication, 2014). There was no association between IBS-subtype and 25OHD status at baseline (figure 2B). However, participants with low 25OHD at baseline reported a greater impact on quality of life (on the basis of the IBS questionnaire) than their replete counterparts (p=0.034) (table 2). There was no difference in other symptom scores between 25OHD deficient and replete individuals (table 2). Data exploration suggested, a trend for lower affected quality of life scores in each IBS subtype in vitamin D replete participants against the non-replete cohort (see online supplementary, figure S1c).

We sought to determine whether potential benefits of replete vitamin D status may be attributed to associations between systemic effects of 25OHD or the potential mucosal action of ingested dietary vitamin D. There was no association between vitamin D intake and symptom severity, suggesting the effect may be systemically mediated (figure 2C, D).

Effect of vitamin D intervention

The distribution of participants by vitamin D status in each treatment arm was compared at baseline and exit. An equal distribution was seen at baseline (table 3) and the distribution was altered by 12 weeks of vitamin D supplementation. Of participants receiving supplementation in combination or alone the percentage of participants who were replete improved from 22.2% and 25.0% to 87.5% and 92.3%, respectively. Participants on placebo also had higher 25OHD levels with 60% of participants replete at exit compared to only 18.5% at baseline (figure 3A).

Participants who received vitamin D alone or in combination with probiotic had significantly higher 25OHD at follow-up compared to baseline (15.8±8.0

	Placebo	Vitamin D	Vitamin D + Probiotic
Participants n	14	6	14
Female n	17	15	15
Mean age (±SD)	36 (±15)	34 (±12)	34 (±14)
IBS subtype n			
Mixed	10	7	11
Constipation	2	5	2
Diarrhoea	6	5	3
Vitamin D status			
Deficient	14	14	12
Replete	4	3	4
Mean serum 25OHD ng/mL (±SD)	15 (±8.4)	14 (±8.3)	16 (±8.0)
Mean IBS severity score (±SD)	243 (67)	244 (92)	237 (67)

25OHD, 25-hydroxyvitamin D; IBS, Irritable bowel syndrome.

Table 1. *Baseline characteristics of participants.*

	Deficient	Replete
Constipation	2	5
Symptom severity	247±70	233±77
Pain severity	44±20	47±24
Pain frequency	37±25	36±24
Distention severity	42±29	40±30
Bowel satisfaction	60±20	58±23
Affected life	62±16	49±21*

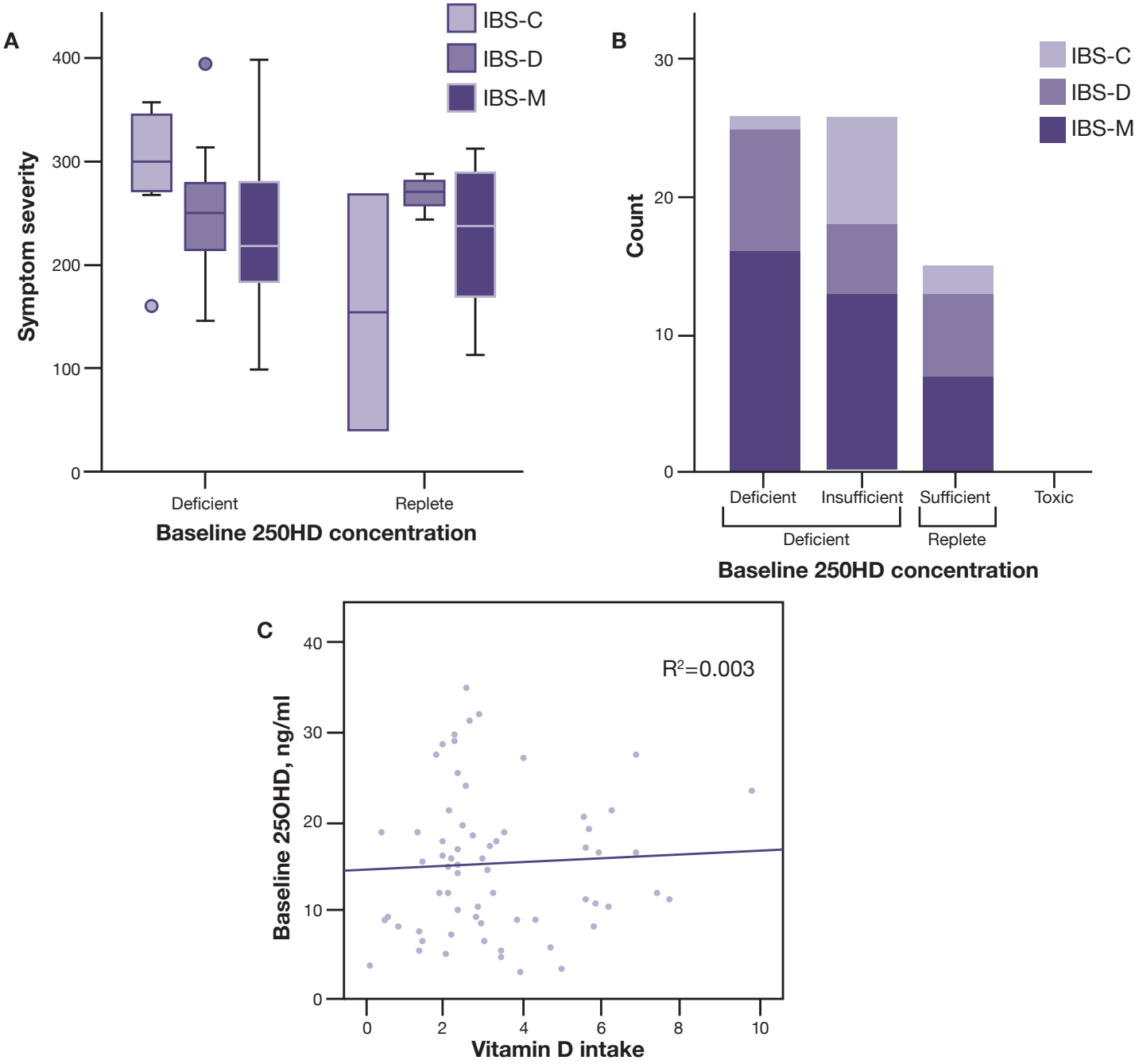
Data are means±SD.
*Denotes p<0.05 w.r.t. corresponding deficient symptom, independent t-test.

Table 2. *Baseline symptoms by vitamin D status*.*

Intervention arm	Baseline (% sufficient)	Exit (% sufficient)
Placebo	18.5	60.0
Vitamin D3	22.2	92.3
Vitamin D3 +Probiotic	25	87.5

Table 3. *Vitamin D status at baseline and exit.*

to 37.2 ±9.3 ng/mL and 14.9±8.4 to 37.1±11.7 ng/mL, respectively, p<0.001). These two groups also had higher levels of 25OHD compared to the placebo group at exit with mean 25OHD levels of 37 ng/mL while the placebo group mean was 25.3±8.0 ng/mL (p=0.008 vitamin D, p=0.005 vitamin D+Probiotic, respectively) (figure 3A). While it appeared that there were improved scores in all reported symptoms from baseline to exit irrespective of the intervention arm, this was not statistically significant for any symptom tested. Additionally, treatment arm did not interact with any of the symptom scores at any of the time point (figure 3B and online supplementary figure S2).



D

	Placebo	VitaminD
Symptom severity	R=-0.25, p=0.838	R=0.38, p=0.838
Pain severity	R=-0.007, p=0.952	R=0.049, p=0.8698
Pain frequency	R=-0.092, p=0.461	R=0.069, p=0.565
Blosting	R=-0.141, p=0.264	R=0.055, p=0.661
Bowel satisfaction	R=-0.107, p=0.39	R=0.062, p=0.748
Quality of life	R=-0.003, p=0.978	R=0.215, p=0.081

Figure 2: *Baseline characteristics of participants (A). Distribution of IBS-subtype across serum 25OHD levels at baseline shows no significant association of IBS subtype with vitamin D status (B). Mean score for symptom severity at baseline in IBS-C, D and M participants stratified by vitamin D status at baseline (C). Correlation of baseline 25OHD with vitamin D intake (D). Table summarising IBS symptom score with vitamin D intake and baseline serum 25OHD. No correlations were detected with in response to vitamin D intake, however, serum 25OHD showed a negative correlation between quality of life and serum 25OHD. IBS, irritable bowel syndrome; FFQ, food frequency questionnaire.*

The analysis was repeated limited to participants who were deficient at baseline. A significant increase was seen between baseline and exit 25OHD in all arms (figure 3C) with no significant relationship of intervention arm on total symptom severity (figure 3D) or individual IBS scores (data not shown).

Discussion

We have presented evidence of high-vitamin D insufficiency in a population with IBS, We have previously reported anecdotal evidence suggesting a benefit of vitamin D in management of IBS.[11] Baseline data from our population would support a need for monitoring vitamin D status and suggests that the impact of vitamin D deficiency is most acute on perceived quality of life.

A correlation indicated a potential relationship between 25OHD and quality of life scores; however, this did not reach significance. Reported quality of life will be influenced by the depression status of participants, a common comorbidity in IBS.[17] Numerous studies have reported that vitamin D levels are correlated with depression. In one such study vitamin D deficiency in young healthy individuals predicted clinical depression across a 4-week period, noting that as vitamin D levels increased due to seasonal difference in sunlight exposure volunteers showed lower levels of depression.[18] A similar pattern was seen in patients suffering post-stroke depression: lower serum vitamin D correlated with depression.[19] The exact mechanism through which vitamin D improves depression is not fully understood. However, vitamin D status has been implicated in neurological development,[14] the vitamin D receptor (VDR) is expressed throughout the nervous system where its activation is linked to neurotransmitter levels and serotonin synthesis.[20] Additionally binding of 25OHD-VDR complex results in expression of 1-α-hydroxylase which converts 25OHD to 1,25-dihydroxyvitamin D.[21] This 25OHD metabolite has been shown to upregulate neurotrophins which in turn promote survival and differentiation of nerve cells.[22] VDR is also expressed in the gut and regulates epithelial barrier function and bowel inflammation[23] suggesting that a vitamin D deficient diet may directly impact bowel function and hence IBS symptomology. Our attempt to distinguish the effects of circulating and ingested vitamin D did not support either model, likely due to underpowering.

Supplementation with vitamin D increased serum 25OHD concentrations from baseline with a significantly greater improvement seen compared to participants receiving placebo. Nonetheless the group receiving placebo showed an improvement in 25OHD levels. This may have prevented us from detecting a significant difference in symptom scores between the placebo and supplemented groups at exit. This general increase in serum 25OHD across intervention arms was likely due to seasonal differences in sun exposure. It has been found in previous studies that the percentage of individuals in the UK deficient for vitamin D ranges from 30–40% (January–March) to 2–13% (July–September).16 Study participants were recruited in the early months of 2014 (January–April) with most follow-up visits taking place from May to August, most likely explaining the rise in 25OHD in all groups.

An exploratory analysis of participants by IBS subtype showed that there was a marked improvement in response to vitamin D supplementation across nearly all IBS symptoms in participants with IBS-C (see online supplementary figure S3). Interestingly the proportion of these participants that were considered severely deficient was low in comparison to the other IBS subtypes (figure 2B). A clear limitation is the very small numbers in groups when the data is split but the observation suggests a future study into vitamin D supplementation in this subgroup may be justified.

Throughout this study we failed to observe a significant interaction with intervention with either vitamin D alone or in combination with probiotic. This is likely explained by a number of limitations in the study. First, we observed an effect of the placebo on symptom scores: IBS symptoms have a highly variable course (compare week-2 and 0 in figures 3B, D for example). It is recommended that active intervention in IBS last a minimum of 4 weeks and potentially up to 6 months for long-term analysis.[24] Furthermore, as a pilot study the current trial is inherently underpowered. In order to detect a statistically significant response in total symptom severity scores we would need larger sample sizes. With the data generated in this study it was calculated that a sample size of 74 per arm would be needed. This was calculated based on change in total symptom severity at exit with a placebo mean of −16, a mean of −54 for vitamin D intervention, a combined SD of 82 and assuming a desired power of 80% with an α error of 0.05.

It is possible that seasonal variations in vitamin D levels may impact IBS in the current study. Our data show a general improvement in symptom score and improved 25OHD over the duration of this study is tempting to speculate that increased sunlight exposure increases vitamin D levels which in turn improved IBS symptoms. Such effects have been previously shown for IBS and IBD symptoms.25 26 However, there was a significantly greater effect of vitamin D intervention than placebo on circulating vitamin D. Taken together, these data suggestion that seasonal variation should also be taken into account in the design of trials to assess the potential application of vitamin D in IBS.

In summary we have shown that IBS sufferers deficient in vitamin D experience poorer quality of lives than their replete counterparts. Our data demonstrate the need for additional studies of more than 8 weeks in duration with recruitment of a larger cohort of IBS patients.

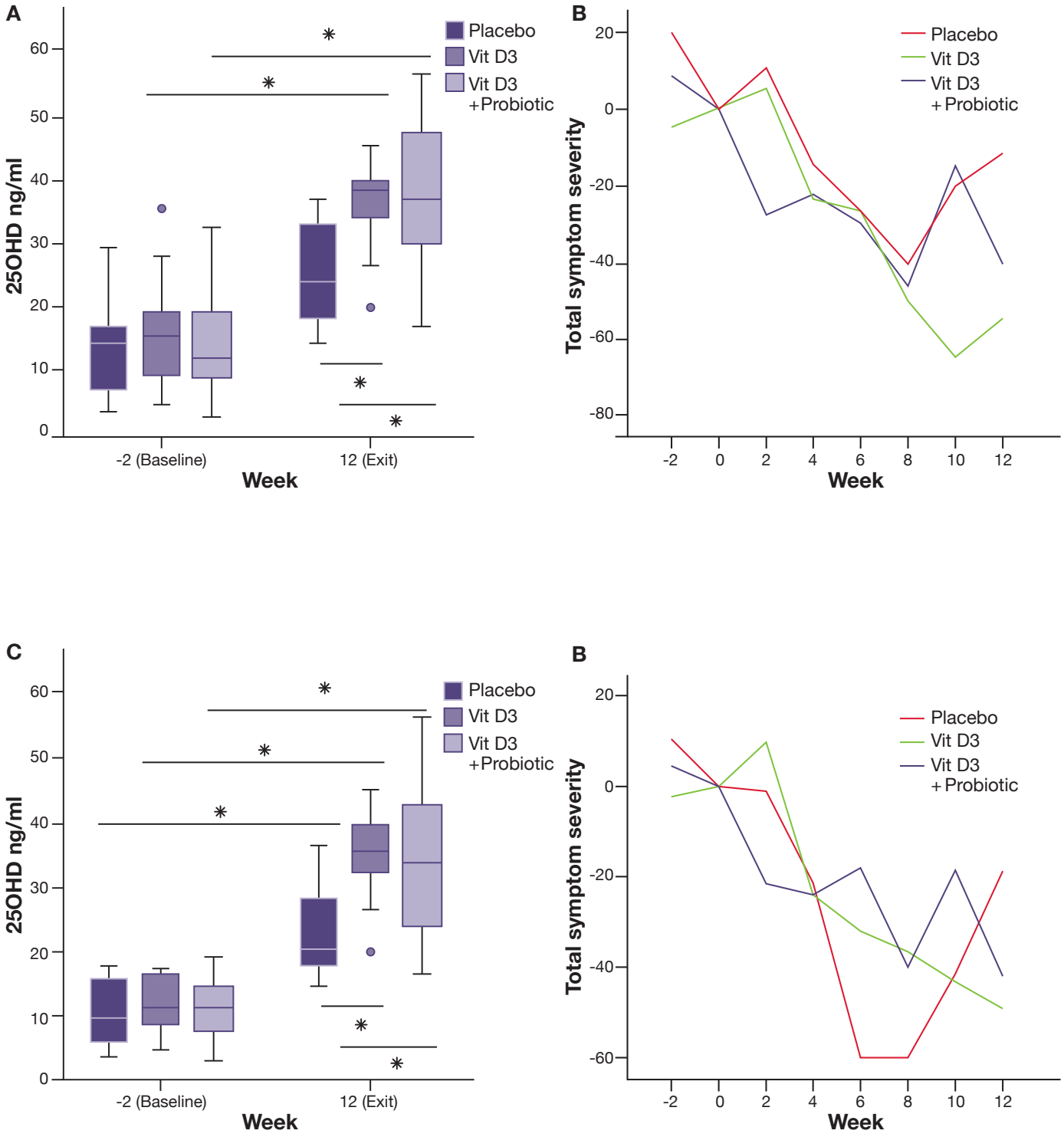


Figure 3: *Effect of intervention on participant serum 25OHD concentration and total symptom severity (A). Box plot serum 25OHD concentrations (ng/mL) before and after intervention with placebo, vitamin D3 or vitamin D3+Probiotic (all participants), a significantly greater increase with vitamin D supplementation alone or with probiotic was seen over placebo group (B). Repeated measure of total symptom severity against time point (all participants) (C). Box plot serum 25OHD concentrations (ng/mL) before and after intervention with placebo, vitamin D3 or vitamin D3+Probiotic. (deplete participants only), a significant increase in serum 25OHD was seen in all groups with a greater response associated with vitamin D supplementation alone or with probiotic when compared to placebo group (D). Repeated measure of total symptom severity against time point (deplete participants only).*

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Contributors

ST completed exit analyses, entered all data, undertook all statistical analyses, wrote the first draft of the paper. NR and ART recruited and consented participants and undertook day-to-day running of the trial in its inception and initial phases and reviewed drafts of the paper. ALE contributed to exit analyses, undertook first-pass data analysis and reviewed drafts of the paper. VAG co-conceived the study and undertook qualitative exit interviews with participants and reviewed drafts of the paper. IG; SFP co-designed the study, advised on dosing and delivery of probiotics and vitamin D, contributed to the analysis and interpretation of the data and reviewed drafts of the paper. EAW co-designed the study and contributed to its direction, supervised and interpreted the nutritional analyses and reviewed drafts of the paper. BMC conceived the study, assumes overall responsibility for the project, is its chief investigator, finalised each draft of the manuscript and produced the final version.

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Competing interests

IG and SFP are employees of Cultech Ltd, a manufacturer of supplements and probiotics.

Patient consent

Obtained.

Ethics approval

University of Sheffield.

Provenance and peer review

Not commissioned; externally peer reviewed.

Data sharing statement

No additional data are available.

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Vitamin D3 supplementation using an oral spray solution resolves deficiency but has no effect on VO2 max in Gaelic footballers: results from a randomised, double-blind, placebo-controlled trial

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Abstract

Purpose: Vitamin D inadequacy is a global health concern in athletes as well as the general population. Whilst the role of vitamin D in skeletal health is well defined, there remains uncertainty over whether vitamin D supplementation has an added benefit beyond bone health. Methods This randomised placebo-controlled trial in healthy male and female Gaelic footballers (n = 42) investigated the effect of vitamin D3 supplementation [3000 IU (75 μg) daily for 12 weeks, via an oral spray solution] on VO2 max which was the primary outcome measure. Secondary outcomes included skeletal muscle and lung function.

Results: Supplementation significantly increased total 25-hydroxyvitamin D concentrations compared to the placebo group. At baseline, 50 and 22% of footballers presented with vitamin D insufficiency (31–49nmol/L) and deficiency (<30nmol/L), respectively. Total 25-hydroxyvitamin D concentration did not significantly correlate with any measure of physical performance. Analysis of covariance (ANCOVA) models demonstrated that vitamin D supplementation over 12 weeks had no significant effect on VO2 max (P = 0.375), vertical jump height, left and right handgrip strength, forced vital capacity or forced expiratory volume at 1 second, after adjusting for confounders. The high prevalence of vitamin D inadequacy observed in this cohort of collegiate Gaelic footballers supports the need for vitamin D supplementation during wintertime to avoid being at risk of poor bone health.

Introduction

Vitamin D insufficiency and deficiency can be defined as a total serum 25-hydroxyvitamin D (25[OH]D) concentration below 50 and 30nmol/L, respectively [1]. Such low total 25(OH) D concentrations are widespread, and a growing number of studies around the globe have identified this health concern in athletes [2–5]. Vitamin D and its metabolites are renowned for their pivotal role in establishing musculoskeletalhealth during childhood and adolescence and in hindering the development of skeletal pathology [6]. Inaddition, a growing body of evidence has demonstrated the importance of vitamin D beyond bone health on immune, cardiopulmonary and skeletal muscle function [7].

Biologically inactive total 25(OH)D is comprised of 25(OH)D2 and 25(OH)D3, compounds that are both formed following hepatic hydroxylation of vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) [8]. Ergocalciferol is derived from fungi exposed to ultraviolet B radiation (UVB) [9]. In humans, cholecalciferol is produced following exposure of cutaneous cells to UVB at a wavelength of 290–315 nm and is also present in oily fish, eggs and liver [10]. Calcitriol (1,25-dihydroxyvitamin D (1,25[OH]2D) is formed subsequent to renal hydroxylation of 25(OH)D, and it is this hormonally active compound that has been implicated in numerous processes involving the immune, cardiovascular and respiratory systems [11]. Binding of 1,25[OH]2D to its nuclear receptor(VDR) results in the formation of a VDR-retinoid X receptor (RXR) heterodimer that enables 1,25(OH)2D to act as a transcriptional regulator through binding to response elements located in the DNA of vitamin D target tissues [12].

In observational studies of both athletes and non-athletes, total 25(OH)D concentration has been positively associated with measures of aerobic fitness although findings have not been consistent [13–19]. One such measure is VO2 max, the maximal volume of oxygen utilised, per minute, during exhaustive exercise, which is considered the gold-standard measure of aerobic fitness and is related to distance covered in field games [20]. VO2 max is determined by a range of overriding factors including cardiac output, oxygen transit time and oxygen saturation [21].A major limitation of existing observational research in this area is its inability to determine causality, and therefore, it is not known whether total 25(OH)D concentration is a cause of increased VO2 max or simply a result of reverse causation, perhaps owing to increased outdoor training time [22]. Despite the paucity of randomised controlled trials investigating this relationship, there is significant evidence of mechanisms by which vitamin D may influence factors that determine VO2 max [23–25]. One such mechanism is the ability of 1,25(OH)2D to suppress mRNA expression of hepcidin, the negative regulator of systemic iron concentration, an action which has been associated with increased expression of ferroportin in hepatocytes and monocytes [24]. Ferroportin is the sole iron export protein in humans and plays a crucial role in maintainingerythropoiesis, a contributory factor to VO2 max [26–28]. Studies in both healthy adults and patients with chronic kidney disease have demonstrated that vitamin D supplementation significantly decreases systemic hepcidin concentrations [24, 25]. Collectively, these findings raise the question as to whether optimising total 25(OH)D concentrations has a beneficial effect on VO2 max in athletes.

The primary aim of the current study was to test whetherathletes who received daily vitamin D3 supplementation at 3000 IU (75µg) demonstrated a significant increase in VO2 max compared to those provided with a placebo. Secondary aims were to determine whether supplementation influenced skeletal muscle and lung function. Experimental methods

This 12-week parallel group, double-blind, randomised, placebo-controlled trial was conducted at the University of Ulster, Coleraine, at a latitude of 55°N between the months of November 2014 and April 2015. All procedures were approved by the University's Research Ethics Committee (REC/14/0087), and the study was registered at www.clinicaltrials.gov (NCT02278172) and conducted in accordance with the Declaration of Helsinki. The protocol consisted of a series of appointments to obtain fasted blood samples and other physical measurements before and after a 12-week intervention. Nutritional and exercise intervention studies in athletes and healthy adults have demonstrated that 12 weeks is a suitable duration to observe a meaningful change over time in VO2 max [29–31]. Yet the dose and duration of vitamin D supplementation required to have a significant impact on VO2 max have not been investigated to date.

Subjects

Apparently healthy male and female athletes over the ageof 18 were considered suitable for inclusion. Exclusion criteria were as follows: not a member of a university sports team; vitamin D supplementation and/or iron supplementation in the 30 days prior to baseline measurements; health concern(s)/physical disabilities identified by the screening questionnaire that would prevent successful completion of the study; consumption of medication(s) known to influence vitamin D metabolism; vegan athletes; sun-bed users; those who had been on a sun holiday in the 30 days prior to baseline measurements; those planning a sun holiday for during the time frame of the study. Gaelic footballers from the university team (n = 72) completed a screening questionnaire in the first instance. A total of 42 Gaelic footballers (n = 18 males and n = 24 females) were deemed eligible for inclusion and provided informed consent before commencing the study.

Supplements and compliance

An independent clinical trials manager used MINIM software [32] to randomise recruited athletes into vitamin D (VD) or placebo (PL) groups, stratified by sex and with an allocation ratio of 1:1. All subjects and researchers were blinded to the allocations until completion of the study and subsequent data analysis. Footballers allocated to the VD group received an oral spray solution providing 3000 IU(75µg) vitamin D3 per spray, whereas those allocated to the PL group received an oral spray solution that did not contain vitamin D but was identical in appearance, smell, taste and from the same brand (BetterYou Ltd, Barnsley, UK). The dose provided was deemed suitable to raise total 25(OH) D concentrations to within the suggested range for extra-skeletal actions of vitamin D (75–100nmol/L) [33–35]. The vitamin D3 content of supplements was verified by an independent laboratory using high-performance liquid chromatography (Eurofins Product Testing, Cheshire, UK). Footballers were instructed to administer a single spray, targeting the buccal membrane, on a daily basis throughout the intervention and to return their used spray bottle at their final appointment. Percentage compliance was determined by applying the following equations.

1. $D = (f - b) - e \div s$

2. $C = D \div d \times 100$

In Eqs. 1 and 2, D is number of days the spray was taken; f refers to the filled weight of the spray bottle, and b is the empty spray bottle weight. e is the weight of the spray bottle upon study completion with s referring to the weight of each spray. In Eq. 2, C is percentage compliance and d represents the number of days on intervention. Filled weights were based upon the manufacturer's specifications and the average weight of a random sample of 10 oral spray bottles from the supplied batch.

Blood collection and processing

Participants were instructed to fast from 10 pm the night prior to blood sampling, and regular water intake was encouraged. Fasted blood samples were obtained from the antecubital fossa using a 21-gauge butterfly needle and 8mL serum and 9mL ethylenediaminetetraacetic acid (EDTA) plasma vacutainer tubes (Greiner Bio-One GmbH, Kremsmunster, Austria). Following inversion, serum sampleswere allowed to clot for <60 min and plasma samples placed in refrigeration until centrifugation. Tubes were centrifuged at 2200rpm for 15min at 4°C to allow separation of whole blood into its respective components. Following separation, serum and plasma samples were pipetted into 0.5mL aliquots and stored at –80°C until further analysis.

Blood analyses

Liquid chromatography-tandem mass spectrometry (LCMS-MS) (API 4000; AB SCIEX) was used to quantify serum 25(OH)D2 and 25(OH)D3 concentrations, using a commercially available assay (Chromsystems Instruments and Chemicals GmbH; MassChrom 25-OH-Vitamin D3/D2). This analysis was undertaken at the Biochemistry Department of St James' Hospital Dublin, a laboratory that complies with the Vitamin D External Quality Assessment Scheme and use of the National Institute of Standards and Technology 972 vitamin D standard reference material. The respective inter- and intra-assay coefficients of variation were 6.5 and 7.5%. Plasma intact parathyroid hormone concentrations were measured, in duplicate, using a commercially available enzyme-linked immunosorbent assay (MD Biosciences Inc., Minnesota, USA). Intra and interassay coefficients of variation were 6.8 and 6.2%, respectively. Calcium, albumin and creatinine concentrations were also measured in duplicate and assessed using an ILab 650 clinical biochemistry analyser (Instrumentation Laboratory, Massachusetts, USA). Intra-assay coefficients of variation were 0.65, 0.85 and 1.65%, respectively. Intact parathyroid hormone and adjusted calcium concentrations were measured in order to ensure there were no adverse effects of the intervention such as hypoparathyroidism or hypercalcaemia [36]. Impaired renal function can lead to deleterious effects on vitamin D metabolism, and therefore, the renal function of athletes' was evaluated by estimating glomerular filtration rate (eGFR) adjusted for fat-free mass [37, 38]. This was quantified from serum creatinine concentrations using the Modification of Diet in Renal Disease (MDRD) equation [39].

Skeletal muscle function

Average handgrip strength (kg) was measured using a dynamometer assembled at handgrip position 2 as default with shoulder flexion at 0º and elbow and wrist fully extended (Jamar Plus+, Patterson Medical, Warrenville, IL, USA) [40]. Footballers held the device alongside their body and gripped maximally a total of 3 times. Average handgrip strength was calculated for analysis. Vertical jump height (cm) was measured using a calibrated electronic jump mat (FSL Electronics Ltd, Cookstown, UK). Footballers performed a counter-movement jump a total of 3 times, with best recorded jump height used for statistical analysis [41]. A standardised rest period of 10 s was given between repeats of both handgrip and vertical jump tests.

Lung function

Footballers' lung function was assessed using a calibrated MicroLab portable spirometer (Carefusion Corporation, San Diego, CA,

USA). Forced vital capacity (FVC) and forced expiratory volume at 1 s (FEV1) were quantified by exhaling maximally into a 1-way disposable mouthpiece, and a minimum of 3 repeats was performed in order to derive average values.

Maximal oxygen consumption

Footballers refrained from heavy training/competition for at least 24h prior to exercise testing, in order to control for last-bout effects. Pre- and post-intervention, subjects completed an incremental exercise test that was designed to elicit a VO2 max response within the capabilities of the treadmill (MERC-C, WOODWAY GmbH, Germany). Gas analysis was performed using a metabolic cart that has been shown to give reliable measurements (Metalyzer 3B, CORTEX Biophysik GmbH, Germany) with calibrations for ambient conditions, analyser volume and concentrations of oxygen and carbon dioxide performed on a daily basis, prior to testing [42]. Footballers wore an FT1 heart rate monitor (Polar Electro Ltd, Warwick, UK) and face maskwith triple V insert. Following a standardised warm-up at 5km/h (1% incline), the test began at a running speed of 8km/h (1% incline) and speed increased by 1km/h every minute until a running speed of 17km/h was attained. At this point, running speed remained constant; however, incline increased by 1% each minute until VO2 max was achieved. The test was terminated upon volitional exhaustion or if any two of the following criteria were met: respiratory exchange ratio >1.15; oxygen plateau observed (i.e. no increase in oxygen consumption despite an increase in workload); heart rate ±10 bpm of age-predicted maximum (208 – 0.7 × age) [43]. Achievement of VO2 max was further verified by determining post-exercise lactate concentrations, using a Lactate Pro device as per the manufacturers' recommendations (Arkray Inc, Kyoto, Japan). Peak lactate concentrations are reached between 3 and 8min post-exercise [44]; therefore, at 5min, following cessation of the VO2 max test, a 6-μL capillary blood sample was obtained for lactate analysis. The lactate response to exercise may also vary according to age and sex. To account for this a blood lactate concentration >9 mmol/L in males and >7 mmol/L in females was considered as evidence of significant anaerobic metabolism [45].

Body composition

Footballers' height and weight were measured using a stadiometer and calibrated scales. Fat mass (FM) and fat-free mass (FFM) were measured by whole-body densitometry pre- and post-intervention (BOD POD, Life Measurement Inc, Concord, CA). The validity of this method has been reported extensively elsewhere [46]. Footballers were asked to refrain from heavy physical activity for 24h, and testing was performed following an overnight fast. Body mass was calculated using a calibrated electronic scale. Body volume was determined by air displacement plethysmography, corrected for predicted thoracic gas volume. The plethysmography chamber was calibrated using a 49.385-L cylinder as per manufacturers' instructions. Percentage fat mass (%FM) was calculated using the Siri equation [47]. Footballers wore tight-fitting clothing (i.e. swimsuit or compression garment with silicone swimming cap), to ensure an accurate measurement of body volume, and were instructed to sit upright and remain still throughout the test. A minimum of two measurements was taken per athlete. FM and FFM (kg) were adjusted for athlete's height (m2) and presented as fat mass index (FMI) and fat-free mass index (FFMI) (kg/m2) [48].

Dietary assessment

In order to estimate dietary vitamin D intake, footballers completed a validated food frequency questionnaire (FFQ) [49]. These data were collected on a single occasion due to the negligible contribution of dietary sources to total 25(OH) D concentration and the low intake of such foods in the Western diet [50, 51], despite a growing range of vitamin D-fortified products. Researchers asked players a series of questions relating to their consumption of food items known to contain vitamin D, with a photographic food atlas used to estimate portion sizes [52].

Physical activity

The validated Recent Physical Activity Questionnaire (RPAQ), capable of assessing physical activity for the previous 4 weeks, was completed pre- and post-intervention to control for a change in moderate-vigorous physical activity during the study [53]. Participants completed RPAQ questionnaires during appointments, and the researcher present queried any ambiguous responses prior to data entry.

Statistical analysis

An a priori power calculation with a two-sided 5% significance level and power at 95% determined that 35 athletes were required to observe a statistically significant 3.5mL/kg/min increase in VO2 max (G Power version 3.1) [54]. The final number of recruited athletes (n = 42) took into account an estimated dropout rate of 20%. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) with significance set at $P < 0.05$ throughout (IBM SPSS Statistics for Windows, version 21.0, IBM Corp, Armonk, NY). In accordance with the Consolidated Standards of Reporting Trials (CONSORT) guidelines, analyses were conducted using the intention-to-treat (ITT) principle thereby including all athletes randomised at baseline (n = 42) [55]. Missing data for physical and biochemical measures were deemed to be missing completely at random, owing to injury or illness unrelated to the intervention, justifying the use of multiple imputation. The Shapiro–Wilk test

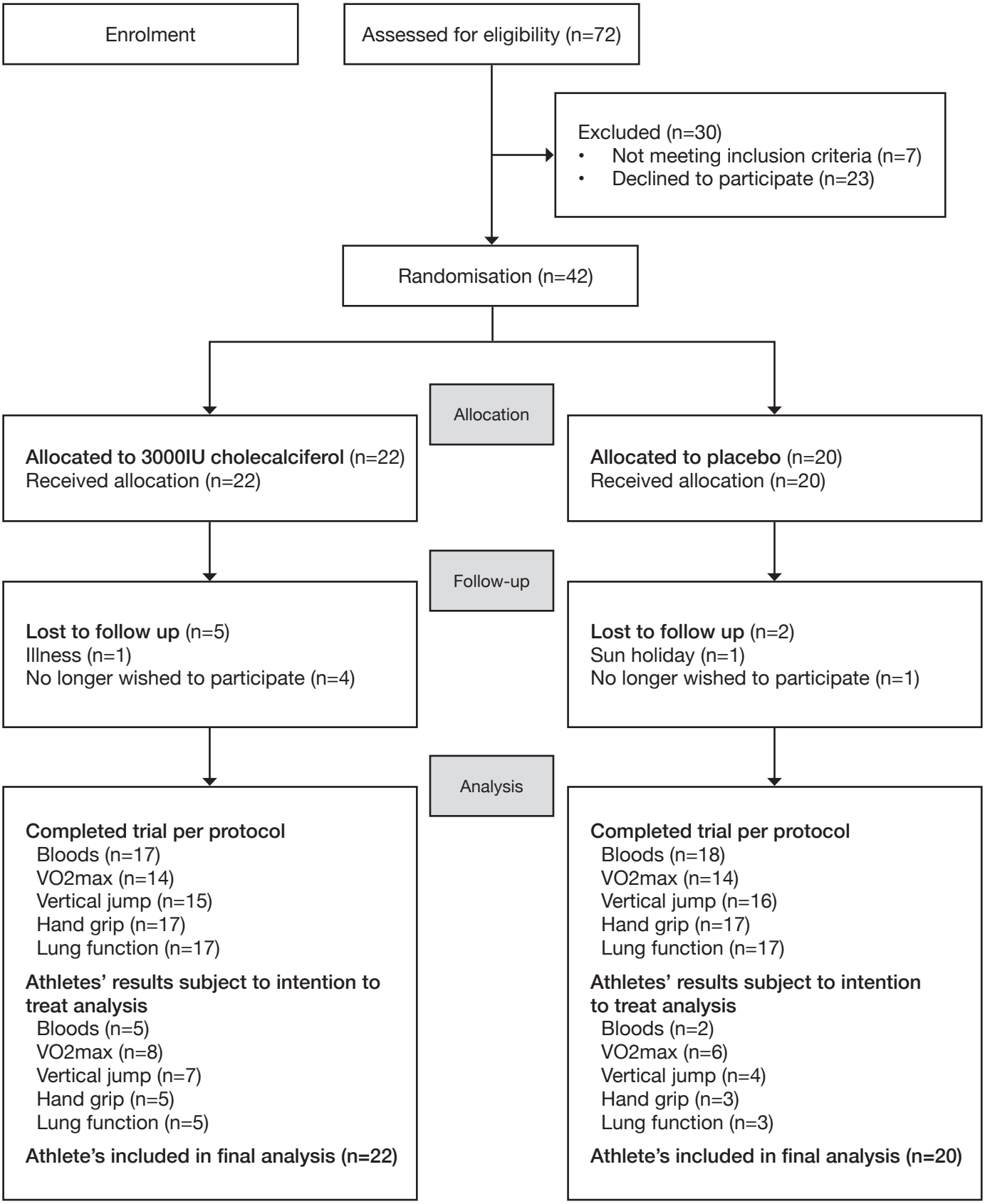

Figure 1: *CONSORT flow diagram. A total of 72 Gaelic footballers were assessed for eligibility with 30 excluded due to not meeting the inclusion criteria (n = 7) or no longer wishing to participate (n = 23). Remaining Gaelic footballers (n = 42) were randomised to receive an oral spray solution containing either 3000 IU of vitamin D3 (n = 22) or placebo (n = 20). A total of 7 athletes were lost to followup owing to illness (n = 1) unrelated to the intervention, sun holiday (n = 1) or no longer wishing to participate (n = 5). A total of 35 athletes completed the study per protocol (vitamin D treatment group n = 18 and placebo n = 17). All footballers randomised at baseline were included in the final analysis (n = 42)*

was used to determine whether data followed a normal distribution and skewed variables were transformed, using the logarithmic function, to attain a normal distribution prior to multiple imputation and further analysis. Transformations were applied to total 25(OH)D, creatinine and PTH concentrations as well as age and change in moderate-vigorous physical activity and FFMI. Multiple imputations consisted of 40 imputed data sets with pooled data used for subsequent analysis [56]. Imputed data are outlined in Fig. 1. Descriptive statistics were used to present participant characteristics at baseline. An independent t-test or a Chi-square test was utilised to test for differences between VD and PL groups at baseline. ANCOVA models were used to assess the effect of intervention on VO2 max and secondary outcome measures. Prognostic covariates were selected a priori for each model based upon evidence of a significant interaction between the covariate and dependent variable in question [57–60]. Reliability of repeated skeletal muscle function tests was assessed by pooling pre- and post-intervention data in order to determine the standard error of measurement (SEM) and intra-class correlation coefficient (ICC) [61]. These analyses were performed using the reliability test function in SPSS and SEM calculated by applying Cronbach's α to following equation. SEM = SD(1−r2xx) where SEM is standard error of measurement, SD refers to the standard deviation of test scores, and rxx refers to Cronbach's α as the reliability measure of test scores [62].

Results

A total of 7 footballers were absent to follow-up (n = 5 VD and n = 2 PL) due to sun holidays or illness unrelated to the intervention. Use of ITT did not change the outcome of the study when compared with per protocol analysis. There was no significant difference in the ratio of male to female subjects between groups (males n = 12 and females n = 10 VD; males n = 6 and females n = 14 PL) P = 0.857. The participant flow throughout the study is summarised in Fig. 1. Footballers' biochemical and physical characteristics at baseline and week 12 are provided in Table 1. The average rate of compliance to the intervention was 95%. At baseline, 50 and 22% of athletes presented with vitamin D insufficiency (31–49 nmol/L) and deficiency (<30 nmol/L), respectively. The effect of the intervention on outcome measures at week 12, after adjusting for covariates, is detailed in Table 2. Supplementation significantly increased total 25(OH)D concentrations in the VD group compared to PL group, P = 0.006, and resolved vitamin D deficiency in all athletes allocated to VD. There was no correlation between total 25(OH) D and measures of physical performance at either time point, P > 0.05. Furthermore, supplementation with vitamin D3 did not significantly increase VO2 max compared to the PL group, P = 0.375. Vitamin D3 supplementation did not significantly increase vertical jump height when compared to the PL group, P = 0.797, and also had no significant effect on left or right handgrip strength, P = 0.146 and P = 0.266, respectively. SEM and ICC results for vertical jump height (SEM = 1.51, r = 0.95), left and right handgrip strength (SEM = 2.06, r = 0.96 and SEM = 2.34, r = 0.95, respectively) indicated a strong internal consistency across repeated skeletal muscle function tests. Supplementation with vitamin D3 did not have a significant effect upon either measure of footballers lung function compared to those allocated to PL, FVC P = 0.573 and FEV1 P = 0.665. There were no adverse health effects to supplementation demonstrated by normal adjusted calcium and PTH concentrations at week 12.

Discussion

This is not only the largest investigation of vitamin D supplementation on VO2 max in athletes to date but also the first randomised controlled trial to successfully optimise vitamin D status using an oral spray solution. In this study, over half of the total cohort presented with vitamin D insufficiency or deficiency at baseline (50 and 22 %, respectively). Such findings corroborate existing literature [3, 5], reiterating that athletes are a population that may be at risk of poor bone health, owing to total 25(OH)D concentrations below 50nmol/L [63, 64]. Our previous research identified this health concern in elite Gaelic footballers, a finding now corroborated in those competing at the collegiate level [3]. Twelve-week vitamin D3 supplementation using a 3000 IU (75μg) oral spray solution resolved vitamin D deficiency and increased mean total serum 25(OH)D concentrations to over 80nmol/L. Conversely, those allocated to the PL treatment group remained at potential risk of poor bone health with a mean total 25(OH)D concentration below 50nmol/L at week twelve [65]. In contrast, Storlie et al. [66] reported no significant change in total 25(OH)D concentrations over 12 weeks in athletes supplemented daily with a 1000 IU (25μg) vitamin D3 oral spray compared to placebo, supporting 3000 IU (75μg) as an effective wintertime dosage. There is ongoing debate over what constitutes an optimal total 25(OH)D concentration for athletes. Some speculate that higher total 25(OH)D concentrations in excess of 100nmol/L may be necessary to trigger the purported extra-skeletal benefits of vitamin D in athletes [67, 68], though there is currently a lack of strong evidence for a benefit of maintaining total 25(OH)D concentrations above this threshold [35]. Furthermore, toxicity manifests itself as hypercalcaemia and increased bone resorption at vitamin D supplementation doses exceeding 10,000 IU/day (250μg) or a total 25(OH)D concentration ≈750nmol/L [69]. Indeed, there was a significant change in FMI over the 12-week intervention period. FMI increased in the VD group but declined in the PL group. Although in-season, this finding may be explained by differences in the footballers'

individual training and conditioning programmes during the winter months in the absence of scheduled training sessions at the university.

Mechanistic studies support the concept that vitamin D may influence VO2 max through direct and indirect actions on iron metabolism [24, 25]. Nevertheless, studies investigating the potential link between total 25(OH)D concentration and VO2 max in vivo have yielded equivocal findings to date with many failing to account for important covariates such as PTH concentrations and participation in moderate-vigorous physical activity [13, 15, 70–75]. Moreover, universal criteria for VO2 max have not been firmly established and therefore this may contribute to variation in study outcomes. Total 25(OH)D concentration was not associated with VO2 max at either time point in the current study, a finding that is supported by Fitzgerald et al. 2014 and research conducted in healthy adults [14, 16]. In contrast, at a latitude of 35°N, Koundourakis and colleagues reported positive bivariate correlations between total 25(OH)D concentration and the VO2 max of elite footballers, before and after a tapering period spanning the months of June and July [17]. During this time, there was a small yet significant decrease in athlete's VO2 max despite a concomitant increase in total 25(OH)D concentrations, likely owing to a reduced training load. Such findings indicate that vitamin D does not play a supportive role in determining VO2 max in athletes, a concept that is substantiated by findings of the current study. Supplementation did not significantly increase VO2 max compared to the PL group despite increasing total 25(OH) D concentration by 77%, a finding that has also been shown by others [76]. This contrasts with some randomised controlled trials of patients with cardiorespiratory pathology, although findings have not been consistent [77, 78]. Established primary determinants of VO2 max include cardiac output and oxygen diffusion capacity [21], and a possible explanation for the disparity between athletes and patients may be that athletes have a smaller ability to improve VO2 max, owing to significant cardiovascular adaptations to aerobic training including enhanced cardiac output and capillary density [79]. It is plausible that such adaptations may outweigh any potential benefit of vitamin D supplementation on VO2 max when compared to patients with diminished cardiac output and/or oxygen diffusion capacity.

The in vitro mechanisms by which vitamin D, specifically 1,25(OH)2D, may impact upon the skeletal muscle function of athletes have been reviewed extensively elsewhere, although less is known about the effects of vitamin D supplementation on skeletal muscle function in vivo [7, 80]. Indeed, the effects of vitamin D supplementation on skeletal muscle function in athletes have been investigated before, albeit without taking into account change in physical activity or body composition, confounders that determine skeletal muscle function and may therefore mask a null effect of treatment [81, 82]. Total 25(OH)D concentration was not associated with any measure of skeletal muscle function in the current study, and supplementation did not significantly increase vertical jump height or handgrip strength when compared to PL, corroborating findings from a recent randomised controlled trial conducted in adolescent swimmers [83]. These results demonstrate that vitamin D3 supplementation does not enhance skeletal muscle function in younger adults after taking into account change in FMI, FFMI and moderate-vigorous physical activity, despite increasing total 25(OH)D concentrations to over 80nmol/L. This contrasts with large studies of elderly patients that have identified a beneficial effect of vitamin D and calcium supplementation on skeletal muscle function and risk of falls [84–86]. Aside from differences in the supplementation regime it is possible to speculate, based on the results of the current study and existing literature, that vitamin D3 supplementation only benefits skeletal muscle in those with diminished function, explaining why no ergogenic effect was observed in this cohort of trained footballers. Another consideration is that the time taken for supplementation to increase total 25(OH)D concentrations to over 50nmol/L, within the 12-week intervention, is not known. Therefore, it is possible that supplementation only increased total 25(OH)D concentrations to above 50nmol/L late in the intervention, leaving little time for ameasurable effect on skeletal muscle function parameters.

Emerging research also posits that total 25(OH) D concentrations may be related to lung function, especially in those with airway disease [87–89]. Potential mechanisms include enhanced innate immunity and downregulation of the T helper 1 cell response, resulting in less airway inflammation and a consequent improvement in overall airway function [90]. Nevertheless, this study did not observe an association between total 25(OH)D concentration and measures of lung function at either time point and, in adjusted analyses, supplementation with vitamin D3 did not significantly impact upon FEV1 or FVC when compared to PL. In unadjusted analyses, there was a significant decrease in FVC over time in the VD group compared to those allocated to PL yet this was not the case in adjusted analyses indicating that the intervention did not have an adverse effect. These findings suggest that the response to vitamin D3 supplementation, in terms of benefits on lung function parameters, may differ between healthy individuals and those with airway disease although larger studies, adjusting for lung function specific confounders are required.

To our knowledge this is the first study to investigate the effects of vitamin D3 supplementation on VO2 max in athletes that has adjusted for key component covariates. Strengths of the study include a gender balance of athletes, accurate recording of compliance, independently

verifying the vitamin D3 content of supplements and being adequately powered to detect any potential significant change in VO2 max. Due to the heterogeneous athlete population it is not known whether our findings translate to non-Caucasian athletes or those from endurance or strength-based disciplines. The VO2 max test used in the current study was not validated. Future studies in this area are encouraged to utilise a validated protocol. The sample size for this study was specific to the primary outcome measure, VO2 max. Further research with larger sample sizes may therefore be required in order to categorically rule out a beneficial effect of vitamin D supplementation on skeletal muscle and lung function in athletes. Future studies may also wish to consider stratifying by weight as well as sex to prevent differences between treatment groups at baseline.

In conclusion, this study observed a high prevalence of vitamin D insufficiency and deficiency in collegiate Gaelic footballers during wintertime and such individuals should consider vitamin D supplementation to avoid being at risk of poor bone health. Twelve-week daily supplementation with a 3000 IU (75µg) vitamin D3 oral spray solution is an appropriate method, dose and duration to resolve deficiency and increase total 25(OH) D concentrations to over 80nmol/L. However, vitamin D supplementation, at the dose provided here for 12 weeks, did not have any beneficial effect on VO2 max, skeletal muscle or lung function in this cohort of Gaelic footballers.

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Compliance with ethical standards

Conflict of interest

The authors have no financial or personal conflicts of interest to declare in relation to this article.

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Efficacy and comparative uptake rates of sublingual and capsular vitamin D preparations

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Abstract

Background: Vitamin D is critical for skeletal health and is increasingly associated with other pathologies encompassing gastrointestinal, immunological, psychological effects. A significant proportion of the population exhibit suboptimal levels of vitamin D, particularly in Northern latitudes in winter. Supplementation is advocated, but few data are available on relative efficacy of preparations, or rates of uptake, or whether serum status may influence uptake. There has been considerable interest in the potential use of sublingual sprays for delivery of nutrient supplements, but data on efficacy remains sparse.

Methods: A randomised, placebo-controlled, 3-arm parallel design study was conducted in healthy volunteers (n=75) to compare uptake rates of vitamin D supplementation in capsule and sublingual spray preparations over a six week period between January and April 2017. Serum 25(OH)D concentrations were measured after day 0, 3, 7, 14, 21 and 42 days of supplementation with 3000IU per diem.

Results: Baseline measurements show 25(OH)D deficiency, insufficiency and sufficiency in 14.9%, 44.6% and 40.5% of the participants respectively. The capsule and spray were equally efficacious with average change in serum vitamin D of 2 nmol/ml/day. The data suggest that uptake rates are higher in individuals with lower serum vitamin D. 71% of the participants preferred the oral spray preparation to the capsule.

Introduction

Vitamin D is essential for the homeostasis of calcium and phosphate and well known for its role in the development and maintenance of bone health. (1). Once vitamin D has been ingested or synthesised via sunlight exposure it requires activation in the liver to form 25 hydroxyvitamin D (25(OH)D) and in the kidney to form 1,25 dihydroxyvitamin D (1,25 (OH)2D (2). 25(OH)D is the most abundant circulating form in the human body and is used to determine vitamin D status (3). Vitamin D levels can be defined as; sufficient (>50nmol/L), insufficient (31-49 nmol/L) of deficient (<30 nmol/L) (4). There is limited research on rates of repletion; one paper reports amounts for maintenance of serum 25(OH) D at 50nmol/L requires around 11-weeks of dosing at study requires 1000 IU vitamin D per day (5). Hypovitaminosis is evident worldwide and is a major public health concern (6) leading to advocacy for supplementation in at-risk groups (7). Research has also shown African Americans may require a higher dose of vitamin D supplementation to reach optimal serum 25(OH)D concentrations compared to the Caucasian participants, perhaps as a result of lower baseline vitamin D levels in this population (8).

Supplementation has classically been with capsule preparations, but sublingual sprays are increasingly available. There are few data available on the relative efficacy of each type of preparation, of uptake and repletion rates, and of any potential interaction between vitamin D status and uptake.

Methods

Study design

This was a 6-week double blind, placebo-controlled 3-arm parallel design study. The participants attended three visits to The Medical School at The University of Sheffield. The initial visit included anthropometrics, issue of first batch of blood test kits and completion of a first self-test blood sample. The second visit occurred approximately two weeks after the initial visit for issue of further test kits and to support participant retention in the trial. The final visit required participants to return their preparation bottles and answer five questions regarding the study.

Sample size and randomisation

There were no data upon which to base a power calculation. 75 healthy male and female participants were recruited between January 2017 and February 2017 and were randomly assigned to one of three arms: (i) active capsules and placebo spray (n= 25); (ii) active spray and placebo capsules (n= 25); (iii) double placebo (n= 25). Participants were according to a computer generated random sequence using block randomisation with a block size of 9, with randomisation undertaken by an independent outside source. The allocation sequence was not available to any member of the team until databases had been completed and locked.

Participants

The University of Sheffield Research Ethics Committee granted ethical approval for this study (Ref: 011865). Participants were recruited via poster advertisements at the University of Sheffield and through a student volunteer email list. All participants were fit and healthy and aged between 18-50 years. Participants who reported any micronutrient supplement use (vitamin D, multi-vitamin, fish oils), recent or upcoming sunny holiday, pregnant or lactating, history of gastrointestinal disease, BMI >30, diabetes, >50 years of age were excluded.

Patient measures

Participant's serum 25(OH)D status was assessed by blood sample using at home finger-prick blood spot kits at 0,3,7,14,21 and 42 days of supplementation. Blood spots were analysed by liquid chromatography tandem mass spectrometry (Waters TQD and Acquity UPLC) for total serum 25(OH)D (25(OH)D2 and 25(OH)D3). LC-MS was undertaken by City Assays, Department of Pathology, Birmingham Sandwell Hospital. Anthropometric measurements included; height, weight, BMI, and body fat percentage.Qualitative opinion of capsules and sprays were assessed via exit questionnaire and focus groups.

Intervention

117 The vitamin D3 and corresponding placebos were manufactured by Cultech Ltd., Port Talbot, UK and provided by Better You Ltd, Barnsley, UK. Preparations of vitamin D3 and corresponding placebos were provided as 15mL sprays and capsule. Each capsule and spray contained 3000 IU (75ug) of vitamin D3 per dose. Volunteers were instructed to ingest one capsule per day with water and one spray orally per day for 6 weeks. Compliance was measured by weighing the spray bottles and counting the remaining capsules at the end of the study. 86% of participants reached 100% compliance with the spray.

Adverse events

Two participants reported that small blisters formed on cheek and tongue after use of the spray began. One participant stopped using the preparation for the duration of the study. The second participant continued to use the spray throughout the intervention despite discomfort.

Statistical analyses

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS)

(IBM SPSS Statistics for Windows, V.23; IBM Corp.). Percentage change in 25(OH)D from baseline was determined by analysis of variance (ANOVA) with Boneferroni correction. Spearman's correlations for rate of change in vitamin D per day was performed. Change in vitamin D over 6 time points were analysed by repeated measures ANOVA (there was a high failure rate in terms of assessment of vitamin D at day 42 leading to the exclusion of this timepoint's data from the main analysis). Comparisons between percentage change in 25(OH)D from baseline in deplete and replete participants were assessed by independent t tests. Two-tailed tests were used in all analyses with the significance value of <0.05.

Results

Baseline demographics are shown in Table 1. The three arms were similar in numbers, age, BMI, body fat, height, weight, skin tone, sex and baseline serum 25(OH)D concentrations. Baseline serum 25(OH)D levels showed 59% of participants had insufficient/deficient levels (<50nmol/L).

Serum vitamin D levels analysed across the time course in all three trial arms by ANOVA showed a significant improvement in vitamin D status in those receiving vitamin D compared to placebo. Post hoc analyses revealed significant differences between each active and placebo (capsules p= 0.003, spray p= 0.001), but no difference between the active preparations at any time point (Fig 1A). As there are few available data on uptake rate of ingested vitamin D, we assessed the inter-individual and inter-preparation difference as change in serum nmol/ml/d Fig1Bi-ii). Whilst there was a range of rates in each dataset, assessment of the distribution of rate showed a monotonic normal distribution for both preparations with similar peak rates (Fig 1Biii-iv). Independent t-test was performed and found no significant difference between mean rates of change for capsule and spray.

In order to investigate a potential homeostatic mechanism for vitamin D status, we investigated the relationship between serum status and uptake rate (Fig 1Bv-vi). We observed inverse relationships between baseline serum 25(OH) D and uptake rates over 21 days using Spearman's correlation for both the spray (r^2 0.26, P= 0.014) and capsule (r^2 0.35, P=0.003).

In an exit interview about preference for either the spray or capsule for delivery, 60% preferred spray, 24% capsules and 16% did not express a preference.

Discussion

Advocacy for vitamin D supplementation for some subpopulations, interest in its use, availability of over-the-counter preparations, and lack of information on the factors predisposing to development of excessive levels collectively identify a need for research on comparative efficacy of preparations and the saturability of uptake. This study used two commonly-available vitamin D preparations;, the widely used capsules and a more novel sublingual spray to investigate these factors.

Our findings show that a sublingual spray is equally effective at raising serum 25(OH) D concentrations with no significant difference between uptake rates compared to capsules in this study population. The study participants reported a preference for the sublingual spray, and this study demonstrates that this delivery platform is of comparable efficacy. Sublingual sprays may be particularly advantageous in people with pre-existing malabsorption conditions or swallowing problems. Our analysis shows for the first time the likely rates of vitamin D uptake and the spread of the uptake rates, albeit in a relatively small, healthy sample. The monotonicity of our rate distribution suggests a limited spread of rates with no suggestions of outliers or subpopulations, however the relatively homogenous profile of the study population, whilst an advantage for this pilot exploration, is a limitation in terms of the prediction of rates in other groups (older adults, different ethnicities). The availability of reference values for rate will allow other populations to be compared to examine the effects of age, ethnicity, BMI, GI function upon rate.

These data also suggest that vitamin D status may influence uptake rate, as a correlation between baseline status and uptake rate exhibited a moderate inverse relationship, furthermore the circulating levels started to saturate towards the end of the intervention. The mechanistic basis of this is unclear, and it is notable that both delivery platforms exhibit this effect, implying control in both enteric and transbuccal absorption. Future work may address the strength of this inferred relationship more thoroughly and identify implied control mechanisms.

Conclusions

In summary, we have shown the capsule and sublingual spray are equally effective at delivery of vitamin D supplement. There was an overwhelming preference (64%) for the spray over capsules for mode of supplement delivery. Absorption rates, reported for the first time, exhibit a monotonic distribution in this population. This study saw a reduction in uptake of vitamin D3 as serum 25(OH)D levels increased over 21 days which suggests vitamin D absorption may be influenced by vitamin D status. This data illustrates the need for further studies to explore uptake rates across mixed population groups, especially those identified as high risk.

Acknowledgements

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Conflict of interest

BetterYou markets vitamin D supplements.

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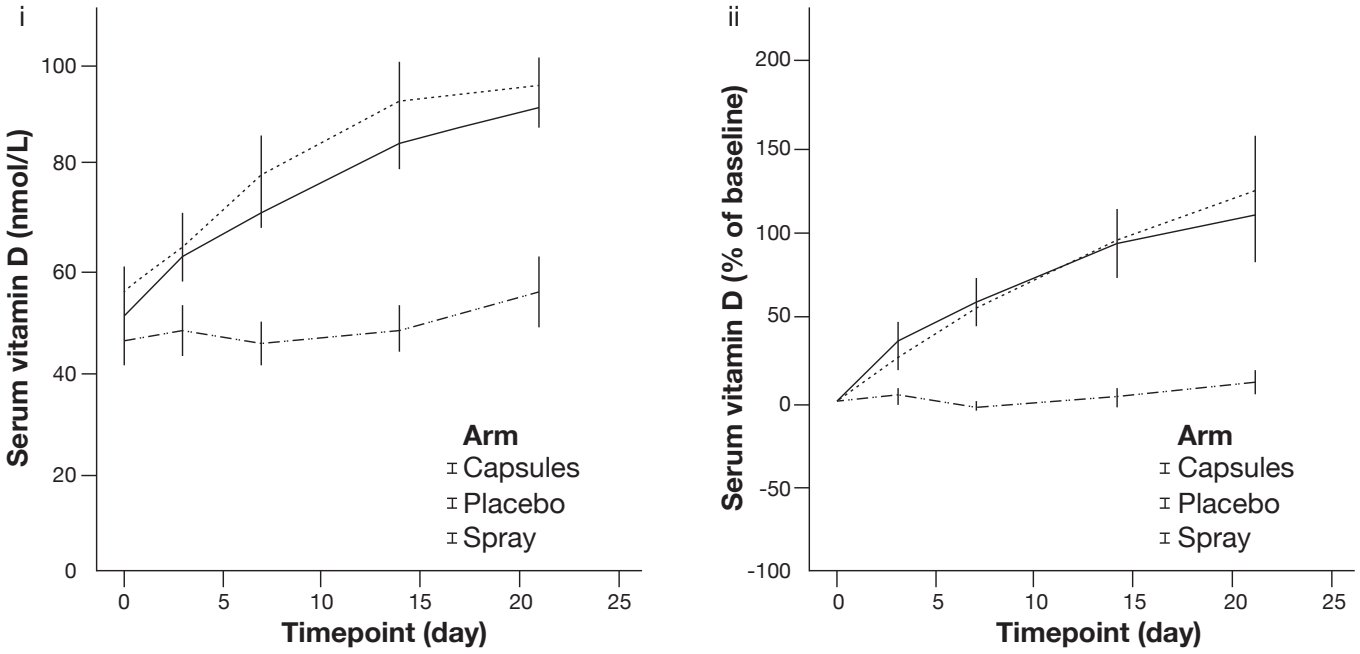
Figure legends

19. **Figure 1. Efficacy and rates of vitamin D uptake with differing delivery platforms.** Panel A shows change in vitamin D circulating levels over time in each of the three study arms, presented as absolute levels (panel Ai) or relative to baseline (Panel Aii). Panel B shows rates of uptake comparing spray (left column) with capsules (right column). Panels Bi and Bii show ladder plots for individuals in each arm of the trial plotting difference in vitamin D between day 0 and day 21 (the abscissa for uptake, based on Panel A). Rates were derived as nmol/ml/day and binned into 5nmol bins (Panels Biii and Biv). KS tests showed the data were normally distributed (capsules p=0.200, spray p=0.200). Finally, the rates for each individual were correlated with the baseline serum concentration for that individual (Panels Bv and Bvi). The r^2 and p values for correlations are indicated.

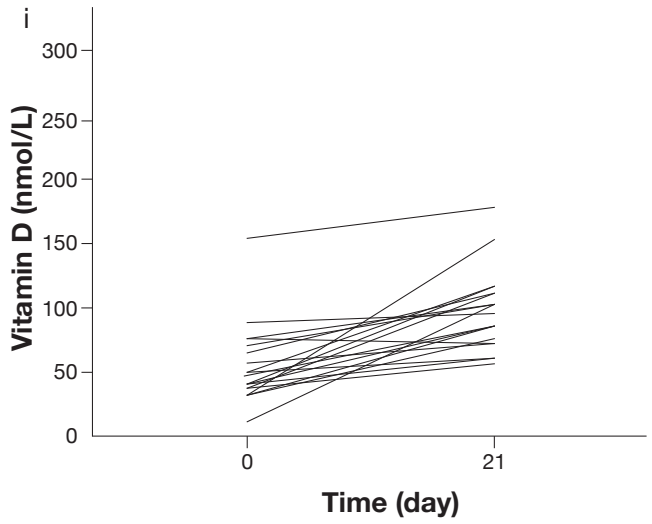
	Capsules	Placebo	Spray	All	P Value
Participants n	25	25	25	75	
Female n	14	10	15	39	0.326
Mean age (±SD)	22.9(±4.62)	22.4(±2.72)	21.7(±3.05)	22.4(±3.65)	0.504
Mean serum 25(OH)D nmol/L	**50.7(**±19.73)	**45.6(**±21.30)	54.2(±27.84)	50.5(±23.24)	0.38
BMI	23.7(±2.95)	22.7(±2.72)	23.8(±2.59)	23.4(±2.77)	0.294
Body fat	23.4(±2.75)	19.1(±5.91)	23.7(±7.65)	22.1(±7.37)	0.043
Height	171.3 ±7.54)	173.5(±10.20)	170.0(±8.35)	171.6(±8.77)	0.357
Weight	69.6(±10.71)	68.8(±12.77)	69.0(±11.32)	69.1(±11.48)	0.958
Skin tone	22/2/1	24/0/1	25/0/0	71/2/2	0.268

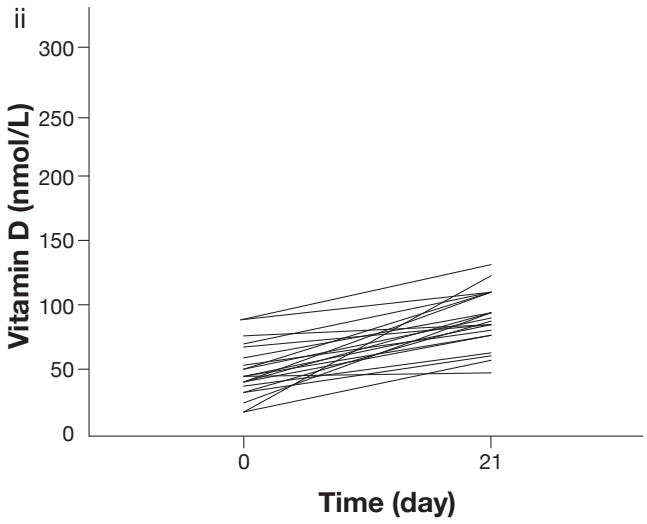
Table 1. *Baseline characteristics of participants.*

A



B


i
Vitamin D (nmol/L)
300
250
200
150
100
50
0
0
21
Time (day)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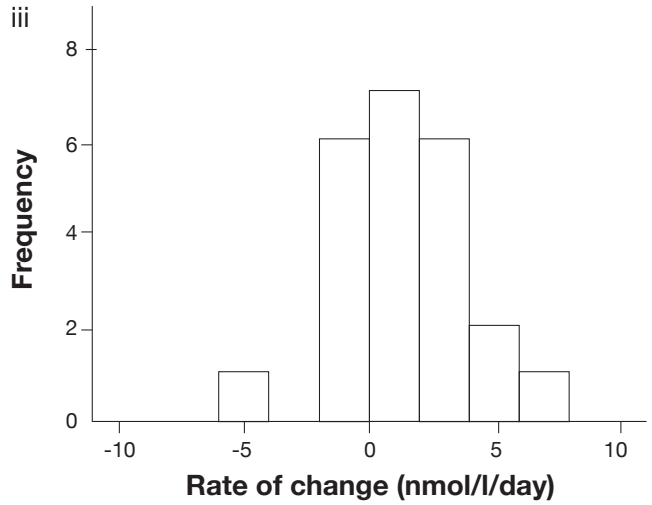
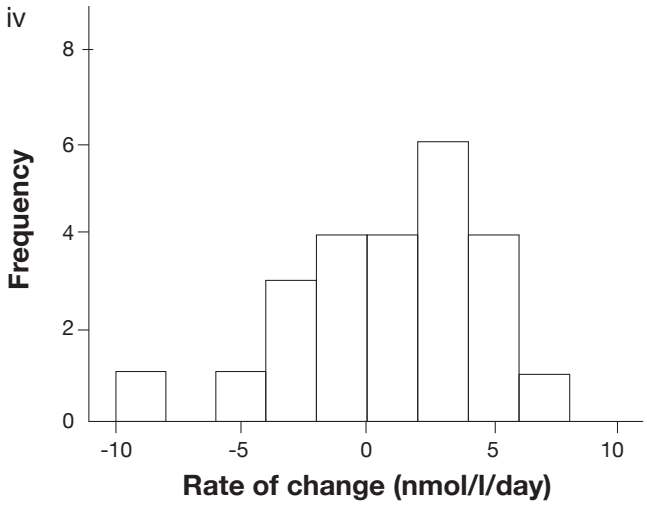
Vitamin D (nmol/L)
300
250
200
150
100
50
0
0
21
Time (day)


iii
Frequency
8
6
4
2
0
-10
-5
0
5
10
Rate of change (nmol/l/day)


iv
Frequency
8
6
4
2
0
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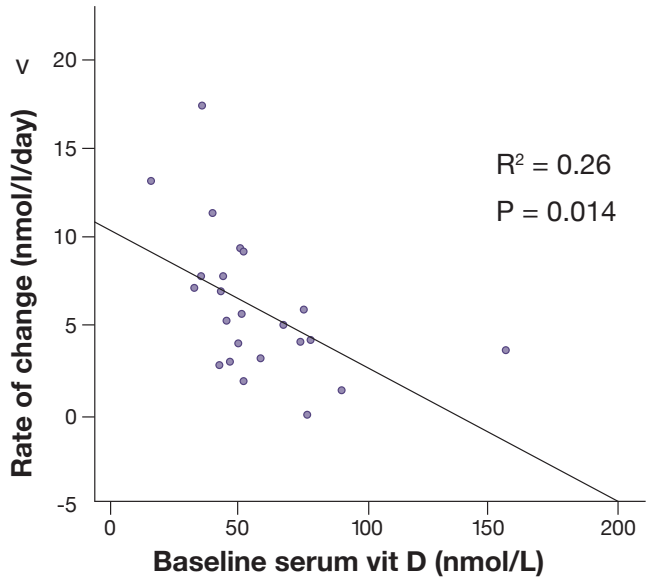
-5
0
5
10
Rate of change (nmol/l/day)


v
Rate of change (nmol/l/day)
20
15
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5
0
-5
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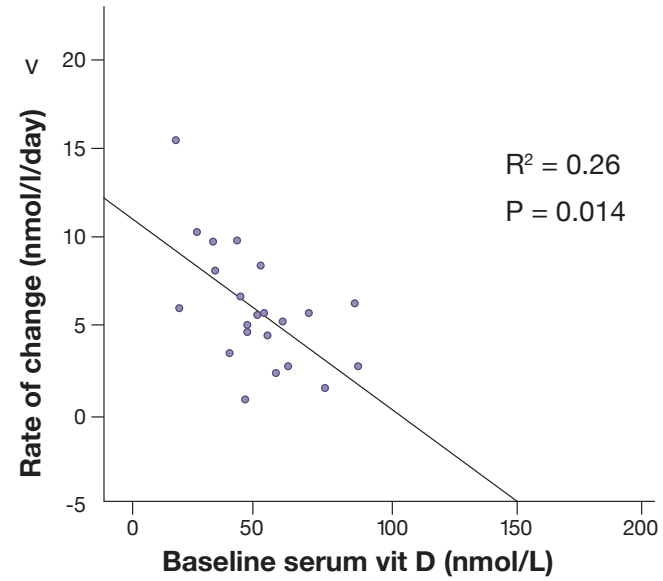
50
100
150
200
Baseline serum vit D (nmol/L)
R² = 0.26
P = 0.014


v
Rate of change (nmol/l/day)
20
15
10
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200
Baseline serum vit D (nmol/L)
R² = 0.26
P = 0.014

Analysis of the oral delivery of curcumin from a spray formulation

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Abstract

Oral mucosal drug delivery is an alternative and promising method of systemic drug delivery which offers several advantages. Sublingual literally meaning is “under the tongue”, administrating substance via mouth in such a way that the substance is rapidly absorbed via blood vessels under tongue. Sublingual route offers advantages such as bypasses hepatic first pass metabolic process which gives better bioavailability,rapid onset of action, patient compliance, self-medicated. Dysphagia (difficulty in swallowing) is common among in all ages of people and more in pediatric, geriatric, psychiatric patients. In terms of permeability, sublingual area of oral cavity is more permeable than buccal area which is in turn is more permeable than palatal area. Different techniques are used to formulate the sublingual dosage forms. Sublingual drug administration is applied in field of cardiovascular drugs, steroids, enzymes and some barbiturates. This review highlights advantages, disadvantages, different sublingual formulation such as tablets and films.

Introduction

1.1 Overview

Sublingual drug delivery utilises the permeability of the mucosal membrane located on the ventral side of the tongue. The sublingual membrane is a preventative barrier for the permeation of many compounds into systemic circulation. The membrane therefore is a difficult route to utilise for the delivery of drugs. However in comparison to other delivery routes this pathway provides several advantages, as discussed later.

This research probed the efficacy of the buccal and sublingual Curcumin supplementation from an oral spray via the In Vitro permeation of Curcumin through excised sublingual and heat separated buccal porcine membranes.

1.2 Curcumin

Plant isolated compounds have been in use for the treatment and prevention of various ailments since ancient times. These bioactive compounds mediate important physiological functions and commercially important for pharmaceutical companies to develop a new drug owing to broad therapeutic applications. Curcumin has been used in the form of turmeric as a dietary supplement for the treatment of various diseases since the turn of the first century.

Historically, turmeric and curcuminoids, have been used for a range of treatments including cancer, inflammation, bacterial infections and chronic wounds, recently there is In Vitro, In Vivo and clinical trial evidence to showing the health benefits of curcumin supplementation. Within the treatment of diabetes and its complications, curcuminoids have been shown to be effective at decreasing fasting blood glucose (De Mola, I.S.V., et al 2017.) and diabetic microangiopathy (Steigerwalt, R., et al 2017). Within cancers preclinical curcuminoids have been shown to mediate anticancer effects by modulating multiple cell signalling pathways regulating expression of microRNA's (Zhou, S., et al., 2017) and as an effective treatment for brain tumours including glioblastoma multiforme (Klinger, N, V., 2017). Also due to the anti-inflammatory properties as the potential use in cardiovascular disease (Grifiths, K., et al. 2016).

The first article referencing the oral administration curcumin was in 1937 showing excellent clinical benefits. More recently curcumin supplementation has taken many forms such as oral tinctures, powders, tablets and sprays with many commercial products available.

1.3 Sublingual Drug Delivery

Sublingual drug delivery is defined as the permeation of a drug through the sublingual mucosal membranes, which cover the ventral side of the tongue and the soft palate; these membranes are part of the non-keratinised epithelia found in the oral cavity.

The total surface area of the oral cavity has been found to be approximately 214.7cm^2 ± 12.9cm^2. Of this surface area, 30% is found to be non-keratinised epithelia, involved in sublingual and buccal delivery. Non-keratinised epithelia line: the inner part of the cheeks (inside the mouth), the ventral part of the tongue and the soft palate (Collins and Dawes 1987). An approximation of the non-keratinised membranes is:

(214 x 30) / 100 = 62.2cm^2

60% of this surface area is represented by the sublingual membranes (the soft lower palate and the ventral side of the tongue) (Wilson 2005; Chen et al. 1999).

(62.2 x 60) / 100 = 37.32cm^2

Of this, 13cm^2 makes up the ventral surface of the tongue (Ong and Heard 2009), with the rest making up the floor and soft palate. Therefore the rest of the surface area, 24.32cm^2 makes up the floor and soft palate of the mouth.

Permeation is easily affected by substances such as alcohols and therefore permeability is classed as selective. This is a limiting factor in the selection of excipients for sublingual formulations.

The permeation of lipophilic compounds is greatly hindered by mucosal membranes, including the non-keratinised membranes, and therefore permeation enhancers are required. curcuminoids are highly lipophilic compound, and the excipients found in oral formulations are to aid permeation, solubility, taste and appearance. Formulations commonly known to be used in sublingual delivery are sprays, the most popular one being the Glyceryl Trinitrate spray (GTN) used to provide relief in angina. (BNF62).

As part of the oral cavity, these membranes are exposed to an abundant supply of saliva which is constantly secreted and continuously flushes the cavity. Continual movement of the tongue, speech and salivary secretions coupled with the swallowing reflex lead to a limited time period of application. Aspects of delivery such as particle size and the physico-chemical nature of a formulation (eg combinations of permeation enhancers (Sudhakar et al. 2006) and mucoadhesives etc) greatly affect the flux of a compound.

1.4 Buccal Delivery

Buccal delivery involves the membranes that line the inner cheek, inside the upper and lower lips in the oral cavity. It forms approximately 40% of the nonkeratinised epithelia found in the oral cavity (Wilson 2005). This can be calculated from the overall surface area:

(40 x 62.2) / 100 = 24.88cm^2

The oral mucosal membrane, comprising of the buccal and sublingual membranes, varies in thickness and permeability. The buccal membrane is thicker, approximately 580μm (in comparison to the sublingual membrane which is approximately 190μm) and is generally less permeable (Squier and Wertz 1996) (Squier and Hall 1985b; Lesch et al. 1989).

This route of delivery is common for muco-adhesive formulations, enabling a longer application time. Similar to sublingual delivery, buccal delivery is affected by salivary secretion and mucus turnover. However, the increased application time in this area is due to the lower susceptibility of tongue movement.

1.5 Advantages of Sublingual and Buccal Delivery

Application of drugs onto the sublingual and buccal membranes have proven to be an easy alternative to those individuals who are incapable of ingesting formulations (i.e. patients who are nil-by-mouth, experiencing episodes of nausea and vomiting) or those that do not like or have difficulty taking tablets or liquid formulations (Narang et al. 2011). This route is non-invasive and is not as intimidating as injectable or rectal and vaginal routes.

The membranes are surrounded by a good vasculature which provides easy access into the systemic circulation bypassing the gastro-intestinal (system); this avoids any lag time of drug activation which is often experienced when dosing orally. The effects of drugs administered through theses membranes are therefore a lot more rapid and are not dependent on factors that commonly affect oral routes (stability of drugs in G.I fluid). These areas are easily accessible for application and can be ideal for sustaining prolonged delivery. In case of any unwanted effects, the dosage form can be easily removed restricting delivery almost immediately.

Sublingual sprays offer a faster onset action in comparison to tablet which would require dissolution (Parker et al. 1986; Marmor 1990)

1.6 Hypothesis and Aims

This study tested the efficacy of the delivery of Curcumin In Vitro through the tissues of the buccal cavity from an oral spray.

1. Evaluation of the permeation of Curcumin from commercially available preparations and compare that to laboratory made formulations through the buccal cavity – sublingual and buccal membranes.

2 Materials and Methods

2.1 Materials

Material/Chemical	Origin
Cyclodextrin encapsulated curcumin	Wacker
Methanol	Fisher
Ethanol	
Acetic acid	
Commercial Turmeric spray	BetterYou Ltd.
High vacuum grease	Dow corning (Mighigan, US
Porcine tissues (tongues)	Local abattoir

Table 1. *Materials used and their site of origin.*

2.2 Materials

In this study In-Vitro analysis of sublingual delivery was carried out separately – ventral membrane of the tongue and heat separated buccal membrane respectively.

2.2.1 Preparation of Porcine Membranes

Porcine sublingual membranes were used to perform in-vitro studies. Human and porcine oral membranes are similar in structure (Squier 1991), composition (Heaney 1978) and permeability (Squier 1996). The sublingual area is comprised of 2 parts: the floor of the mouth and the ventral surface of the tongue. Previous studies conducted have shown that permeation via the ventral surface of the tongue is greater than through the floor of the mouth (Ong and Heard 2009).

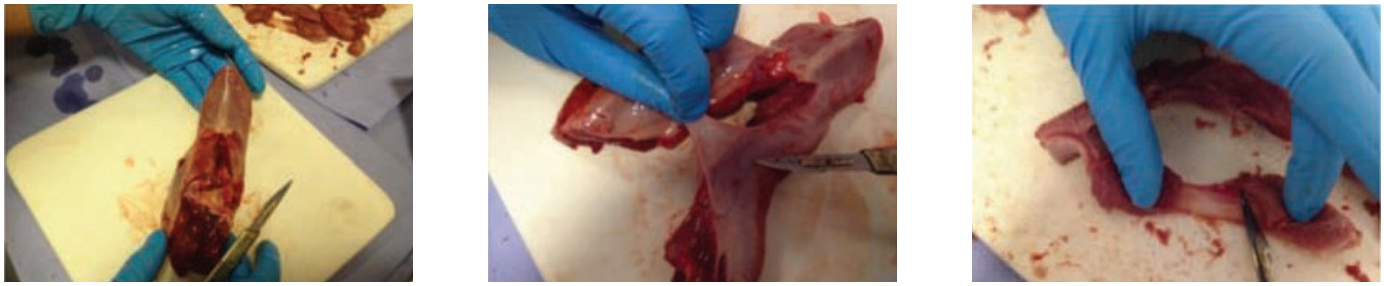

Figure 2: *Sublingual membranes and display of the blunt discussion technique (right).*

2.2.1.1 Sublingual membranes – Blunt dissection technique

Porcine tongues were collected from the local abattoir as soon as they were excised and transported immediately to the laboratory for membrane extraction. The ventral surfaces of the porcine tongues were excised using blunt dissection. Separation of the membrane required careful scalpel dissection from the ventral surface before the membrane was cut into approximately 1cm^2 pieces ready to be used on Franz-diffusion cells (FDC) for permeation studies as shown in Figure 1. Each piece was microscopically examined to ensure its full intactness.

2.2.1.2 Buccal membranes – Heat separation technique

The buccal membranes were cut and separated using heat separation. The porcine cheeks were excised from the inner cheek region of the porcine head and were placed in DI H_2O at 80°C for 60s. This allowed the membrane to be peeled away from the muscle using a forceps. This must be done carefully in order to extract large sections of the membrane for use on FDC's. Cells with a larger diffusional area were used because these membranes are thicker and tougher. The membranes extracted were cut up into approximately 2.5 cm^2 pieces, and microscopically examined before use.

2.3 Preparation of Donor and Receptor Phase solutions

2.3.1 Donor Phase Solutions

The donor phases consisted of 200µL of: One commercially supplied formulation and one simple Di H_2O saturated solution of Cyclodextrin encapsulated Curcumin.

2.3.2 Receptor Phase Solution

50/50 H_2O DMSO solution was used for the receptor phase. The solution was stirred using a magnetic stirrer. This was added to each FDC together with a magnetic stirrer and allowed to equilibrate within the water bath for 15min prior to application of the donor phases.

2.4 In Vitro Permeation Studies

The permeability of both membranes to the formulations was determined using all-glass FDC's, with a receptor volume of 3.9mL and a diffusional area of 1.1cm^2. The cell flanges for both the cells were greased with high performance vacuum grease prior to the mounting of the membranes.

Prepared membranes were then mounted in between the receptor and donor compartments covering the diffusional area. They were positioned with the mucosal surface facing the donor compartment, with metal clamps holding the membrane in place between the cell top (donor compartments) and cell body (receptor compartment) together.

The receptor compartment was filled to the calibration mark with the donor phase before adding magnetic stirrers and the sampling arm capped. The complete cells were placed in a water bath set at 37°C for 15 minutes to allow for equilibration before the addition of 200µL of donor phase.

Receptor phases were drawn at 10 20 30 and 60 min time intervals from the sampling ports and replaced with fresh receptor solution. 1mL of the samples drawn, were then placed into HPLC vials for testing.

2.5 HPLC Analysis

Reverse phase HPLC was used for curcumin analysis. An Agilent 1100 fitted with Gemini NX C18 column was used; the UV detector was set at 425nm. A mobile phase of 50:50 (v/v) mixture of acetonitrile and 2% acetic acid in water at a flow rate of 1.2mL min^{-1}.

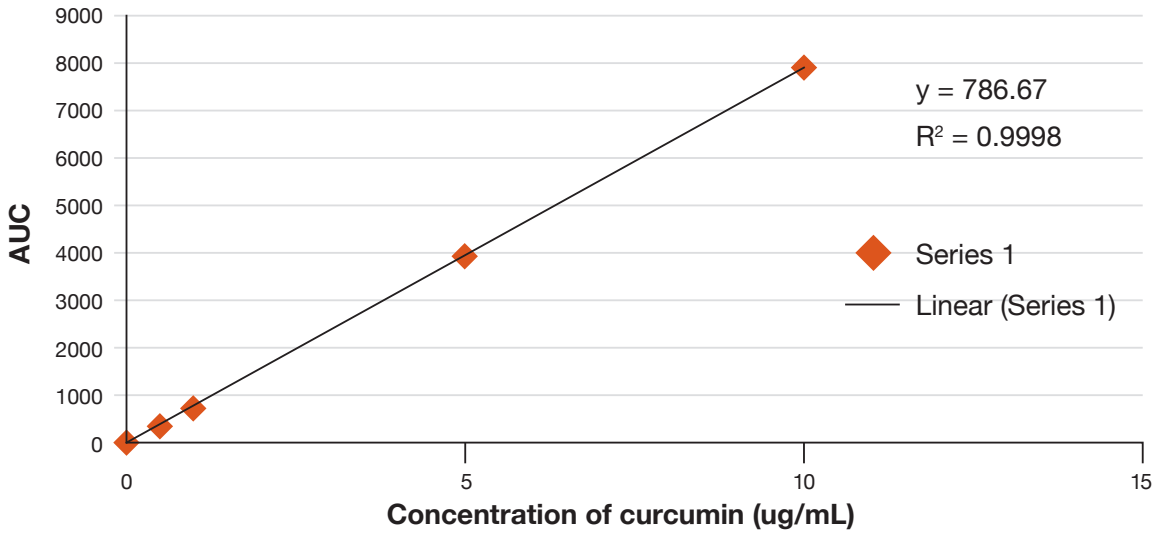

Figure 2: *HPLC curcumin calibration curve*

2.6 Data Processing and Statistical Analysis.

For each sample and each tissue cumulative amounts of curcumin permeated per unit area were plotted against time over 60min. Flux values were calculated using the linear portions of these graphs.

Statistical analysis was completed using InStat 3 for Macintosh GraphPad Software, Inc. (Hercules, CA, USA). A one way ANOVA with post t-test was used to investigate differences between the data sets of the various tissues. To be considered a significant p-value of < 0.05 must be achieved. (Squier 1996).

3 Results

3.1 Sublingual permeation

Each preparation was tested on porcine sublingual membranes, the ventral part of the tongue. The volume of donor phase applied each time was the same.

The permeation profiles of curcumin for the commercial product and simple formulation is shown graphically in Figure 3.

This analysis shows that both formulations permeated the sublingual membrane, with the commercial formulation shown to have delivered the highest amount of curcumin over 60 min achieving a total mass of 0.201μgcm^{-2} ± 0.043μgcm^{-2} with an average Jss of 0.0047 (x10^{-3}μgcm^{-2}h^{-1}). The curcumin water formulation showed significantly less at 0.12μgcm^{-2} ± 0.028μgcm^{-2} with and average Jss of 0.0027 (x10^{-3}μgcm^{-2}h^{-1}).

3.1.1 Buccal permeation

Each preparation was tested on porcine heat separated buccal membranes. The volume of donor phase applied each time was the same.

The permeation profiles of curcumin for the commercial product and simple formulation is shown graphically in Figure 4.

This analysis shows that both formulations permeated the buccal membrane, with the commercial formulation shown to have delivered the highest amount of curcumin over 60 min achieving a total mass of 0.348μgcm^{-2} ± 0.035μgcm^{-2} with an average Jss of 0.0065 (x10^{-3}μgcm^{-2}h^{-1}). The curcumin water formulation showed significantly less at 0.073μgcm^{-2} ± 0.015μgcm^{-2} with and average Jss of 0.0011 (x10^{-3}μgcm^{-2}h^{-1}).

4 Discussion

4.1.1 Comparison of the oral membranes involved in the delivery of curcumin.

Sublingual and buccal deliveries are both forms of topical delivery, although permeation across each of the membranes varies. Studies on the different membranous regions of the oral cavity have shown this. Sublingual membranes have the highest permeability followed by the buccal membrane that is the least permeable (Lesch et al. 1989). The buccal membrane does not provide the same rapid absorption and bioavailability which is seen with the other two membranes (Singh et al. 2011). This

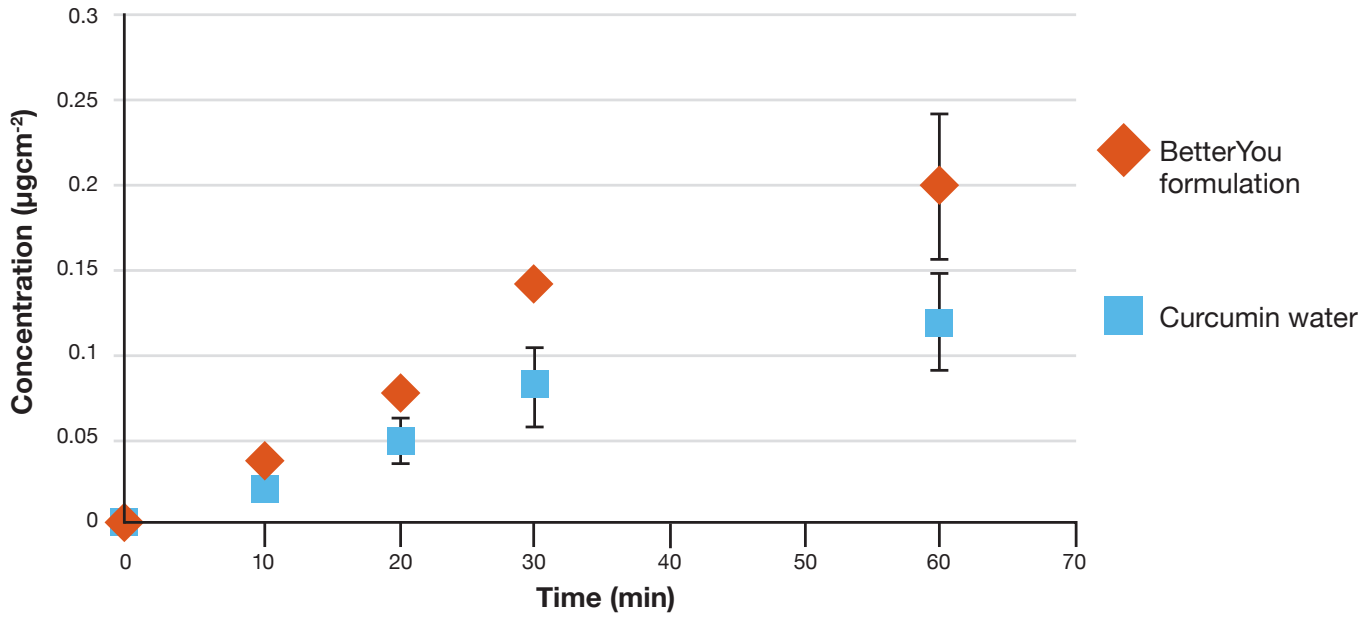

Figure 3: *Plot showing the cumulative delivery of Curcumin across the ventral surface of the tongue for a commercial spray and a curcumin water formulation.*

however can be argued based on the variation seen in this study, where the buccal permeation is greater than the sublingual, as shown in Section 3.

The difference in permeability can be seen with reference to the flux values of the commercial spray between the ventral membrane of the tongue and the buccal membrane. The flux across the buccal membrane is approximately 30% greater than that of the ventral membrane of the tongue. This difference can be attributed to glucosylceramide, which is an important mucosal membrane constituent. Studies have shown that the greater the glucosyleceramide content the poorer the membrane permeability (Squier et al. 1991). The amount of glucosylceramide present in the buccal mucosa is almost 3 times more than in the sublingual membranes. However in this case having a high glucosylceramide (a membrane lipid) content, may increase the permeation of highly lipophilic drug encapsulated in cyclodextrin within an emulsion formulation such as commercial curcumin supplement in this study.

The commercial spray is a micro-emulsion of cyclodextrin encapsulated curcumin whilst the other is a simple water preparation of cyclodextrin encapsulated curcumin. The main difference being the types of excipients used and thusly the concentration of curcumin applied, with the commercial formulation containing the greater concentration. Although the greatest level of delivery was from the commercial formulation across both membranes it is noted that the increase of delivery was significantly greater across the buccal membrane in comparison to the sublingual.

5 Conclusion

The current study has shown that curcumin permeates both the sublingual and buccal membranes, however the permeation across buccal membrane greater than that across the sublingual.

Due to the low concentrations of curcumin delivered across any membrane from either formulation this work could only be carried out with maximal delivery. Analysis of finite delivery proved to be lower than the limit of detection (results not shown).

Comparing the different types of preparations, this work has shown that the micro-emulsion commercial preparation has a higher degree of permeation in comparison to the simple water preparation. It is unclear as to whether this is due to a greater loading of curcumin in the commercial preparation or that the emulsion provides a better delivery environment for the cyclodextrin encapsulated curcumin.

The formulation provided by BetterYou of an emulsion containing cyclodextrin encapsulated curcuminoids successfully permeates the membranes of the buccal cavity and is a superior delivery method than that of the water based formulation.

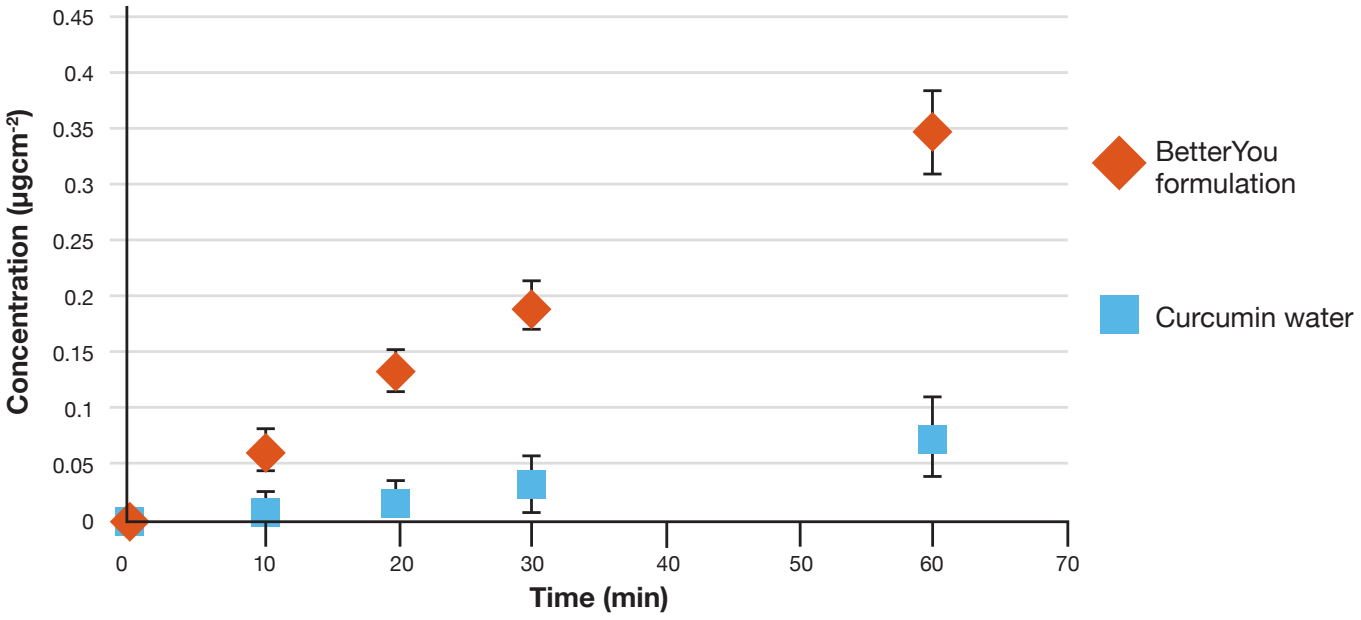

Figure 4: *Plot showing the cumulative delivery of Curcumin across heat separated buccal membrane for a commercial spray and a curcumin water formulation.*

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Vitamin D status in Irritable Bowel Syndrome and the impact of supplementation on symptoms: what do we know and what do we need to know?

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Abstract

Low vitamin D status is associated with risk of colorectal cancer and has been implicated in inflammatory bowel disease. Irritable Bowel Syndrome (IBS) is a chronic, relapsing, functional bowel disorder. A nascent literature suggests a role for vitamin D in IBS, but this has not been collated or critiqued. To date seven studies have been published: four observational studies and three randomised controlled trials (RCTs). All observational studies reported that a substantial proportion of the IBS population were vitamin D deficient. Two intervention studies reported improvement in IBS symptom severity scores and Quality of Life (QoL) with vitamin D supplementation.

There are limited data around the role of vitamin D in IBS.

The available evidence suggests that low vitamin D status is common among the IBS population and merits assessment and rectification for general health reasons alone. An inverse correlation between serum vitamin D and IBS symptom severity is suggested and vitamin D interventions may benefit symptoms. However, the available RCTs do not provide strong, generalisable evidence; larger and adequately powered interventions are needed to establish a case for therapeutic application of vitamin D in IBS.

Introduction

The reported health benefits of vitamin D have recently extended from musculoskeletal health to focus on the potential relationships in systemic diseases, such as Multiple Sclerosis (MS), colorectal cancer (CRC), Inflammatory Bowel Disease (IBD) (1). Vitamin D is a hormone that has two key roles within the body; i) to aid the absorption of calcium and phosphate ii) control the secretion of parathyroid hormone (2). The principal circulating form of vitamin D is 25-hydroxyvitamin D (25(OH) D; calcifediol; ChEBI:17933), which is used clinically to determine vitamin D status (3). There is no universally agreed optimal level of vitamin D, however the National Academy of Medicine (USA and Canada) has asserted that serum 25(OH) D levels need to exceed 50nmol/L (20ng/ml) to be adequate to meet the needs of 97.5% of the population (4) and by extension levels <50nmol/L (<20ng/mL) are considered insufficient (5, 6). Poor vitamin D status is of major public health concern with low vitamin D status affecting 8-24% of children and 20% adults in the UK (7). Consequently SACN guidelines recommend an intake of 10µg/d for anyone aged 1 year and older (8). Vitamin D has increasingly been implicated in the pathobiology of colorectal diseases. A meta-analysis and systematic review of observational studies in inflammatory bowel disease (IBD) suggested that patients were 64% more likely to be vitamin D deficient compared to controls without IBD (p=0.0001) (9). Similarly, a recent review and a meta-analysis of the potential relationship between vitamin D and colorectal cancer identified an association between vitamin D intake and colorectal cancer prevalence: a significant inverse association between dietary vitamin D intake, 25(OH)D status and colorectal cancer risk was reported (10) (11). The potential for vitamin D as a secondary preventive of adenoma recurrence has also been investigated in several trials both alone and in combination with calcium (12)

Irritable bowel syndrome is one of the most common functional bowel disorders seen globally (10-20% of some populations (13) with significant healthcare cost (14). The pathogenesis of the disease remains unclear and is categorised primarily by the symptoms experienced ``z(15-26 17). Symptoms of IBS include bloating, abdominal pain, diarrhoea and / or constipation; the ROME III criteria incorporate assessment of these symptoms to diagnose the condition (18). There are three recognised sub-types of IBS: diarrhoea-predominant (Type D), constipation-predominant (Type C) and alternating diarrhoea and constipation (Type A) (19). Other common features of this syndrome not covered in the diagnostic criteria are bloating, passing of mucus from the rectum, irregular stool habits and urgency of evacuation (20). These symptoms have a serious impact on the person's every day quality of life and appear to have strong links to mental health issues such as anxiety and depression (21). A number of reports linking vitamin D and IBS have received significant media attention, this review aims to collate and contextualize this research. The literature was searched systematically (See Supplementary Online Information Section I) to identify the full scope of publications in this area; 7 reports were identified, comprising of 4 observational studies and 3 randomised control trials (RCTs).

Summary of the literature to date

Observational Studies

Four intervention trials were identified that assessed vitamin D status in IBS (see Table 1).

A case study reported that a high dose supplementation (50-75mcg per day throughout the year) of vitamin D significantly improved one woman's IBS symptoms (22), including a return to almost-normal bowel patterns and decreased anxiety and depression. This paper also systematically identified analysed social media (blogs by people with IBS), noting that 70% of 37 individuals' blogs reported that vitamin D supplementation resulted in an improvement of symptoms. This case resided in the UK (hence a Northerly latitude), however blogs were from those living internationally and exact locations were not reported. Deficiency thresholds were not defined and serum 25(OH)D levels were not stated. Although in agreement with some intervention trials (23, 24), case studies are not generalisable or statistically significant.

A case control study reported vitamin D serum concentrations in patients with IBS attending a gastroenterology clinic in Saudi Arabia (International Medical Centre)(5). Cases had a confirmed diagnosis of IBS using ROME III criteria and healthy controls were gender and age matched staff members from the medical centre. This study defined deficient serum 25(OH)D concentrations as <50nmol/L (23, 25); mean serum 25(OH)D concentrations in patients with IBS was 21±12nmol/L which was significantly different to 31±16nmol/L reported for the control group. It should be noted that this study only reported serum 25(OH)D concentrations retrospectively from medical records.

A second observational study in Saudi Arabia reported recruitment of subjects (n=498) with both Crohn's Disease (CD) and IBS and compared these to a control group of staff and students (n=442) (26). The study reported insufficiency of serum 25(OH)D concentrations in 67.3% of the patients, however it is difficult to ascertain whether the insufficiency of vitamin D was a result of the IBS, CD, a combination of both or a common issue among this general population. This study neglected to define their threshold of 'vitamin D insufficiency'. Both studies

were conducted in Saudi Arabia known for its year-round sunshine which should have a positive effect on serum 25(OH)D levels. However, for religious reasons the population avoid direct exposure of their skin to sunlight and a recent systematic review (27) of 13 studies (n=24,399) found that 81% of different Saudi Arabian populations (e.g. pregnant/lactating women, children, adults) had serum concentration levels of 25(OH)D <20ng/ml (<50nmol/L).

In a US-based study (Atlanta, Georgia) medical records of 1,000 IBS patients were reviewed (28). The mean serum concentration of 25(OH)D of the population studied was 25.05nmol/L. It was also reported that 72% of women and 3% of men with IBS had a serum concentration <30 nmol/L. There were no controls used for comparison. Furthermore, this research is only available in abstract form and as such a full analysis is unavailable.

A retrospective case-controlled study (6) analysed the medical records of 55 children and adolescents aged 6-21 diagnosed with IBS living in Massachusetts, USA. This research shows that only 7% of the IBS cohort had sufficient vitamin D levels compared to 25% of BMI-matched healthy controls attending a well-child clinic. This study suggested prevalent vitamin D insufficiency in both the IBS and control populations, albeit with a limited study design.

Intervention studies

Three intervention trials were identified that investigated the possible beneficial effect of vitamin D on IBS symptoms (see Table 2).

Tazzyman et al. (2015) conducted a 12 week randomised double-blind three-arm parallel pilot study in people with IBS which compared placebo to either vitamin D supplementation (75µg/d) or combination of vitamin D (75µg/d) plus probiotic (two strains of Lactobacillus acidophilus per capsule). The trial was conducted in the UK in January-April 2015. Analysis of baseline data illustrated that participants with low vitamin D (<50nmol/L) had lower QoL (using the single question in the Total Symptom Severity IBS questionnaire (29) compared to their replete counterparts (p=0.034)). Improvements were reported in all treatment arms, but no significant difference between the treatment arms was observed. The study provides valuable data on which to base power calculations for future randomised control trials.

A RCT conducted in Iran with 85 participants with IBS (23) found significant improvement of IBS symptoms (p < 0.001) and quality of life (p <0.001) following very high dose (1250µg fortnightly for 6 months) vitamin D3 supplementation compared to a placebo over a period of 6 months. Separate tools measured symptom severity (29) and quality of life (30) at baseline and exit of the study.

A second Iranian study (24) used a 2x2 factorial design to conduct a blinded randomised control trial with women aged 18-75 to investigate the effects of vitamin D, soy isoflavones or both on IBS symptoms and quality of life. One hundred participants were randomly assigned to one of four possible arms of the intervention; vitamin D and placebo (D+P), soy isoflavones and placebo (S+P), soy isoflavones and vitamin D (S+D) or both placebo vitamin D and placebo soy isoflavones (P+P). 50 000 IU (1250µg) of vitamin D was administered fortnightly and 2 31 x 20mg of soy isoflavones capsules daily. The length of study was a restrictive 6 weeks with a follow-up at 4 weeks post intervention. This study reported significant improvements in IBS symptom severity score and quality of life in participants randomised to either vitamin D isoflavones. Both S+P and the D+P groups significantly improved IBS total score (p=0.004, p=0.015 respectively). The combination effect of vitamin D and soy on IBS-TS was also significant (p<0.05).

Both the Abbasnezhad and Jalili studies showed extraordinarily low standard deviations of IBS symptom severity scores (around 10% around the mean; our ongoing work suggests that the majority of such studies report the SD of symptom severity in the range of 20-70% of the mean (Corfe, unpublished). This suggests a significantly more homogenous population than comparable publications, the reasons for this are unclear.

All three intervention studies reported low mean baseline vitamin D serum concentrations in the IBS populations studied, ranging from 14ng/mL-21.23ng/mL (35nmol/L-53nmol/L). Vitamin D deficiency is present in the general populations of both the UK and Iran (31, 32) populations and as such, no causal link with IBS can be inferred without control population data. Two (23, 25) out of the three studies showed an increase in the mean 25(OH) D levels from deficient (<20ng/mL or <50nmol/L) status to replete (>20ng/mL or >50nmol/L) in the active arm. Dosages of vitamin D supplement varied between the studies. The preparations were either in the form of one 50 000IU (1250ug) oral capsule fortnightly or a daily 3000IU (75ug) sublingual spray. Although optimal dosing strategy is not known, research suggests that both larger, less frequent doses and daily preparations are equal in effectiveness in their repletion of 25 (OH)D (33, 34). Despite small losses to follow up, final sample sizes from previous studies appear to be relatively similar.

Conclussions and directions

There is a nascent body of literature associating vitamin D status and the pathobiology and management of colorectal conditions including IBD and cancer. Four papers and one abstract report cross-sectional studies. A consistent limitation of these was that vitamin D status of the wider population is not reported. Cause and effect are difficult to determine as it might be argued that individuals with severe IBS may exhibit behaviour changes, for example elevated time indoors consequent to symptoms,

that may impact on vitamin D status.

Two of three interventions studies report a positive benefit of vitamin D supplementation in people with IBS, however the low variation in the study populations and unusual dosing regime in these two studies raises questions about the generalisability of the data.. All three RCTs reported a relationship, either at baseline or in response to intervention, between vitamin D and QoL, a symptom domain of particular importance to the patient population.

Collectively the studies reviewed, although restricted, offer enough justification for further work in this subject area. In particular, future research may benefit from adequate powering (Tazzyman et al. suggests 74 subjects / arm), now that effect size data are in the public domain, to assure generalisability and conclusiveness. Future studies should include a broader spread of participant, or multiple studies should address the potential benefits in defined populations and limit claims to these populations.

Less equivocally, the body of evidence accrued across multiple populations already suggests that vitamin D status assessment should be incorporated as a routine assessment alongside IBS diagnosis in routine practice to identify individuals at risk and likely to benefit from vitamin D intervention for general health as much as for IBS symptoms.

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Conflict of interest

The authors authored two of the systematically reviewed papers. BetterYou markets vitamin D supplements.

Authorship

CEW undertook the searches, collated literature and wrote the first draft. EAW co-conceived the study, reviewed and edited all drafts. BMC co-conceived the study, undertook the searches, collated the literature and edited all drafts. All authors agreed the final version of the manuscript.

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Legends

Table 1. Observational studies identified linking IBS symptoms and vitamin D status. Papers are in order of publication, showing populations used in the study.

Table 2. Intervention Studies identified testing the effect of vitamin D supplementation on IBS symptoms. Papers are in order of publication, the study size, population and principle outcomes are shown. Abbreviations: IBS-SSS = Irritable Bowel Symptom Severity Score, TSS = Total Severity Score, QoL = Quality of Life, D = vitamin D, S=soy isoflavones, P=placebo.