

THE MEDICINAL PROPERTIES AND APPLICATIONS OF A CARBOHYDRATE DERIVED FULVIC ACID

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DECLARATION

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ABSTRACT

Background: Carbohydrate derived fulvic acid (CHD-FA) is a synthetic heavy metal free fulvic acid. Although CHD-FA has been suggested as a nutritional supplement and even a medication, there is currently no data available with regards to the systemic kinetics of CHD-FA when ingested orally. Fulvic acid has shown equivalence when compared to diclofenac sodium and betamethasone in a murine hypersensitivity model when applied topically with no adverse side effects. Data from several animal studies indicate that fulvic acid administered topically or orally is safe and effective as an anti-inflammatory agent. Human *in vivo* safety and efficacy as well as potential *in vitro* genotoxicity still need to be assessed.

Aim: To determine whether carbohydrate derived fulvic acid shows any genotoxic effects and whether it is safe, clinically effective as an anti-inflammatory and attempt to establish a suitable CHD-FA marker for kinetic studies.

Methods: Genotoxicity was determined via an *in vitro* micronucleus assay. Systemic and topical safety and efficacy of CHD-FA was established via two limited participant, double blind, randomised, placebo controlled clinical trials. One to determine safety and efficacy in 30 adult male volunteers with a predetermined atopic hypersensitivity, using a skin prick test, and the second trial to test topical safety and efficacy in 40 patients suffering from atopic dermatitis. LC-MS/MS assays were conducted in order to determine whether a unique or dominant ionic analyte could be found in plasma.

Discussion: In the *in vitro* genotoxicity tests, CHD-FA compared closely to that of the negative control with respect to the number of micronuclei observed. All tested *in vivo* safety parameters proved to remain constant throughout both the clinical trials. A significant decrease in flare was observed in CHD-FA treated patients following a skin prick challenge. Due to the complexity of the mass spectral fingerprints of both plasma and the fulvic acid no suitable CHD-FA markers were found.

Conclusion: No genotoxicity was observed for CHD-FA treated cells. No severe adverse events occurred in either the oral or topically administered CHD-FA trials, proving CHD-FA to be systemically and topically safe. A significant decrease in wheal formation in the skin prick test, and a significant clinical improvement in atopic dermatitis compared to placebo

were observed implicating that CHD-FA may act as an anti-inflammatory agent *in vivo* in humans. No suitable CHD-FA markers for pharmacokinetic studies in human plasma could be identified.

Keywords: carbohydrate derived fulvic acid, atopic dermatitis, safety, suppressed wheal formation, efficacy, anti-inflammatory, genotoxicity, LC-MS/MS



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List of Abbreviations

µL	microlitre
µm	micrometer
µM	microMolar
µmol/l	micromoles per litre
°C	Degree Celsius
%	Percentage
AD	Atopic Dermatitis
AE	Adverse Event
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ANCOVA	Analysis of co-variance
ART	Antiretroviral Therapy
AST	Aspartate aminotransferase
BW	Body Weight
CBMN	Cytokinesis Block Micronucleus Assay
CD4	Cluster of differentiation 4
CE	Collision energy
CHD-FA	Carbohydrate derived fulvic acid
CO₂	Carbon dioxide
COX	Cyclooxygenase
cp	Copies
CR3	Complement Receptor 3
CRF	Case Report Form
Cyt-B	Cytochalasin-B
Da	Dalton
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNFB	2,4-Dinitrofluorobenzene
DP	Declustering potential
ECG	Electrocardiography
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
FA	Fulvic Acid
FBC	Full Blood Count
g	Gram
<i>g</i>	Relative Centrifugal Force
GIT	Gastrointestinal
GGT	Gamma glutamyltransferase
HA	Humic Acid
HIV	Human Immunodeficiency Virus
Hb	Haemoglobin
IC₆₀	Inhibitory concentration that results in 60% cell death
ICAM	Intracellular Adhesion Molecule
ICH	International Conference on Harmonisation
IGA	Investigators global assessment
IL	Interleukin
ITT	Intent- to-treat
KCl	Potassium chloride



L	Litre
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDH	S- Lactate Dehydrogenase
LMWCHD-FA	Low Molecular Weight Carbohydrate Derived Fulvic Acid
m	Milli
MCC	Medical Control Council
MCV	Mean corpuscular/ cell volume
MCHC	Mean corpuscular haemoglobin concentration
MNi	Micronuclei
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)
MRM	Multiple reaction monitoring
NaCl	Sodium chloride
nm	nanometer
OECD	Organisation for Economic Cooperation and Development
PBS	Phosphate buffered saline
pH	Negative logarithm of hydrogen ion concentration: a measure of the acidity of a solution
PHA	Phyto-hemagglutinin
PPT	per-protocol-population
prn	as require
PMA	Phorbol 12-myristate 13-acetate
RCC	Red blood cell count
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SD	Standard Deviation
TIC	Total ion chromatograms
UEA	Ung Emulcificans Aqueosum
UP	University of Pretoria
USA	United States of America
VAS	Visual Analogue Scale
WBC	White blood cell count
XIC	Extracted ion chromatograms

CHAPTER 1: LITERATURE REVIEW

1.1 THE DRUG DEVELOPMENT PROCESS

For patients, new medicines offer fewer side effects, fewer hospitalizations, improved quality of life, increased productivity, and importantly, extended lives. But developing medicines is a long, complex process. The rapid pace of scientific advances is enabling a greater understanding of diseases at the molecular level. In turn, scientific, technical, and regulatory challenges related to drug development create complexities as companies often focus their research and development where the science is difficult and the risks of failure are higher.

Modern medicine makes extensive use of drugs to control physiological processes that in turn affects the symptoms and improves the patient's well-being. The pharmaceutical industry is however highly regulated to ensure the safety and best interests of the patient. To achieve this there is a worldwide consensus that drugs must go through a systematic review of the safety and efficacy parameters during the pre-registration phase. There is a common drug development and review process applied in almost all countries around the world and this is summarized in Figure 1.

Biopharmaceutical companies perform basic research independently and in partnership with researchers and others from across the biomedical research ecosystem, including disease foundations and patient groups, venture capital, and pre-competitive consortia. After learning more about the underlying disease pathway and identifying potential targets, researchers then seek to narrow the field of compounds to one lead compound, a promising molecule that could influence the target and, potentially, become a medicine.

Lead compounds can be found through various processes that include *in silico* drug design based on knowledge of the target receptor or protein, modifying the structure of existing drugs to improve selectivity or pharmacokinetic properties, finding new indications for drugs already on the market (off-label use), diverse high throughput *in vitro* screening or the targeted use of traditional or herbal medication that is investigated for which compounds are active. Each of these approaches has met with varying success.

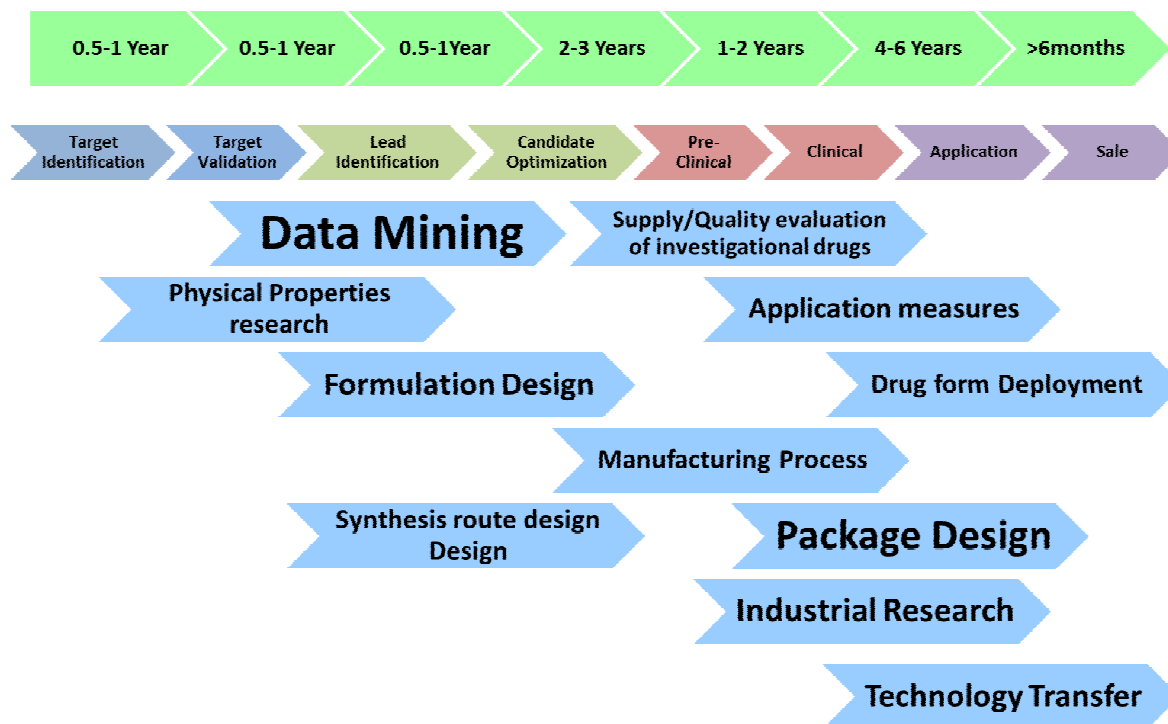


Figure 1. A summary of the many aspects of the Drug Development Process indicating the approximate timelines, the technical and regulatory features of the process.

Any new products identified have in each case, except off label use, required that the product go through the full preclinical safety and efficacy testing followed by the compulsory sequence of clinical testing phases and review of the data by the regulatory authorities.

Preclinical testing involves several aspects, starting with *in vitro* tests to assess the targeted effects of the drug using model systems that mimic the *in vivo* drug target.

Once the proof of concept with respect to the drug efficacy has been established and the stability of the compounds are confirmed, the initial safety data can be collected, normally using well established rodent toxicity models. These tests often include dose incremental assays and assess the toxicity profile at various levels, the pharmacokinetics, tissue accumulation and metabolic products before being used on a diseased model to assess the actual efficacy. Other testing that is often performed at this early stage of drug development are the carcinogenic, mutagenic, teratogenic and long term chronic exposure tests. These assays are repeated in at least two other animal species to establish the possibility that the compound does not have a species specific differential toxicity effect.

If the lead compound demonstrates reasonable safety and efficacy effects and can be shown to have an acceptable benefit to risk ratio it may be taken further into clinical trials but first needs to be registered with the relevant registering authorities.

At this stage the compound needs to be registered as a new molecular entity or a new chemical entity before application can be made to the regulatory bodies to approve the initial Phase I clinical trials where the compounds would be administered to humans for the first time under very controlled conditions. “First in man” studies are where low doses are administered to a small number of healthy individuals and the pharmacokinetics and metabolite profiles are assessed while monitoring potential toxicity to confirm the data collected during animal studies. During the Phase I trial/s, many physiological effects are monitored to be able to measure as many possible effects, whether good or bad, to be able to provide data for the design of Phase II clinical trials where the drug would be administered under controlled conditions to patients with the disease or condition to be treated.

Phase II trials are generally aimed at determining the efficacy of the new lead compound and to perform dose ranging studies, tolerability studies, monitoring side effects, confirming safety and pharmacokinetics and determining the best route of administration. These studies are generally performed as a double blind test to be able to evaluate the true unbiased drug effects. There are numerous Phase II clinical trial designs that impacts on the number of patients required, the comparator to be used (placebo or best available treatment), the method of determining the efficacy, the duration of treatment as well as the statistical methods to be used to assess the data. If the lead compound still shows potential and the benefit/risk profile is acceptable Phase III studies will be carried out on large patient populations to confirm a broad efficacy and safety profile without a high adverse side effect profile.

On successful completion of a phase III trial the drug would be registered for a particular indication and the industrialisation process would be put in place for the manufacture, formulation and packaging of the drug.

Phase IV trials would be run once the drug has been released onto the market to provide post-marketing safety data that would monitor any side effects within the general population when taking the drugs under uncontrolled conditions. This data is reviewed by the registering authorities at set intervals or if a particular side effect becomes obvious or when a life threatening side effect is identified. Therefore when any research is to be conducted, it is both relevant and pertinent as to where and at which stage this is commenced within the drug development lifecycle as there are various considerations required that need to be taken into consideration.

1.2 HUMIC SUBSTANCES AND MEDICINAL APPLICATIONS

Humic substances are ubiquitous in nature and arise from the decay of plant and animal residues in the environment (MacCarthe *et al.*, 1990). These substances can be divided into fulvic acid, humic acid and humin on the basis of the solubility in water as a function of pH. Fulvic acid is the fraction that is soluble in water under all pH conditions and is generally lower in molecular size and weight and shows less colour intensity than the larger and darker humic acids.

Humic acids are macro-colloidal molecules with supramolecular character that are natural components of drinking water, peat, soil and brown coal and have been defined as “a general category of naturally occurring, biogenic, heterogeneous organic substances that can generally be characterized as being yellow to black in colour and of high molecular weight”. The average molecular weight of humic substances may range from 500 to 5000 Da for fulvic acids and 3000 to 1000 000 Da for humic acid (Sparks, 2003).

Medicinal use of humic substances has been reported in many old traditional medical reports including Ayurvedic traditions and Eastern medicine. In Europe mud baths, rich in humic and fulvic acids were used for the treatment of various ailments, such as rheumatic conditions during the 19th century (Baatz, 1988; Lent, 1988; Kleinschmidt, 1988; Kovarik, 1988). Peat was also used during the First World War to treat wounds and amputations in field hospitals to prevent infections, relieve pain and facilitate healing (Haanel, 1942; Van Beneden, 1971)

There are currently several humic acid based topical and oral medications commercially available. Their therapeutic properties have been summarized by Schepetkin *et al.*, (2002) as antibacterial, antitoxic, anti-ulcerogenic, anti-arthritic, anti-allergic and anti-inflammatory.

Studies by Kleinschmidt (1988) and Iubitska and Ivanov (1999) have shown that sodium humate therapy improves the clinical condition of osteoarthritis patients (Soliev, 1983) possibly due to its reported anti-inflammatory and bone regenerating properties (Kelginbaev *et al.*, 1973; Suleimanov, 1972).

Humic substances' beneficial effects have been partly ascribed to the ability of humic acids to absorb or chelate toxic compounds or metals (Stackhouse and Benson, 1989; Shanmukhappa and Neelaktan, 1990; Nifant'eva *et al.*, 1999; Sauvant *et al.*, 1999; Marx and Heumann, 1999) to absorb xenobiotics (Nielsen *et al.*, 1997; Prosen and Zupancic-Kralj, 2000), mutagens (Ferrara *et al.*, 2000) and mycotoxins (Jansen van Rensburg *et al.*, 2002). There are several mechanistic studies that were done on potassium humate, derived from bituminous coal, during the early 2000's. Van Rensburg *et al.*, (1999 and 2000) and Jooné *et al.*, (2003) found that this product stimulates lymphocyte proliferation by an increased production of the cytokine, interleukin-2 (IL-2). This response was even more striking in the case of lymphocytes from human immunodeficiency virus (HIV)-infected individuals (Jooné *et al.*, 2003).

It was found that potassium humate inhibits degranulation of inflammatory cells as well as the expression of CR3, a pro-inflammatory adhesion molecule, expressed by human neutrophils, leading to a decreased binding to intra-cellular adhesion molecule (ICAM-1), the ligand of CR3 present on activated endothelial cells (Jooné *et al.*, 2001). Leonardite derived humate resulted in similar inhibition of CR3 expression (unpublished data). Exposure of phorbol 12-myristate 13-acetate (PMA) activated, but not resting human neutrophils to CHD-FA resulted in a dose-dependent decrease in expression of CR3. Inhibition of the expression of adhesion molecules by activated phagocytes and the inhibition of granule polypeptide release which are both responsible for tissue damage during inappropriate inflammatory processes are attractive targets for anti-inflammatory drugs.

Leonardite derived humate compared favourably with prednisolone in suppressing contact hypersensitivity in a rat model. Although the anti-inflammatory properties of humate derived from peat, sapropels and mumie have been described in humans, no controlled clinical trials have been done on the anti-inflammatory effects of humate derived from different coal sources.

In a study conducted by Van Rensburg *et al.*, (2007) it was determined that 2,4-dinitrofluorobenzene (DNBF)-induced contact hypersensitivity in rats was reduced by oral administration of brown coal derived humate at a dosage of 61 mg/kg body weight (BW), which was administered by gavage directly after sensitization. The anti-inflammatory effects were suggested to have been due to either an inhibition of the sensitization or the inhibition of the specific challenge. The possibility that humate adsorbed pro-inflammatory mediators such as cytokines and complement factors was also suggested.

In that same study it was also found that brown coal derived humate at an oral dosage of 1000 mg/kg BW per day had no effect on any of the commonly measured safety parameters when administered for one month, nor were there any effects observed on pups after pregnant female rats were treated with doses of 500 mg/kg BW from Day 5 through 17 of pregnancy (Van Rensburg *et al.*, 2007).

According to a report by the European Agency for the Evaluation of Medicinal Products on toxicity studies (Feb 1999), humic acids extracted from brown coal have no toxic effects at oral dosages as high as 1000 mg/kg BW. It has no teratogenic effects when administered to pregnant rats nor does it have any *in vitro* mutagenic effects based on standard tests for mutagenicity. The LD₅₀ in rats, after oral administration of humic acids, is greater than 11 g/kg BW, which was confirmed by Van Rensburg *et al.*, (2007).

CHD-FA is a relatively new synthetic heavy metal free carbohydrate derived fulvic acid with a metal content comparable to that of Pretoria's (South Africa) municipal drinking water. CHD-FA is a complex mixture consisting mainly of organic compounds of carbohydrate origin, having mostly an acidic supramolecular character.

Although CHD-FA has been suggested as a nutritional supplement and even as a medication, there is currently no data available with regards to the bioavailability or

systemic kinetics of CHD-FA when ingested orally. The measured effect of CHD-FA could be either direct or indirect as a result of binding to a non-target zone. The current hypothesis is that due to the acidic and ionic nature of CHD-FA, it is likely to be highly protein bound after oral absorption.

An animal study using CHD-FA in a topically applied toxicity and sensitization study used 60 female 6-8 weeks old BALB/c mice. The mice were randomized into 3 groups, consisting of 20 mice per group receiving UEA only, 8 mg fulvic acid/mouse or 8 mg fulvic acid neutralized to a pH of 5.5/mouse. From day 8 onwards they were treated topically with CHD-FA on their left ears twice a day for 30 days (up to day 38). Morbidity was determined daily by evaluation of food and water intake, weight loss, abnormal movement (particularly as it pertained to the ability of the animals to obtain food and water) and behaviour and ease of breathing. Blood samples (500 µl/mouse) were drawn, at the end of the study after termination to determine the serum concentrations of kidney and liver enzyme levels (creatinin and alanine amino transferases, aspartate transaminases, gamma-glutamyl transferases). The data suggested that topically applied CHD-FA, did not produce any hypersensitivity reactions and is non-toxic with regards to liver and kidney function in mice over a period of one month (Sabi, 2008).

Studies to determine the long term effects (6-weeks and 6-months) of CHD-FA oral ingestion (150 mg/kg body mass/day) as well as the effects of CHD-FA ingestion on pregnant rats and their off-spring where investigated. An acute toxicity study in which rats received a single oral dose of CHD-FA (150 mg/kg BW) and then observed for a period of 7 days, followed by a 6-week chronic study in which the animals received 100 mg/kg BW daily for the duration of the experiment. Results obtained in all studies showed that CHD-FA is safe for topical use, as well as oral use at the doses tested. In addition, it was shown to be safe for use during pregnancy (Sabi, 2008).

In healthy volunteers fulvic acid had no significant effect on tested safety parameters and did not induce any sensitization when applied topically. Fulvic acid cream (4.5%) inhibited an induced inflammatory reaction, and this anti-inflammatory effect was comparable to that of topically applied hydrocortisone (Snyman *et al.*, 2002).

The new CHD-FA product was developed for systemic administration and was synthesised to have low heavy metal content. An investigation of the *in vivo* safety of this product administered by gavage in rats (175 mg/kg) was performed (Sabi, 2008).

The rats showed no sign of discomfort and results obtained from blood samples tested by Clinical Pathology Laboratories for hepato- and renal- toxicity markers indicated that the fulvic acid, which was administered orally for a period of six weeks did not show any significant increase in any of the tested markers and therefore claimed to be safe for oral use.

In a study by Gau *et al.*, (2000), using human umbilical vein endothelial cells (HUVEC), they investigated the effects of humic acids (HA) on the expression of adhesion molecules and also the activation of NF-kB, both processes being induced by LPS. All humic acid samples obtained from various sources demonstrated an ability to inhibit the LPS-induced expression of adhesion molecules and also to inhibit NF-kB activation, which is a key factor in the innate immune response, in a dose- and time-dependent manner.

On-going research by Cornejo *et al.*, (2011) suggests that fulvic acid is likely to provide new insights to develop potential treatments for Alzheimer's Disease as these substances have multiple nutraceutical properties with added potential activity to protect against cognitive impairment. They provided evidence that the aggregation process of the tau protein, forming paired helical filaments *in vitro*, is inhibited by fulvic acid, affecting the length of fibrils and their morphology. In addition, Maccioni *et al.*, (2012) investigated whether fulvic acid is capable of disassembling pre-formed paired helical filaments. They showed that by means of analysis of aggregation, atomic force microscopy and electron microscopy that the fulvic acid is an active compound against pre-formed fibrils affecting the whole structure by diminishing length of paired helical filaments and probably acting at the hydrophobic level. These observations led to the conclusion that fulvic acid inhibits heparin-induced tau aggregation *in vitro* (Maccioni *et al.*, 2012).

Furthermore, due to the inappropriate or misuse of broad spectrum antimicrobials that are ineffective against recalcitrant biofilms, *Candida* biofilms are becoming clinically important (Niimi *et al.*, 2010). CHD-FA is a microbiocidal compound that acts non-specifically on the cell membrane and therefore it may serve as a potential novel

antiseptic agent for the treatment of oral candidiasis and other *candida* biofilm infections (Sherry *et al.*, 2012).

The Department of Pharmacology at the University of Pretoria (South Africa) has been involved in researching the novel medicinal properties and applications of humic acid and fulvic acid since the late 1990's. Oxifulvic acid, so called because it is derived from controlled wet oxidation of lignic coal, was used for topical application research (Van Rensburg 2015).

The topical fulvic acid creams (4.5% and 9%) showed equivalence when compared to diclofenac sodium (1%) and betamethasone (0.1%) in a murine model when applied topically to suppress the cutaneous inflammatory response with none of the adverse side effects seen in the above mentioned anti-inflammatory agents (Van Rensburg *et al.*, 2001). In feline and canine models, fulvic acid showed anti-inflammatory properties by inhibiting the accumulation of pus in pyotraumatic dermatitis when applied topically (Dekker and Medlen 1999).

1.3 MICRONUCLEUS AND GENOTOXICITY

In the previous section a review has been done into most aspects relating to humic substances, the complexity of the respective molecules, potential uses, mechanisms of actions and current data and trials that have explored these further. There is however literature where humic acid in well water used by the inhabitants for drinking is one of the possible etiological factors for Blackfoot disease, a peripheral arterial occlusive disease found in the inhabitants of the southwest coast of Taiwan in the 1970s. However, the underlying pathophysiological mechanisms are still not established (Lu, 1990).

In a study conducted by Hseu *et al.*, 2007, they concluded that their work suggested that humic acid can induce genotoxicity in human lymphocytes as evidenced by DNA damage and HA-induced changes in Ca^{2+} -homeostasis and ROS/RNS production appear to be the main pathways of genotoxicity induction (Hseu *et al.*, 2007).

Several *in vitro* mammalian cell systems are widely used and can be considered sufficiently validated: the *in vitro* metaphase chromosome aberration assay, the *in vitro*

micronucleus assay and the mouse lymphoma L5178Y cell thymidine kinase gene mutation assay. These three assays are currently considered equally appropriate and therefore interchangeable for measurement of chromosomal damage when used together with other genotoxicity tests in a standard battery for testing of pharmaceuticals, if the test protocols recommended in this guidance are used. *In vivo* test(s) are included in the test battery because some agents are mutagenic *in vivo* but not *in vitro* and because it is desirable to include assays that account for such factors as absorption, distribution, metabolism, and excretion. Lymphocytes cultured from treated animals can also be used for cytogenetic analysis, although experience with such analyses is less widespread (OECD 2007).

In vitro and *in vivo* tests that measure chromosomal aberrations in metaphase cells can detect a wide spectrum of changes in chromosomal integrity. Breakage of chromatids or chromosomes can result in micronucleus formation if an acentric fragment is produced; therefore, assays that detect either chromosomal aberrations or micronuclei are considered appropriate for detecting clastogens. Micronuclei can also result from lagging of one or more whole chromosome(s) at anaphase and thus micronucleus tests have the potential to detect some aneuploidy inducers.

The DNA located in the nucleus of every cell carries the genetic blueprint of the organism. A common event is replication of selected genes within a living cell during the normal activity and functioning of a cell, with complete replication taking place during cell proliferation. The integrity of the DNA is very important to the cell and there are various mechanisms in place to ensure that the DNA is properly maintained and to repair any errors detected. It has however been observed that chromosome damage can be caused by exposure to ionising radiation or carcinogenic chemicals due to physical or chemical alterations to the DNA of eukaryotic cells (Evans *et al.*, 1977). Chromosomal breaks may result from unrepaired double strand breaks in DNA and chromosome rearrangements may result from miss-repair of strand breaks in DNA (Savage, 1993).

It was proposed independently by Schmid (1975) and Heddle (1973) that a simple approach to assess chromosome damage *in vivo* was to measure micronuclei (MNi), in dividing cell populations such as the bone-marrow. The micronucleus assay in bone-

marrow and peripheral blood lymphocytes is now one of the best established *in vivo* cytogenetic assays in the field of genetic toxicology.

MNi are found in dividing cells that either contain chromosome breaks lacking centromeres (acentric fragments) and/or whole chromosomes that are unable to migrate to the spindle poles during mitosis. At telophase during the cell division, a separate nuclear envelope also forms around the lagging chromosomes and fragments. These then uncoil and gradually assume the morphology of an interphase-like nucleus, with the exception that they are smaller than the true nuclei in the cell, hence the term “micronucleus”. This is reported to provide a convenient and reliable index of both chromosome breakage and chromosome loss within a cell (Fenech, 2000).

Because MNi are only visible in cells that have completed nuclear division, they are ideally scored in the binucleated stage of the cell cycle (Fenech and Morley, 1985). Several methods have been proposed based on stathmokinetic, flow cytometric and DNA labelling approaches, but the method that has found most favour due to its simplicity and lack of uncertainty regarding its effect on baseline genetic damage is the cytokinesis-block micronucleus (CBMN) assay (Fenech, 2007). In the CBMN assay, cells that have completed one nuclear division are blocked from performing cytokinesis using cytochalasin-B (Cyt-B) and are consequently readily identified by their binucleated appearance (Fenech, 2007).

Cyt-B is an inhibitor of actin polymerisation required for the formation of the microfilament ring that constricts the cytoplasm between the daughter nuclei during cytokinesis (Carter, 1967). The use of Cyt-B enables the accumulation of virtually all dividing cells at the binucleate stage in dividing cell populations regardless of their degree of synchrony and the proportion of dividing cells. MNi are then scored in binucleated cells only, which enables reliable comparisons of chromosome damage between cell populations that may differ in their cell division kinetics. It was proposed that the micronucleus assay be used instead of metaphase analysis for genotoxicity testing of new chemicals (OECD, 1997).

1.4 INFLAMMATION

Inflammation is a normal response to tissue invasion or injury and is a critical process to remove foreign, dead or damaged tissue and to destroy any invading organism or object. The inflammatory response is a well-orchestrated sequence of events controlled through the immune system and involves cells with immune function which in addition to the phagocytic activity, release chemical mediators to attract and activate further migrating immune cells. The most strongly implicated endogenous chemical mediators are the cytokines, prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor and interleukin-1 (Lawrence, 2009).

The inflammatory response is characterized by coordinated activation of various signalling pathways that regulate initial expression of pro-inflammatory mediators in resident tissue cells and recruitment of leukocytes from the blood causing a strong localised response, but is shut down by anti-inflammatory mediators being released from monocyte derived immune cells when the required effect has been achieved. This inflammatory response is normally self-limiting and resolves through the release of endogenous anti-inflammatory mediators and the clearance of inflammatory cells from the affected area.

This initial recognition of infection or injury leading to inflammation is mediated by tissue resident macrophages and mast cells which then leads to the production of a variety of inflammatory mediators, including chemokines, cytokines, vasoactive amines, eicosanoids and products of proteolytic cascades. The immediate effect of these mediators is to elicit an inflammatory exudate locally. Plasma proteins and leukocytes that are normally restricted to the blood vessels now gain access, through the postcapillary venules, to the extravascular tissues at the site of infection or injury. The activated endothelium of the blood vessels allows selective extravasation of neutrophils while preventing the exit of erythrocytes. This selectivity is afforded by the inducible ligation of endothelial-cell selectins with integrins and chemokine receptors on leukocytes, which occurs at the endothelial surface, as well as in the extravascular spaces (Poerber and Sessa, 2007).

When these mediators reach the afflicted tissue site, neutrophils become activated, either by direct contact with pathogens or through the actions of cytokines secreted by tissue-resident cells. The neutrophils attempt to kill the invading agents by releasing the toxic contents of their granules, which include reactive oxygen species (ROS) and reactive nitrogen species, proteinase, cathepsin G and elastase (Barton 2008, Nathan 2006). These highly potent effectors do not discriminate between microbial and host targets, so collateral damage to host tissues is unavoidable (Nathan 2002).

A successful acute inflammatory response results in the elimination of the infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages. The switch in lipid mediators from pro-inflammatory prostaglandins to lipoxins, which are anti-inflammatory, is crucial for the transition from inflammation to resolution. Lipoxins inhibit the recruitment of neutrophils and, instead, promote the recruitment of monocytes, which remove dead cells and initiate tissue remodelling (Serhan and Savill, 2005).

Resolvins and protectins, which constitute another class of lipid mediator, as well as transforming growth factor- β and growth factors produced by macrophages, also have a crucial role in the resolution of inflammation, including the initiation of tissue repair (Serhan and Savill, 2005, Serhan 2007). If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics. The neutrophil infiltrate is replaced with macrophages, and in the case of infection also with T cells. If the combined effect of these cells is still insufficient, a chronic inflammatory state ensues, involving the formation of granulomas and tertiary lymphoid tissues (Drayton *et al.*, 2006).

The characteristics of this inflammatory state can differ depending on the effector class of the T cells that are present. In addition to persistent pathogens, chronic inflammation can result from other causes of tissue damage such as autoimmune responses, owing to the persistence of self-antigens or undegradable foreign bodies. Unsuccessful attempts by macrophages to engulf and destroy pathogens or foreign bodies can lead to the formation of granulomas, in which the intruders are walled off by layers of macrophages, in a final attempt to protect the host (Majno and Joris, 2004, Kumar *et al.*, 2003).

The mechanisms of systemic chronic inflammatory states in general are poorly understood, but it is clear that they do not seem to fit the classic pattern of transition from acute inflammation to chronic inflammation.

As mentioned above, in some cases an inappropriate inflammatory response occurs, and this does not progress to the self-limiting stage, remaining in a phase typical of the initial response where immune cells tend to accumulate and express large amounts of pro-inflammatory mediators. Persistent accumulation and activation of pro-inflammatory leukocytes is a hallmark of chronic inflammation (Lawrence *et al.*, 2001). The recruitment of circulating leukocytes to a specific target area plays an important role in inflammatory and immune responses (Gonzalez-Amaro *et al.*, 1998) and requires a critical early process of leukocyte adhesion to vascular endothelial cells (Gau *et al.*, 2000) followed by active migration to the inflammatory area.

Chronic inflammatory responses have even been implicated as one of the risk factors in the formation of atherosclerotic lesions (Hansson and Hermansson, 2011). Abnormal elevation of homocysteine levels in the blood have been reported in patients with hyperhomocysteinemia (Ghassibe-Sabbagh *et al.*, 2011). Severe hyperhomocysteinemia (plasma levels of homocysteine greater than 100 mmol/L) is found in patients with extremely premature atherosclerosis and early occlusive vascular disease (Lentz 2005).

The release of pro-inflammatory mediators by monocyte-derived macrophages may play a vital role in atherosclerotic inflammatory responses (Seneviratne *et al.*, 2011). It has been demonstrated that cyclooxygenase (COX)-2 in activated monocytes is of particular relevance in inflammation and atherosclerosis (Beloqui *et al.*, 2005). The activation of macrophages has been previously correlated with the induction of COX-2. Macrophages expressing COX-2 are known to produce prostaglandins that have pro-inflammatory effects, including activating chemotaxis, increasing vascular permeability, and promoting cell proliferation (Ricciotti and FitzGerald, 2011).

Another widely believed systemic inflammatory disease is psoriasis which is also believed to drive the process of atherosclerosis (Boehncke and Boehncke 2012). In a study conducted by Ahmed *et al.*, (2015), it was concluded that psoriasis patients have a hypercoagulable state which increases the risk to develop atherothrombosis, and this is

likely related to psoriasis linked inflammation which cause endothelial dysfunction and oxidative stress, and is also related to hyperhomocysteinemia which as above is considered an established risk factor for atherosclerosis and thrombosis, as it may cause direct endothelial injury followed by facilitated thrombosis causing oxidative damage to the endothelium (Ahmed *et al.*, 2015).

A large proportion of the global burden of disease involves nonresolving inflammation that appears to be chronic from the outset, in that the first cellular signs of inflammation involve infiltration of the tissue by monocytes, dendritic cells, and macrophages. Examples include atherosclerosis (Galkina and Ley, 2009), obesity (Nathan, 2008), and some cancers (Mantovani *et al.*, 2008).

In many cases acute and chronic inflammation coexist over long periods, implying continual reinitiation. Examples are found in rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, multiple sclerosis, Crohn's disease, ulcerative colitis, and cancers whose stroma is infiltrated both by macrophages and immature myeloid cells (Mantovani *et al.*, 2008). For example, in rheumatoid arthritis, the synovium presents a striking picture of chronic inflammation, with extensive infiltration by macrophages and lymphocytes and activation of synoviocytes. In contrast, the synovial fluid is a sea of neutrophils. The effusion in an affected joint of an untreated patient with rheumatoid arthritis can be invaded by over a billion neutrophils per day that have a half-life of about 4 hours (Hollingsworth *et al.*, 1967). Neutrophils contain cytosolic peptidyl arginine deiminase type 4, an enzyme whose activity depends on the levels of Ca²⁺ found in extracellular fluid. When neutrophils die, this enzyme may be released and activated. It may then convert the guanidino side chains of L-arginine residues to ureido residues, generating citrulline in some proteins in the joint. The autoantibodies most closely associated with the pathogenesis of rheumatoid arthritis react with citrullinated proteins (Uysal *et al.*, 2009). Thus, dying neutrophils may help sustain an ongoing antigen-antibody reaction that attracts and activates more neutrophils, whose secretion of oxidants and proteases is synergistically destructive (Han *et al.*, 2005).

At the cellular level the biochemical pathways involving the transcription factor Nuclear Factor (NF)- κ B signalling is commonly activated and this has been found to have a central role in the processes leading to cellular expression of diverse pro-inflammatory mediators.

NF- κ B is a dimeric transcription factor that governs the expression of many genes involved in immune and inflammatory responses, including interleukins (Liou and Baltimore, 1993), nitric oxide synthase (Xie *et al.*, 1994), and many adhesion molecules (Collins *et al.*, 1995). NF- κ B pathways may be activated by many different stimuli, such as reactive oxygen species, inflammatory cytokines, viruses or ionizing radiation.

Within human vascular endothelial cells, NF- κ B appears to play a major role in the expression of the adhesion molecules ICAM-1, VCAM-1, and E-selectin (Collins *et al.*, 1995). The prototypical pro-inflammatory cytokines such as tumour necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β), initiate the consensus pathway for NF- κ B activation resulting in release of other pro-inflammatory mediators, and have important roles in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease (IBD), asthma, and chronic obstructive pulmonary disease (Holgate 2004, Chung 2006, Williams *et al.*, 2007).

Previous studies of mice genetically deficient in components of the NF- κ B pathway have shown that Rel proteins are important in the recruitment of leukocytes in the innate immune response. Several studies have shown pro-inflammatory cytokine and chemokine production within diseased tissue is NF- κ B- dependent; for example, using fibroblast like synoviocytes from RA patients (Aupperle *et al.* 1999; Aupperle *et al.* 2001). Similar studies have shown that pro-inflammatory cytokine production in human atherosclerotic plaques is also NF- κ B-dependent (Monaco *et al.*, 2004).

Despite a correlation of NF- κ B activation with inflammatory diseases in animal models of arthritis (Miygkov *et al.*, 1998) and allergic airway disease (Poynter *et al.*, 2002) being reported, the association of NF- κ B activity and inflammatory disease is not easy to interpret because both pro- and anti-inflammatory mediators are produced during inflammation and the balance between these mediators is likely to dictate disease progression (Lawrence and Gilroy, 2007).

Most treatment options for treating inflammation rely on the inhibition of pro-inflammatory mediator production or mechanisms that initiate the inflammatory response. For this reason much attention has focused on the development of anti-inflammatory drugs targeting NF- κ B (Karin *et al.*, 2004).

Currently most knowledge of signalling in inflammation is gained from studying members of the IL-1 β and TNF receptor families. IL-1 β and TNF α represent the typical protein based pro-inflammatory cytokines that are rapidly released following tissue injury or infection (Lawrence *et al.*, 2001).

1.5 ATOPIC DERMATITIS

Atopic dermatitis (AD) or eczema is a chronic inflammatory response in the skin with symptoms such as itchy, red, swollen, and cracked skin. It is sometimes associated with an exudate. The term atopic is used to indicate that the syndrome has a hyper-allergic character yet often the initiating cause is unknown, although it appears that there is a genetic link (Cookson and Moffatt 2000).

This is a condition that generally starts during childhood although adult onset is also recorded. In 45% of children, the onset of AD occurs during the first 6 months of life, during the first year of life in 60%, and before the age of 5 years in at least 85% of affected individuals (Kay *et al.*, 1994).

Approximately 20% of children with onset before the age of 2 years will have persisting manifestations of the AD, and an additional 17% will still have intermittent symptoms by the age of 7 years (Illi *et al.*, 2004). In adults with AD, only 16.8% had onset after adolescence (Wang *et al.*, 1998, Ozkaya, 2005).

Different stages of atopic dermatitis are associated with a range of skin pH levels. Acute lesions that are erythematous with exudate and crusts tend to be alkaline and chronic lesions with lichenification and scale are only slightly more alkaline than normal skin (Knor *et al.*, 2011). The pH is also an important factor in other skin diseases. Some diseases are associated with an increase in skin pH e.g. atopic and seborrhoeic dermatitis,

where increased skin pH exacerbate the symptoms e.g. in contact dermatitis, diaper dermatitis and acne vulgaris. In many of these diseases the pathogenesis of alkalinisation is not clear, but the higher pH likely predisposes the skin to secondary infections (Chikakane *et al.*, 1995).

Topical agents are the mainstay of AD therapy. In more severe cases requiring systemic or phototherapy, they are often used in conjunction with these modalities. In a review of the currently available highest level of evidence, an expert work group acknowledged that although much is known about the use of non-pharmacologic and pharmacologic topical therapies for AD, much has yet to be learned (Lawrence *et al.*, 2014).

A number of clinical trials have shown that topical treatments improve symptoms and signs of AD, including pruritus, erythema, fissuring, and lichenification. However a standard treatment, dose and frequency per patient has yet to be determined, as there the variation of patients and variety of treatments are vast with varying results. Also one has to take into consideration the surface area affected as well as the treatment and respective potential side effect.

Currently there is no “golden” standard of treatment and each case is treated individually trying various treatments in order to relieve symptoms, however the failure rate is high due to cost implications and potential side effects (Lawrence *et al.*, 2014).

1.6 LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY AND BIOMARKER DISCOVERY

LC-MS/MS holds enormous potentials for improvements in pharmaceutical fields and laboratory medicine mainly including therapeutic drug monitoring, endocrinology, toxicology and metabolomics analyses. It is generally accepted as the preferred technology for quantitating small molecule drugs, metabolites, and other xenobiotic biomolecules in biological matrices as like plasma, blood, serum, urine, and tissue (Xu *et al.*, 2007).

Development of standard techniques of analytical detection in clinical chemistry relies on indirect characteristics of an analyte, e.g. its absorption of light, chemical reactivity or physical interaction with macro-molecules (Vogeser and Kirchhoff, 2011). Analytes can be detected directly from molecular characteristics using molecular mass and molecular disintegration patterns in mass spectrometric methods. Thus, mass spectrometric technology is very attractive for the quantification of biomarkers or chemicals in the context of diagnostic procedures.

During the past decade, LC-MS/MS technologies have substantially extended the methodologic armamentarium of clinical laboratories (Vogeser and Seger, 2008). Over the past decade atmospheric pressure ionization techniques and liquid chromatography-mass spectrometry (LC-MS) has undergone vast technological improvement allowing for its applications to endogenous components such as proteins, peptides, carbohydrates, DNA, and drugs or metabolites. Furthermore, powerful new technologies of ion-analyses substantially increased the sensitivity and selectivity capabilities of MS analyzers with respect to specificity and to the extent of data read out. These developments have led to a more widespread use of MS techniques superior to other analytical methods in routine laboratory medicine.

The field of metabolomics, aiming at global analysis of numerous targeted or non-targeted low molecular compounds (metabolites) in a biological sample, has recently found its application in diverse research areas (Kaddurah-Daouk and Krishnan, 2008; Wishart, 2008) including food quality and authenticity assessment (Cevallos–Cevallos *et al.*, 2009); environmental & biological-stress studies (Rosenblum *et al.*, 2005), biomarker discovery (Griffiths *et al.*, 2010), functional genomics (Saito & Matsuda, 2010) and integrative systems biology (Oliver *et al.*, 1998).

Rapid growth of metabolomics has been enabled by substantial advances in analytical techniques such as mass spectrometry coupled to liquid chromatography (LC), gas chromatography and nuclear magnetic resonance, all techniques facilitating analysis of a wide range of metabolites with diverse physicochemical properties, occurring at different concentration levels (Dunn and Ellis, 2005). Therefore these techniques are ideal to attempt to establish a biomarker in a complex compound such as fulvic acid.

1.7 STUDY AIM

The vast research that has been done and published with respect to fulvic acid (FA) and with recent reports of its nutraceutical properties and physiological action on the human body, makes this compound a most interesting naturally occurring phytochemical. An array of properties ranging from its neuroprotective effect (Cornejo *et al.*, 2011), antimicrobial and anti-inflammatory properties (Van Rensburg *et al.*, 2001), as well as the antioxidant activity and inhibitory effect on chemical mediator release in basophils (Yamada *et al.*, 2007) makes it a compound that warrants further investigation. Although there are a number of studies on the effect of FA on cellular and biological functions, the detailed mechanisms underlying the regulatory effect of FA remain unclear.

When it comes to research and potential new drugs one of the key cornerstones is to first do no harm, with this in mind the current study set out not only to determine the safety of a new carbohydrate derived fulvic acid using both *in vitro* as well as *in vivo* methods, but also the potential efficacy and mechanism of action via two small pilot clinical trials and then attempting to establish a potential biomarker for future reference.

In vitro genotoxic safety will be determined via the micronucleus test, where the toxicological relevance of the micronucleus is well defined as a multi-target genotoxic endpoint, assessing not only clastogenic and aneugenic events but also some epigenetic effects, which is simple to score, accurate and applicable in different cell types. In addition to this, it is predictive for cancer, amenable for automation and allows good extrapolation for potential limits of exposure or thresholds and it is easily measured in experimental both *in vitro* and *in vivo* systems. Implementation of *in vitro* micronucleus assays in the battery of tests for hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified.

In vivo safety was determined in two relatively small yet significant pilot clinical trials where the aim of this research was to establish the acute and subacute safety of fulvic acid in an anticipated systemic dose range in participants with a pre-determined atopy as well as in a topical formulation for participants suffering from atopic dermatitis. Inflammatory conditions like atopic dermatitis are often recurrent and difficult diseases to treat, and treatments usually include glucocorticoids and in chronic conditions, calcineurin

antagonists that may even be supplemented with systemic immunosuppressive therapies in severe cases.

Assessing the potential anti-inflammatory properties in the two clinical trials would determine whether or not fulvic acid could be efficacious in the treatment of selected inflammatory disorders, and whether or not further investigation into the use of CHD-FA was warranted. Although fulvic acid has been suggested as a nutritional supplement and even a medication there is currently no data available with regards to the systemic kinetics when ingested orally.

The current hypothesis is that due to the acidic and ionic nature of fulvic acid, it is likely to be highly protein bound after oral absorption. Due to limited information regarding systemic kinetics and the complexity of fulvic acid as a compound, LC-MS/MS was used in order to determine whether or not an unbound fulvic acid derived plasma soluble analyte exists in plasma after administration that could be used to determine the *in vivo* kinetics of orally administered fulvic acid.

In summary, even with the vast amount of information and data regarding fulvic acid available there are still apparent gaps that require further research and this study attempts to fill some of those gaps. This study attempts to answer some of these questions by using a carbohydrate derived fulvic acid and determining genotoxicity *in vitro* via a micronucleus assay, systemic and topical safety and efficacy of in two double blinded, randomised, placebo controlled clinical trials and use of LC-MS/MS to determine whether a unique CHD-FA ionic analyte could be found in plasma.

CHAPTER 2: GENOTOXICITY TESTING

2.1 BACKGROUND

In a study by You-Cheng *et al.*, (2008), the genotoxic potential of humic acid (HA) is assessed using human peripheral blood lymphocytes, using comet and sister chromatid exchange assays respectively. Their study suggested that HA can induce genotoxicity in human lymphocytes as evidenced by DNA damage and that HA induced changes in Ca^{2+} homeostasis and ROS/RNS production appear to be the main pathways of this genotoxicity induction. This demonstration of HA genotoxicity lead to their hypothesis that HA could induce cancer by disrupting genetic integrity (You-Cheng *et al.*, 2008).

As described earlier, humic substances are divided into fulvic acids, humic acids and humin on the basis of the solubility in water as a function of pH. Fulvic acid is the fraction that is soluble in water under all pH conditions and is in general lower in molecular size and has lower colour intensity than humic acids. The average molecular weight of humic substances may range from 500 to 5000 Da for fulvic acid and 3000 to 1000 000 Da for humic acid (Sparks, 2003). The potential genotoxicity of CHD-FA was tested using the *in vitro* micronucleus test as part of the battery tests required to determine potential genotoxicity in combination with a bacterial reverse mutation test (Ames test) which was conducted by Dr G Jooné (data on file Department of Pharmacology, University of Pretoria, South Africa).

The presence of micronuclei (MNI) in cultured human cells was reported as early as the 1960s (Elston, 1963). The *in vitro* micronucleus test is a robust quantitative assay of chromosome damage due to the development of the cytokinesis-block technique which has eliminated the confounding effects on MNI expression by the cytostatic effects caused by poor culture conditions, treatment effects, cell senescence and variability in mitogen response in the lymphocyte test system (Fenech and Morley, 1985, 1986).

In the cytokinesis-block micronucleus (CBMN) assay, scoring of MNI allows for the differentiation of post-divided cells that are accumulated and recognised by their binucleated appearance from the mononucleated cells that did not divide during the *in vitro* culturing period.

This technique has become an attractive tool for genotoxicity testing because of its capacity to detect not only clastogenic and aneugenic events but also some epigenetic effects and its simplicity of scoring, accuracy, wide applicability in different cell types and amenability to automation (OECD 1997).

This *in vitro* micronucleus assay is a mutagenicity test system that assesses chemicals for their ability to induce the formation of small membrane-bound DNA fragments such as micronuclei in the cytoplasm of interphase stage cells during cell division. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes that are unable to migrate to the poles with the rest of the chromosomes during the anaphase of cell division (Kirsch-Volders, 1997).

The advantages of this technique are well defined:

1. It is a multi-target genotoxic endpoint, predictive for cancer (Bonassi *et al.*, 2010)
2. It is amenable for automation (Decodier *et al.*, 2010)
3. It allows good extrapolation for potential limits of exposure or thresholds (Elhajouji *et al.*, 2010)
4. MNi can be scored easily, measured in a variety of systems, *in vitro* (Hayashi *et al.*, 2007).

There is an extensive amount of data to support the validation of the *in vitro* micronucleus assay using various cell lines or human lymphocytes, which include, in particular, the international validation studies co-ordinated by the French Society of Genetic Toxicology (Bonassi *et al.*, 2010; Decodier *et al.*, 2010 Elhajouji *et al.*, 2008, 2010 Hayashi *et al.*, 2007; Dertinger *et al.*, 2010; Fenech 2000) and the reports of the international *in vitro* micronucleus assay working group (Fenech and Morley, 1985, 1986). Implementation of this assay in the battery of tests, such as sister chromatid and comet assays, for the hazard and risk assessment of potential mutagens/carcinogens is therefore fully justified.

2.2 METHODS

CHD-FA buffering and concentration determination

The initial pH of CHD-FA was measured at pH 1.89 and in order to reach pH of 5.5, 79.425 g of sodium acetate was used to buffer the solution, with an initial volume of 210 mL and a final volume of 251 mL. Therefore the final pH adjusted concentration of CHD-FA with an initial raw material concentration of 3.9% was now 3.3% CHD-FA post buffering. This percentage reflects the solid/dry mass of CHD-FA in solution post ultrafiltration.

Low molecular weight CHD-FA (LMWCHD-FA) buffering and concentration determination

The initial pH of LMWCHD-FA was 1.95 and in order to adjust this to pH of 5.5, 80.599 g of sodium acetate was used, with an initial volume of 210 mL and a final volume of 260 mL. Therefore the final pH adjusted concentration of the LMWCHD-FA with an initial raw material concentration of 3.6% was now 2.9% post buffering. This percentage reflects the solid/dry mass of CHD-FA in solution post ultrafiltration.

Determination of IC₆₀ on human lymphocytes

- 500 ml of heparin was added into a 500 mL blood collection bag.
- 500 mL of blood was collected and mixed well to prevent coagulation.
- 15 mL of Histopaque® was added into 50 mL graduated, centrifuge tubes.
- 30 mL uncoagulated blood was carefully layered onto the Histopaque.
- Tubes were centrifuged GS6 (Beckman Coulter, USA) for 25 minutes at 1600 x g in a centrifuge at ambient temperature.
- The separated plasma (the top layer) and the overlying Histopaque was removed from above the distinct mixed lymphocyte band near the top of this Histopaque layer with a plastic pipette and discarded into a plastic container, together with a 5% hypochlorite solution.
- The lymphocyte layer, ± 3 mm was carefully removed and pipetted into a clean sterile 50 mL tube which was filled with ice cold 0.84% ammonium chloride (NH₄Cl). It was then mixed gently but well and left to stand for 10 minutes in ice. Any contaminating red blood cells present in the cell suspension were lysed during this step.

- The cells were then centrifuged for 10 minutes at 900 x *g*
- The resultant supernatant fluid was discarded and the pellet kept.
- The pellet was resuspended in 50 mL phosphate buffered saline (PBS) by vortex mixing (Scientific Industries, USA) and the tube centrifuged at 300 x *g*
- The resultant supernatant fluid was discarded.
- The cells were resuspended in 1 mL cold PBS and vortex mixed.
- A 50 µl aliquot of the mixed lymphocyte suspension was counted and the suspension adjusted to a standardised cell concentration of 2×10^6 /mL.
- One hundred microliters of the cell suspension were plated out per well in a 96 well plate.
- pH adjusted CHD-FA was added to duplicate wells to achieve the following final concentrations : 7.5, 15, 30, 60, 100, 200, 300, 400 and 500 µg/mL.
- Lymphocytes were then stimulated to divide by adding phytohaemagglutinin (PHA), final concentration 2.5µg/mL.
- The cells were incubated for 3 days in a CO₂ incubator at 37°C in a water saturated atmosphere.
- After 72 hours incubation, 20 µl of a sterile MTT solution (5 mg/mL in PBS) was added to each well.
- The plate was then re-incubated for 4 hours in a CO₂ incubator at 37°C.
- The cells were then centrifuged at 1800 x *g* for 10 minutes.
- The supernatant was removed without disturbing the pellet.
- The pellets were then washed with 150 µl PBS, centrifuged again and the cell pellet retained and allowed to dry.
- One hundred microliters of DMSO was added to each cell pellet and the plate shaken gently for an hour on a shaker.
- The plate was then measured spectrophotometrically at a wavelength of 540 nm with a reference wavelength of 630 nm (Bio-Tek Instruments Inc., Vermont, USA).

Micronucleus assay

The micronucleus assay was used in order to determine whether or not CHD-FA and LMWCHD-FA displayed any genotoxic characteristics. The assay was performed on lymphocytes obtained from two different, non-smoking, healthy 25 year old males, thus two separate cell culture experiments.

The micronucleus assay technique uses the same lymphocyte isolation technique as above until the step where the CHD-FA is added. In this assay however, 25 cm² culture flasks were used, and three concentrations of CHD-FA or a specifically prepared low molecular weight CHD-FA (LMWCHD-FA) were used.

The MNi which was determined by using the IC₆₀ which was 50 µg/mL, 100 µg/mL and 150 µg/mL as the low, intermediate and upper cytotoxic levels respectively. After forty-four hours of PHA stimulation, a 100 mL aliquot of Cyt-B stock solution in DMSO (600 µg/mL) was thawed, 900 mL culture medium added and mixed. Seventy-five microliters of the mixture was added to each 1 mL of culture to give a final concentration of 4.5 µg Cyt-B/mL. Twenty-eight hours after adding Cyt-B cells were harvested. The assay was run twice using two different healthy non-smoking volunteers. A positive and a negative control were also used for each assay, with the negative being the medium only and the positive being Aflatoxin B1 (AFB1) (Sigma-Aldrich Inc., St. Louis, MO). A primary stock solution of 1,000 mg of AFB1/L of methanol was prepared, then diluted with medium so that the final concentration would be 16 µg/mL.

Twenty microliters of the cell suspension was then plated out onto a cover slip and allowed to dry. The cells were then fixed using 1 mL of a 100% methanol for 10 minutes.

Acridine orange staining (Sigma-Aldrich, St Louis, USA)

- **Stock solution:** 0.1 g acridine orange powder was dissolved in 100 mL distilled water.
- **Acridine orange buffer:** 1 PBS buffer tablet (Sigma-Aldrich, St Louis, USA) was dissolved in 1 L of distilled water.
- **Working solution:** A 0.1 mL stock solution was added to 10 mL acridine orange buffer.

- **Staining:**

- The cover slips were immersed in acridine orange working solution for one minute.
- The cover slips were then quickly rinsed in distilled water to remove excess stain.
- The cover slips were then immersed in acridine orange buffer for one min.
- The cover slips were then inverted onto a microscope slide and the edges sealed with transparent nail polish.

Analysis:

- All slides, including those of positive and negative controls, were independently coded before the microscopic analysis.
- Micronucleus frequencies were analysed in 1000 binucleated cells per culture, with two cultures per CHD-FA concentration tested.
- Slides were examined at 1000 x magnification using a light or fluorescence microscope.
- The distribution of binucleated cells with zero, one or more than one micronuclei were scored.
- Scoring criteria were as follows:
 - Cells had a round or oval appearance with an intact cytoplasm
 - Nuclei similarly were round or oval with an intact nuclear membrane
 - Only cells having undergone one nuclear division were scored for the presence of micronuclei
 - Micronuclei were counted only if they were one third or less the size of the main nuclei
 - Micronuclei stained similar to the main nuclei
 - Micronuclei were clearly separated from the main nuclei

Statistical differences between controls and treated samples were determined with the Fisher exact test for micronuclei frequencies using Graphpad 4.0 statistical analysis software.

The results and statistical analysis of the two experiments were calculated independently and the means thereof represented in Table 2.2. Cells on each microscope slide were counted until a total of 1000 binucleated cells was reached, which was on average just over 3000 cells in total. The ability of AFB1 to induce MNi in cytokinesis blocked cells has been described at concentrations as low as 3 µg/mL (Türkez *et al.*, 2009), but in this study 16 µg/mL AFB1 resulted in a significant MNi increase of 3.3 fold versus the negative control (summarized in Table 2.2), which is similar to the fold increase found by Türkez *et al* (2009). When comparing the six different treatment preparations versus that of the negative control, it was observed that there was no significant increase in MNi, however there was a significant decrease in MNi in two of the preparations, namely CHD-FA 150 µg/mL and CHD-FA 50µg/mL respectively.

2.3 RESULTS

IC₆₀ Determination

The IC₆₀ was determined to set the upper limit of the CHD-FA concentration that could be used to treat the lymphocytes in the micronucleus assay, as per OECD guidelines. Due to the safety of CHD-FA, the concentration had to be increased significantly to achieve and IC₆₀. A summary of the results is shown below and using statistical analysis and a dose response was observed (GraphPad Prism 4.0, Survival Proportions), the IC₆₀ was determined to be 149.2 µg/mL, therefore 150 µg/mL was used as the upper limit, with 100 µg/mL and 50 µg/mL being used as the intermediate and low cytotoxic levels respectively (Table 2.1).

Table 2.1: Summary of the IC₆₀ determination using lymphocytes treated with varying concentrations of CHD-FA or LMWCHD-FA.

	60 µg/mL	100 µg/mL	120 µg/mL	140 µg/mL	160 µg/mL
% Survival	99.1	80.8	65.7	65.9	47.7
±SD	0.07	0.03	0.03	0.04	0.03

Table 2.2: Summary of Micronuclei with varying concentrations of CHD-FA and LMWCHD-FA

Samples	Total Cells Counted		Binucleates		Cells Containing MNi		%Cells Containing MNi		Fold increase of Cells containing MNi vs Control	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Negative Control	3278	393.151	1000	0	104	90.5	10%	9%	1.0	0
Positive Control (AFB1 16ug/mL)	3022	393.2	1000	0	**252	96.2	25%	10%	3.3	1.9
LMWCHD-FA 150ug/mL	3520	746.7	1000	0	130	104.7	13%	10%	1.3	0.1
LMWCHD-FA 100ug/mL	3440	192.3	1000	0	114	87.7	11%	9%	1.2	0.2
LMWCHD-FA 50ug/mL	3235	247.5	1000	0	96	90.5	10%	9%	0.9	0.1
CHD-FA 150 ug/mL	2880	0	1000	0	**58	2.8	6%	0%	0.9	0.7
CHD-FA 100ug/mL	3116	124.5	1000	0	130	116	13%	12%	1.2	0.0
CHD-FA 50ug/mL	3504	854.2	1000	0	*76	33.9	8%	3%	0.9	0.5

MNi: micronuclei

*p=0.0015

**p<0.0001;

2.4 DISCUSSION

People are routinely exposed to harmful chemicals of which some are potentially carcinogenic, including polycyclic aromatic hydrocarbons, pesticides, herbicides, toxins and other carcinogenic compounds, both accidentally or occupationally, which may lead to the development of cancer. Mutagens such as aflatoxins, mycotoxins, benzo(a)pyrene and pesticides are the most common types of the carcinogenic compounds to which people are exposed (Bofetta *et al.*, 1997).

The Human Micronucleus project, an international collaborative project aimed at determining the variables affecting MNi frequency in humans and the pathological significance of this biomarker (Fenech *et al.*, 1999) completed a cohort study involving a total of 6,718 subjects from ten countries, screened for MNi frequency between 1980 and 2002; where a significant increase of all cancer incidence was found for subjects in the groups with medium and high MNi frequency relative to those with low MNi frequency (Bonassi *et al.*, 2006). Results from a case–control study of lung cancer in smokers showed that both spontaneous and nicotine-derived nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced MNi are associated with increased lung cancer risk. These initial results indicated the potential predictive value of the CBMN assay with respect to cancer risk and validates its use as a test for detecting nutritional, environmental and genetic factors that are potentially carcinogenic (El-Zein *et al.*, 2006).

Over the past 17 years, the CBMN assay has evolved into a comprehensive method for measuring chromosome breakage, DNA misrepair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis (Fenech 2007). Most chemical agents and different types of radiation have multiple effects at the molecular, cellular and chromosomal level, which may occur simultaneously and to varying extents depending on the exposure dose. The CBMN assay has already been proven to be an effective tool for the study of cellular and nuclear dysfunction caused by *in vitro* or *in vivo* aging, micronutrient deficiency or excess, genotoxin exposure and genetic defects in genome maintenance. More recent studies indicate that this method is likely to also prove fruitful in the emerging fields of nutrigenomics and toxicogenomics and their combinations, as it becomes increasingly clear that nutrient status also impacts on sensitivity to exogenous genotoxins (Beetsra *et al.*, 2005).

MNi formation resulting from chromosome breakage and/or disturbance of the chromosome segregation machinery requires a mitotic or meiotic division and assessment of cell division in the presence of the test substance is compulsory. When using primary cultures (e.g. peripheral blood lymphocytes), it is essential to use the cytokinesis-block method to score MNi in once-divided cells only and thus avoid confounding of the observed MNi frequency caused by cell division inhibition due to cytotoxicity or poor culture conditions. It is possible to generate false-negative results if MNi are not specifically scored in cells that have completed at least one nuclear division after or during the exposure to the genotoxin (Umegaki and Fenech, 2000). The MNi assay is a valuable approach for identifying the ability of a chemical to induce chromosome loss and non-disjunction because it allows detection of both clastogenicity (chromosome breakage) and aneugenicity (chromosome lagging due to dysfunction of mitotic apparatus) (Elhajouji *et al.*, 1998).

Humic acid and fulvic acid have been reported to possess cancer preventive properties (Peña-Méndez *et al.*, 2005). They demonstrated that these compounds can inhibit mutagenesis and have free radicals scavenging, photoprotective, anti-inflammatory and toxin removing properties that can all contribute to inhibition of cancer development.

In accordance with previous reports, and similar to what was found in this study, it has been reported that frequencies of sister chromatid exchanges in human lymphocytes were significantly increased by AFB1 exposure (Geyikoglu and Turkez 2005, Turkez and Sisman, 2007). The genotoxic effects of AFB1 were also established using human, mouse and rat liver preparations (Wilson *et al.*, 1997).

According to the findings reported for this study it revealed firstly that there is a certain background mutagenesis that people are exposed to on a daily basis resulting in MNi in the negative control. However when a known carcinogen is added e.g. AFB1, this mutagenesis significantly increased the number of cells showing MNi. Although multiple studies have indicated that both humic and fulvic acids may inhibit mutagenesis, it was important that this new source of CHD-FA be tested in order to ensure that it is not mutagenic, which was confirmed when comparing the six CHD-FA preparations tested to that of the negative and positive controls.

What was however noteworthy is that out of the six CHD-FA preparations, two of the concentrations namely CHD-FA 150 µg/mL and CHD-FA 50 µg/mL respectively, actually demonstrated a significant decrease in MNi, thus suggesting that CHD-FA may inhibit mutagenesis or have a protective property, however this warrants further investigation with larger sample size and more targeted assays. Historically it has been suggested that polyphenolic components, namely caffeic acid derivatives and flavonoids in particular, are of interest for their antioxidant property (Gregoris and Stevanato 2010). Caffeic acid exhibited antimutagenic properties and this positive effect was assumed to be a result of its ability to scavenge reactive oxygen species (ROS) (Belicova *et al.*, 2001, Benkovic *et al.* 2009).

2.5 CONCLUSION

Registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. It is however clear that no single test is capable of detecting all relevant genotoxic agents and therefore, the usual approach should be to carry out a battery of tests for genotoxicity. Along with the CBMN assay the potential genotoxicity of CHD-FA was also tested in combination with a bacterial reverse mutation test (Ames test) which was conducted by Dr Jooné, where it also proved that CHD-FA did not show any genotoxic or mutagenic potential (data on file, Department of Pharmacology, University of Pretoria, South Africa).

With the distinct differences versus the positive and negative control respectively, CHD-FA proving to be no more genotoxic than that of the negative control and coinciding with other CHD-FA genotoxic data, this study therefore concluded that the CBMN assay indicated no genotoxicity above background for both CHD-FA and LMWCHD-FA, using concentrations of 50, 100 and 150 $\mu\text{g}/\text{mL}$ in an *in vitro* micronucleus assay.

Of note was the significant decrease in MNi, when lymphocytes were exposed to two of the preparations namely CHD-FA 150 $\mu\text{g}/\text{mL}$ and CHD-FA 50 $\mu\text{g}/\text{mL}$ respectively, which indicates that there may even be a protective property to CHD-FA and that these results may be attributed to an antioxidant activity of CHD-FA as other research has suggested.

Further targeted studies into confirming this finding and determining the potential mechanism of action would need to be conducted but lies outside the scope of this study. CHD-FA does not prove to be genotoxic, and may even be desmutagenic.

CHAPTER 3: CLINICAL SAFETY AND EFFICACY

3.1 INTRODUCTION AND BRIEF STUDY OUTLINE

The *in vivo* human data demonstrating topical efficacy and safety as well as significant animal data supporting *in vivo* efficacy and safety after systemic administration, as well as observational data confirming safety of oral administration of this complimentary or alternative medicine in humans motivated this *in vivo* safety study. The aim of including this clinical trial in the study was to establish the acute and sub-acute safety of CHD-FA within the anticipated dose range and also to determine if CHD-FA has potential anti-inflammatory efficacy following oral administration that would warrant further investigation. Participants were recruited into the study after approval was obtained from the University of Pretoria (South Africa) Research Ethics Committee (Protocol no: 52/2007).

The clinical trial was a pilot study following a double-blind, randomised study design to assess the acute safety, tolerability, and potential anti-inflammatory efficacy using 30 male volunteers with predetermined atopy who were randomly assigned to Group A or Group B on arrival by random allocation of an alpha numeric, eg., A06 or B11 from a computer-randomized list, each consisting of 15 participants. Only male volunteers, aged between 19 and 24 were recruited in order to statistically have a 90% power of detecting a 20% difference in regimens during the proof of concept phase. A biostatistician was consulted.

Part 1: Acute exposure safety

A sequential initiation and escalation of dose administered, with 72 hours between dosages, using a single oral dosage of either 5 mL escalating to 20 mL (Group A) or 10 mL escalating to 40 mL (Group B) of a 3.8% CHD-FA solution was administered with the volunteers being monitored at various intervals for 72 hours, with a final visit 7 days after last oral dosage. The following parameters were monitored: vital parameters, clinical investigation, patient perception of general health questionnaire, electrocardiography, and haematology and biochemistry, including full blood count and liver and kidney functions. After successful completion of Part 1, ie, no adverse events that were of clinical relevance, Part 2 was commenced.

Part 2: Sub-acute toxicity

The sub-acute toxicity test formed Part 2 of the clinical toxicity assessment and followed a 3-day CHD-FA exposure using the same volunteers used in Part 1. In this study, 20 mL or 40 mL of the 3.8% CHD-FA solution was administered orally twice per day, with the following parameters being monitored at various intervals: vital parameters, clinical investigation, patient perception of general health, electrocardiography, haematology and biochemistry, including full blood count and liver and kidney function.

After successful completion of Part 2, ie, no adverse events that were of clinical relevance, Part 3 was commenced again with the same participants.

Part 3: Proof of concept

This was a 7-day exposure using the same volunteers, ie, 40 mL of 3.8% CHD-FA solution or a placebo administered twice per day, followed by a two-week washout period and subsequent crossover, with the following parameters being monitored at various intervals: skin prick challenge to elicit the atopic responses as identified during initial screening, vital parameters, clinical investigation, patient perception of general health, electrocardiography, and haematology and biochemistry, including full blood count and liver and kidney functions. For each participant, the initial skin prick test was done as part of the screening process and from the result the allergen to which the participant was most allergic was selected along with the positive histamine and negative control to be repeated at the start and end of each respective stage of the clinical trial. Wheals developing during the skin prick challenge were measured using a digital calliper 10 minutes after initiating the skin prick test.

Outcomes

Outcomes from each part were measured and collated to included safety, assessed by clinical parameters, vital functions and wheal size changes as efficacy measure. Biochemistry tests included urea, uric acid, creatinine, chloride, potassium, sodium, gamma-glutamyl transferase, alkaline phosphatase, aspartate aminotransferase, and alanine aminotransferase. Haematology included a full blood count and differential counts, with haemoglobin and white and red blood cells. All biochemistry and haematology measurements were done in an ISO (International Organization for Standardization) accredited laboratory using a Roche P800 and Siemens Advia 2120,

respectively. Electrocardiograms were read by an independent clinician who reported on any measurable changes from baseline.

A significant ($P < 0.05$) reduction in size of wheal formation post CHD-FA treatment was accepted as “Proof of Concept” and the outcome proved that the CHD-FA treatment did show a positive anti-inflammatory effect on an induced atopic reaction.

Inclusion criteria

Male patients aged 18 years and older who had a pollen allergy and were not being administered any anti-allergic treatment were recruited into the study. Patients gave their written informed consent. Patients were otherwise healthy with no other systemic disease that could influence the immune response. Normal haematology and biochemistry values, electrocardiograms, and clinical investigations were confirmed before randomization.

Exclusion criteria

Women and subjects with systemic diseases, such as diabetes mellitus, kidney failure, liver failure, or autoimmune, haematologic, psychiatric, gastrointestinal, cardiovascular, or lung disease that indicated compromised health were excluded. Any immune system disease other than atopy was also an exclusion criterion, as were an inability to understand or give written informed consent, any abnormality in haematology or biochemistry values, alcoholism or drug addiction, and any type of immunosuppressant therapy.

Treatment

In the acute toxicity study, Group A received 5 mL escalating to 20 mL of CHD-FA 3.8% in solution as single doses and Group B received 10 mL escalating to 40 mL of CHD-FA 3.8% as a single dose. In the sub-acute toxicity study, Group A received 20 mL of CHD-FA 3.8% twice daily for 3 days and Group B received 40 mL of CHD-FA 3.8% twice daily for a 3-day period. For proof of concept with respect to the anti-inflammatory response to treatment, both groups received either 40 mL of CHD-FA 3.8% or placebo for a 7-day period, followed by a 7-day washout period, then a crossover to the alternative treatment. All treatments were taken without food.

Statistical analysis

The results were initially analyzed using GraphPad Prism 4.0. The Wilcoxon rank test was used to assess the safety data collected during the study. For the proof of concept results, an analysis of covariance was used, with $P < 0.05$ regarded as statistically significant when comparing the before and after results of the treated versus placebo groups, respectively.

3.2 HYPOTHESES

- (a) CHD-FA is safe at the oral dosages tested?
- (b) CHD-FA demonstrates anti-inflammatory properties in the atopic dermatitis?

3.3 STUDY OBJECTIVES

- To determine safety and toxicity by comparing cardiac, haematological and biochemical results, before, during and after the clinical trial.
- To determine participants' subjective impression of their health status i.e. discomfort during the course of the study using a general health questionnaire.
- To determine changes in patient's health with full clinical assessments.
- To establish proof of concept pertaining to anti-inflammatory properties related to atopic dermatitis.

3.4 STUDY PLAN AND EVALUATION

Study design

Prospective, investigational, single centre, randomized, double blind, placebo controlled, phase one safety study.

Sample size

30 subjects. (15 per arm to determine clinical significance in a homogeneous cohort)

Study duration

43 days.

General outline

Study population

After ethics committee approval and informed consent obtained, volunteers were screened and 30 male, otherwise healthy volunteers with atopic hypersensitivity, determined via a skin prick test, were recruited into this double blind study and randomly assigned to Groups A or B, consisting of 15 participants each.

In Part 1 of the study, a dose ranging assessment was performed to establish tolerability and safety criteria where the groups were administered two sequential increasing doses of CHD-FA ranging a starting dose of 5 mL or 10 mL then increased to 20 mL or 40 mL of a 3.8% CHD-FA buffered solution, with the proviso that no adverse events occurred.

In Part 2, Group A received 20 mL 3.8% CHD-FA solution 2 x per day for a period of 3 days and were monitored twice daily for a week. As no adverse events were reported, Group B then received an increased dose of 40 mL 3.8% CHD-FA solution 2 x per day for a period of 3 days and were monitored in the same way as Group A for a week following the last dose.

In Part 3 a placebo controlled cross-over trial was performed where either 40 mL 3.8% CHD-FA solution or placebo were administered 2 x per day for a period of a week, then after a one week washout period the treatments were switched for each group. Skin prick test were done on commencement and completion of each of the respective phases, thus 4 in total.

The main outcomes were as follows for each part of this study:

Part 1: Acute exposure safety:

A sequential escalation, with 72 hours between dosages, of a single oral dosage of either 5 mL and 20 mL (Group A) or 10 mL and 40 mL (Group B) (3.8%) CHD-FA was administered and the volunteers were monitored at various intervals (30 min, 1 hour, 24 hours and 72 hours post ingestion, with the final visit 7 days after the first oral dose.

The following parameters were monitored:

- Vital parameters
- Clinical investigation
- Patients perception of general health
- ECG
- Haematology and Biochemistry: FBC, Liver and kidney function

After successful completion of Part 1 i.e. no adverse events that are of clinical relevance as judged by an independent safety board consisting of all healthcare practitioners involved, Part 2 commenced.

Part 2: Sub acute toxicity:

A 3 day exposure in the same volunteers i.e. 20 mL or 40 mL of 3.8% CHD-FA solution administered 2 x per day, with the following parameters being monitored:

- Vital parameters
- Clinical investigation
- Patients perception of general health
- ECG
- Haematology and Biochemistry: FBC, Liver and kidney function

After successful completion of Part 2 i.e. no adverse events that are of clinical relevance as judged by an independent safety board, Part 3 commenced.

Part 3: Proof of concept:

A 7 day exposure in the same volunteers i.e. 40 mL of 3.8% CHD-FA solution or placebo administered 2 x per day, with the following parameters being monitored:

- Skin Prick wheal size
- Vital parameters
- Clinical investigation
- Patients perception of general health
- ECG
- Haematology and Biochemistry: FBC, Liver and kidney function

Outcomes:

Safety

- Clinical parameters and vitals.
- Biochemistry: Urea, uric acid, creatinine, Cl^- , K^+ , Na^+ , γGT , Alkaline phosphatase, AST and ALT.
- Haematology: Full blood count and differential: Haemaglobin, white blood cells and red blood cells.

Efficacy

- Change in diameter of wheal and flare on forearm after allergen skin prick test.

Visits to study site

There were 53 visits to the investigators' site:

Visit 1: Investigator obtained written informed consent from the patient. Demography of patient: Initials, date of birth, weight, height. Habits: smoking, illicit drugs, alcohol. Medical history. Participants' perception of general health questionnaire General physical examination plus ECG. Skin prick test. Laboratory tests. Urea, uric acid, creatinine, Cl^- , K^+ , Na^+ , γGT , Alkaline phosphatase, AST and ALT. Full blood count (differential and morphology). Verify eligibility of the patient – inclusion and exclusion criteria. Randomization into either group A or group B.

Commenced Part 1: Acute safety

- Visit 2: Clinical examination. Ingestion of a once off oral dosage of either 5mL (3.8%) CHD-FA for group A or 10 mL (3.8%) CHD-FA for group B.
- Visit 3: 30 min after CHD-FA ingestion the participant underwent a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 4: 1 hour after CHD-FA ingestion the participant underwent a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 5: 8 hours after CHD-FA ingestion the participant underwent a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 6: 24 hours after CHD-FA ingestion the participant underwent a clinical exam, ECG and blood drawn for biochemistry markers.
- Visit 7: 72 hours after CHD-FA ingestion the participant underwent a clinical exam, ECG and blood drawn for biochemistry markers.
- Visit 8: 7 days after CHD-FA ingestion the participant underwent a clinical examination, ECG, participants' perception of general health questionnaire, blood drawn for haematology and biochemistry markers. Ingestion of a once off oral dosage of either 20 mL (3.8%) CHD-FA for group A or 40 mL (3.8%) CHD-FA for group B.
- Visit 9: 30 min after CHD-FA ingestion the participant underwent a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 10: 1 hour after CHD-FA ingestion the participant underwent a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 11: 8 hours after CHD-FA ingestion the participant underwent a physical examination, ECG and blood drawn to test the biochemistry markers.

- Visit 12: 24 hours after CHD-FA ingestion the participant underwent a clinical examination, ECG and blood drawn for biochemistry markers.
- Visit 13: 72 hours after CHD-FA ingestion participant underwent a clinical examination, ECG and blood drawn for biochemistry markers.
- Visit 14: 7 days after CHD-FA ingestion participant underwent a clinical exam, ECG, participants' perception of general health questionnaire, blood drawn for haematology and biochemistry markers.

Commenced Part 2: Sub-acute safety and proof of concept (See Annexure C)

- Visit 14: 14 days after initial CHD-FA ingestion, 20 mL (3.8%) CHD-FA was administered to group A and 40 mL (3.8%) CHD-FA was administered to group B.
- Visit 15: 6-8 Hours after previous ingestion of CHD-FA, 20 mL (3.8%) CHD-FA was administered to group A and 40 mL (3.8%) CHD-FA was administered to group B.
- Visit 16: 1 Hour after previous visit, blood drawn to check potassium and ECG done.
- Visit 17: 15 days after initial CHD-FA ingestion, physical exam, ECG and 20 mL (3.8%) CHD-FA was administered to group A and 40 mL (3.8%) CHD-FA was administered to group B.
- Visit 18: 6-8 Hours after previous CHD-FA ingestion, blood drawn for biochemistry markers, 20 mL (3.8%) CHD-FA was administered to group A and 40 mL (3.8%) CHD-FA was administered to group B.
- Visit 19: 16 days after initial CHD-FA ingestion, physical exam, 20 mL (3.8%) CHD-FA was administered to group A and 40 mL (3.8%) CHD-FA was administered to group B.
- Visit 20: 6-8 Hours after previous ingestion of CHD-FA, participant underwent a clinical exam, ECG, participants' perception of general health questionnaire, blood drawn for haematology and biochemistry markers, 20 mL (3.8%) CHD-FA was administered to group A and 40 mL (3.8%) CHD-FA was administered to group B.

Commenced Part 3: Proof of concept:

- Visit 21: 23 days after initial CHD-FA ingestion, physical exam, ECG, participants' perception of general health questionnaire, blood drawn for haematology and biochemistry markers, skin prick test and 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 22: 6-8 Hours after previous ingestion of CHD-FA/placebo, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 23: 24 days after initial CHD-FA/placebo ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 24: 6-8 Hours after previous CHD-FA/placebo ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 25: 1 Hour after previous visit, blood drawn to check potassium levels and ECG done.
- Visit 26: 25 days after initial CHD-FA ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 27: 6-8 Hours after previous CHD-FA/placebo ingestion, 40 mL (3.8%) CHDFA/placebo administered.
- Visit 28: 26 days after initial CHD-FA ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 29: 6-8 Hours after previous CHD-FA/placebo ingestion, physical examination, participants' perception of general health questionnaire and 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 30: 27 days after initial CHD-FA ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 31: 6-8 Hours after previous CHD-FA/placebo ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 32: 1 Hour after previous visit, blood drawn to check potassium levels and ECG done.
- Visit 33: 28 days after initial CHD-FA ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 34: 6-8 Hours after previous CHD-FA/placebo ingestion of, 40 mL (3.8%) CHD-FA/placebo administered.

- Visit 35: 29 days after initial CHD-FA ingestion, 40 mL (3.8%) CHD-FA/placebo administered.
- Visit 36: 6-8 Hours after previous CHD-FA/placebo ingestion, physical examination, ECG, participants' perception of general health questionnaire, blood drawn for haematology and biochemistry markers and skin prick test.

A 7 day washout period then commenced, followed by the cross-over phase of proof of concept part, where the groups receiving the placebo now received the CHD-FA and *visa versa*.

Additional visits were arranged with patients as dictated by clinical need.

3.5 SELECTION OF VOLUNTEERS

Inclusion criteria

The following criteria were met at the initial visit:

- (a) Male patients aged 18 years and over.
- (b) Had pollen allergy and not on medication.
- (c) Patients gave written informed consent.
- (d) Patients were able to complete the questionnaire and understand all aspects pertaining to the trial as to discuss it with the trialist/investigator.
- (e) Patients were otherwise healthy with no other systemic disease that could influence the immune response.
- (f) Normal haematology or biochemistry values, ECG and clinical investigation were available before randomisation.
- (g) Not on a clinical trial during the previous 2 months.

Exclusion criteria

- (a) Females
- (b) Systemic diseases such as diabetes mellitus, kidney failure, liver failure, haematologic, psychiatric, GIT, cardiovascular or lung disease that significantly compromised the patients' health and any immune system disease other than atopy.
- (c) Patients not able to understand and give written informed consent.
- (d) Any abnormality in haematology or biochemistry values
- (e) Alcoholism or drug addiction.
- (f) Participation in a clinical trial during the last 2 months prior to enrolment.
- (g) Patients already included in another study
- (h) Patients intending to donate blood during the study
- (i) Patients on any type of immunosuppressants
- (j) Medications prohibited throughout the study. Observe the listed washout periods below.

Washout periods required

- systemic corticosteroids short course (up to 14 days) 1 month
- systemic corticosteroids for long term (14 days) use 3 months
- topical corticosteroids 14 days
- H₁ antihistamines (i.e. loratadine, cetirizine and fexofenadine) 2 days
- decongestants topical no washout
- decongestants oral 2 days
- β_2 agonists for mild asthma only when needed on a prn base was it allowed (eg once or twice a week)

Criteria verified at randomisation visit (Visit 1):

Laboratory test results

- Kidney function
- Liver function
- Washout periods for drugs

3.6 TREATMENTS

CHD-FA: Participants were dosed as follows:

Acute toxicity study: Group A received a single dose of 5 mL and if well tolerated were given a further once off dose of 20 mL of a 3.8% CHD-FA 7 days after treatment one, in solution form and Group B received a single dose of 10 mL and if well tolerated were given a further once off dose of 40 mL of a 3.8% CHD-FA after 7 days post treatment in solution form.

Sub-acute toxicity: Group A received 20 mL 3.8% CHD-FA 2 x per day for 3 days and group B received 40 mL 3.8% CHD-FA 2 x per day for a 3 day period.

Proof of concept: Both groups received either 40 mL of a 3.8% CHD-FA or placebo solution for a 7 day period, and then had a 7 day washout period and crossed-over to the alternative treatment.

Patient identification

Participants' initials and number identified each participant.

A unique number identified each investigational product package.

3.7 WITHDRAWALS

Withdrawals from the study

- ineligibility – inclusion/exclusion criteria
- death
- concomitant medication not allowed
- personal reasons
- Later withdrawals after visit 2
- therapy failure – intolerant symptoms
- any abnormalities in haematology or biochemistry

Code breaking

In case of a medical emergency, the investigator was allowed to open the sealed envelope to identify the patient's therapy if necessary. Reason for opening, time and date would have been entered on the Adverse Event Form.

Patients lost to follow-up

The investigator contacted patients to come for a final follow-up visit. If the follow-up visit is refused, the reason for drop-out was ascertained and the CRF completed.

3.8 ADVERSE EVENTS

Definition of adverse events (AE)

An adverse event is any untoward medical occurrence in a patient or clinical investigational patient administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An adverse event can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of a medicinal (investigational) product, whether or not related to the medicinal (investigational) product.

In order to ensure complete safety data collection, all AE or undesirable experiences occurring during the trial (i.e. after signature of the Informed Consent document), including any protocol required pre- and post-treatment periods, were reported even if no investigational product was taken. These included all AEs not present prior to the initial visit and all AEs, which reoccurred or worsened after this initial visit.

Procedures for reporting and recording adverse events

Recording/Collection of AE information

The trial participant was given the opportunity to spontaneously report Adverse Events. A general prompt was also given to detect adverse events.

“Did you notice anything unusual about your health (since your last visit)?”

In addition, the investigator reviewed any self-assessment procedures (i.e. diary cards) used in the trial.

Description of AEs

The investigator for the description of AEs used the following guidelines and definitions when reporting information:

Nature of the AE:

Preferably an overall diagnosis or syndrome, rather than individual symptoms or signs. The investigator reported adverse events using standard medical terminology. The same terms were used in the Source Documentation and in the CRF.

Date of onset (and time): Date (and time) the AE started.

Pattern:

Intermittent: The AE reoccurred with the same intensity at various intervals throughout the entire time period specified. There were intervals within the specified time period when the AE was not present
continuous: The AE was present at the same intensity for the entire time period specified. There was no time at which the event abated or was not present during the time period specified.

Intensity:

Mild: the patient was aware of the sign or symptom (syndrome), but it did not interfere with his usual activities and/or is of no clinical consequence

Moderate: The AE interferes with the usual activities of the patient or it is of some clinical consequence.

Severe: The patient was unable to work normally or to carry out his usual activities, or the AE is of definite clinical consequence.

Action taken with investigational product:

- **Not applicable:** For AE occurring during the pre-treatment period
- **Dosage changed:** Investigational product dose was increased or decreased because of this AE
- **No change :** Investigational product dose remained the same in spite of AE being present
- **Temporarily discontinued:** Investigational product was temporarily discontinued because of this AE, either because the patient chose to discontinue the study drug or the physician felt it was in the patient's best interest to temporarily discontinue the investigational product.
- **Permanently discontinued:** Investigational product was permanently discontinued because of this AE, either because the patient chose to discontinue the study drug or the physician felt it was in the patient's best interest to discontinue the investigational product.

Other actions taken

All actions taken are to be noted

- **None:** No other action was taken for this AE
- **Medication:** The patient took a medication (either prescription or non-prescription) specifically for this AE
- **Hospitalisation or prolongation of hospitalisation:** Patient was hospitalised for this AE or patient's stay in hospital was prolonged because of this AE.

- **Therapeutic or diagnostic procedure:** Patient used other therapeutic measures (e.g. ice, heating pad, brace, cast, etc.) or patient underwent a diagnostic procedure (e.g., additional lab test, x-ray, etc.) for this AE.

Date of outcome (and time):

Date (and time) and AE abated.

If the AE consisted of several signs and symptoms (syndrome), the sign or symptom with the longest duration determined the duration of the AE.

Outcome:

- **Resolved:** The AE is no longer present at any intensity – completely abated.
- **Resolved with sequelae:** The AE is resolved but residual effects are still present.
- **Worsened:** The AE is still present but at a heightened intensity. The rule of repetition of AE reporting should be applied.
- **Fatal:** This AE caused or directly contributed to patient’s death.
- **Ongoing:** The AE is still present at the last contact with the patient.

Relationship to investigational product:

- **None:** Only applicable when no investigational product was taken or when the patient is taking single blind placebo, or when the AE can be ascribed with reasonable certainty to another cause, e.g. a gunshot wound.
- **Unlikely:** There were good reasons to think that there is no relationship, e.g. the AE was a known adverse drug reaction of a concomitant medication, or the same AE did not reappear after re-administration of the investigational product.
- **Possible:** Equally valid arguments were considered for or against an implication of the investigational product, e.g. the AE:
 - Followed a reasonable temporal sequence from the administration of the investigational product;
 - Followed a known or expected response pattern to the investigational product; but could readily have been produced by a number of other factors.

- **Probable:** The relationship is likely, e.g. the AE:
 - Followed a reasonable temporal sequence from administration of the investigational product
 - Followed a known or expected response pattern to the investigational product; is confirmed by improvement of the AE by stopping or reducing the dosage of the investigational product;
 - Could not be reasonably explained by the known characteristics of the patient's clinical state.

- **Highly probable:** There was a strong relationship, e.g. the AE:
 - Followed a reasonable temporal sequence from administration of the investigational product or in which the investigational product level had been established in body fluids or tissues;
 - Followed a known or expected response pattern to the investigational product; was confirmed by improvement on stopping or reducing the dosage of the drug, and reappearance of the reaction on repeated exposure (rechallenge).

Follow-up of adverse events ongoing at the end of the study

- If an AE was still present at the time the CRF was collected, a follow-up report was provided later on.
- If no follow-up report was provided, the investigator provided a justification.
- A serious AE or an AE leading to premature discontinuation from the study was always followed-up until it was resolved or the Principal Investigator no longer felt it was clinically significant.

Rule for repetition of an AE

An unexpected increase in the intensity of an AE that was not related to the natural course of the disease lead to the repetition of the AE reporting with:

- The outcome date of the first AE being the same as the start date of the repeated AE.
- The investigator's original description of the AE being the same for the first and repeated AE.

3.9 STATISTICAL METHODOLOGY AND ANALYSIS

Study Population

- The intent-to-treat population (ITT) consisted of all randomised patients except those for whom it was confirmed that they didn't take any study medication.
- The per-protocol-population (PPT) was a subject of the intent-to-treat population, consisting of those patients who did not have any major protocol deviations.
- During the pre-analysis meeting (before unblinding of the study) it was decided whether a patient was excluded from the ITT or PPT population. Major deviations excluded patients' data from the safety assessment.

The primary safety parameters

Haematological and biochemical results, before, during and after the trial were compared to determine safety and toxicity.

General Health Questionnaire. Subjects' subjective feelings i.e. discomfort during the course of the study.

Clinical assessment of patient's health was determined.

3.10 ETHICAL CONSIDERATION

- This study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (South Africa) Protocol number 52/2007.
- This study was conducted according to best Good Clinical Practice Guidelines and the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects (last update: Edinburgh Scotland October 2000).
- Informed Consent and Information document was signed by all recruited patients.

3.11 MEDICAL CONTROL COUNCIL (MCC)

Natural Product

Natural products are currently listed at the MCC but not approved or disapproved. The natural product fulvic acid falls in this category (MCC code 4625). This development of a natural product is therefore in line with the MCC's current approval system which did not mandate submission of clinical trials on natural products in 2009.

The research project was registered with the National Research Ethics Committee of the Department of Health registration no 52/2007.

3.12 RESULTS

Twenty one of the participants successfully completed the trial, one failed to comply after screening, one failed to comply at visit 1, one subject experienced an adverse event, namely hypertension, which was not drug related, two subjects failed to comply during Part 1.

Four subjects were withdrawn due to either an arrhythmia or bradycardia that was present at baseline. This decision was made due to a previous CHD-FA trial where an acute increase in serum potassium was found at much higher dosages and in a sweetened formulation. The causal link remains elusive as this was not repeatable.

3.12.1 Acute Toxicity

Safety

All safety parameters remained within normal limits for the duration of the acute phase of the trial, with only one of the participants having elevated AST and ALT levels after the 40 mL dosing, which was more likely associated to a concomitant viral infection rather than a side effect of the fulvic acid and recorded in the source documentation for follow up and verification. These values did return to within normal limits. The parameters of interest are tabulated below (Tables 3.1-3.4).

Table 3.1: Mean potassium levels and standard deviations at baseline and 1 hour post dosing with a 40 mL dose of 3.8% fulvic acid.

	Potassium (mmol/L)	
	Mean	SD
Baseline	4.492	0.214
1 Hour	4.323	0.224

Table 3.2: Mean ALT levels and standard deviations at baseline and 1 hour post dosing with a 40 mL dose of 3.8% fulvic acid.

	ALT (U/L)	
	Mean	SD
Baseline	22.154	4.879
1 Hour	21.538	4.994

Table 3.3: Mean AST levels and standard deviations at baseline and 1 hour post dosing with a 40 mL dose of 3.8% fulvic acid.

	AST (U/L)	
	Mean	SD
Baseline	31.462	13.794
1 Hour	22.846	4.862

Table 3.4: Mean urea levels and standard deviations at baseline and 1 hour post dosing with a 40 mL dose of 3.8% fulvic acid.

	Urea (mmol/L)	
	Mean	SD
Baseline	4.831	0.930
1 Hour	4.977	1.031

Table 3.5: Mean creatinine levels and standard deviations at baseline and 1 hour post dosing with a 40 mL dose of 3.8% fulvic acid.

	Creatinine ($\mu\text{mol/L}$)	
	Mean	SD
Baseline	94.231	15.347
1 Hour	86.846	12.812

Side effects

Throughout this part of the trial the following side effects were experienced:

- No side effects at the 5 mL dosage
- One report of diarrhoea at the 10 mL dosage
- One report of diarrhoea at the 20 mL dosage
- At the 40 mL dosage
 - Two subjects experienced appetite suppression,
 - One reported headache after each dose of fulvic acid
 - Four reports of malaise
 - Three subjects complained that the fulvic acid burned their throats and that it left them with a sore throat
 - One report of diarrhoea

3.12.2 Sub-Acute Toxicity

Safety

All safety parameters remained within normal limits for the duration of the acute phase of the trial, with only one of the participants having elevated AST and ALT levels at baseline, but returned to within normal limits, which was more likely associated to a viral infection rather than a side effect of the fulvic acid based on the haematology results. These values did return to within normal limits. The parameters of interest are tabulated below (Tables 3.6-3.10).

Table 3.6: Mean potassium levels and standard deviations at baseline and end of Part 2, 2 x 40mL 3.8% fulvic acid dosing per day for 3 days.

	Potassium (mmol/L)	
	Mean	SD
Baseline	4.400	0.309
Final	4.120	0.187

Table 3.7: Mean ALT levels and standard deviations at baseline and end of Part 2, 2 x 40mL 3.8% fulvic acid dosing per day for 3 days.

	ALT (U/L)	
	Mean	SD
Baseline	19.800	8.257
Final	18.000	6.566

Table 3.8: Mean AST levels and standard deviations at baseline and end of Part 2, 2 x 40mL 3.8% fulvic acid dosing per day for 3 days.

	AST (U/L)	
	Mean	SD
Baseline	19.700	5.579
Final	20.500	4.453

Table 3.9: Mean urea levels and standard deviations at baseline and end of part 2, 2 x 40mL 3.8% fulvic acid dosing per day for 3 days.

	Urea (mmol/L)	
	Mean	SD
Baseline	5.210	1.584
Final	4.200	0.935

Table 3.10: Mean urea levels and standard deviations at baseline and end of part 2, 2 x 40mL 3.8% fulvic acid dosing per day for 3 days.

	Creatinine ($\mu\text{mol/L}$)	
	Mean	SD
Baseline	90.100	8.850
Final	87.700	9.661

Side effects

No side effects were reported during this phase.

3.12.3 Proof of concept

Safety

All safety parameters remained within normal limits for the duration of the acute phase of the trial, with only one of the participants having elevated AST and ALT levels at baseline, but returned to within normal limits, which was more likely associated to a viral infection rather than a side effect of the fulvic acid. These values did return to within normal limits. The parameters of interest are tabulated below (Tables 3.11-3.15).

Table 3.11: Mean potassium levels and standard deviations at baseline and end of part 3, 2 x 40mL 3.8% fulvic acid dosing per day for 7 days.

	Potassium (mmol/L)	
	Mean	SD
Baseline	4.185	0.303
Final	4.370	0.285

Table 3.12: Mean ALT levels and standard deviations at baseline and end of part 3, 2 x 40mL 3.8% fulvic acid dosing per day for 7 days.

	ALT (U/L)	
	Mean	SD
Baseline	20.150	7.809
Final	20.400	9.087

Table 3.13: Mean AST levels and standard deviations at baseline and end of part 3, 2 x 40mL 3.8% fulvic acid dosing per day for 7 days.

	AST (U/L)	
	Mean	SD
Baseline	22.450	8.140
Final	23.250	10.647

Table 3.14: Mean urea levels and standard deviations at baseline and end of part 3, 2 x 40mL 3.8% fulvic acid dosing per day for 7 days.

	Urea (mmol/L)	
	Mean	SD
Baseline	4.835	1.266
Final	5.215	1.026

Table 3.15: Mean creatinine levels and standard deviations at baseline and end of part 3, 2 x 40mL 3.8% fulvic acid dosing per day for 7 days.

	Creatinine (µmol/L)	
	Mean	SD
Baseline	96.700	12.897
Final	102.950	11.936

Efficacy

With statistical analysis, using an ANCOVA, adjusting for baseline, a significant difference was observed with a $p < 0.05$ when comparing the before and after results of the treated versus placebo groups respectively, with 81% of the participants having a decreased wheal after fulvic acid treatment versus that of the 62% when treated with placebo (Table 3.16).

Table 3.16: Measurements of wheal (diameter in mm) before and after the study of the treated vs placebo groups with respect to their skin prick tests.

	Diameter of wheal (mm)			
	Before		After	
	Mean	SD	Mean	SD
CHD-FA	6.63	3.64	*4.97	3.26
Placebo	7.03	4.15	6.73	3.98

* Indicating statistical significance ($p < 0.05$) $n = 21$

Side effects (Figure 3.1)

During this phase the following side effects were reported:

- Eight reports of diarrhoea.
- Six reports of a burning or sore throat after dosing.
- One report of abdominal discomfort
- Two reports of nausea post dosing
- Four reports of a headache post dosing
- Four reports of malaise

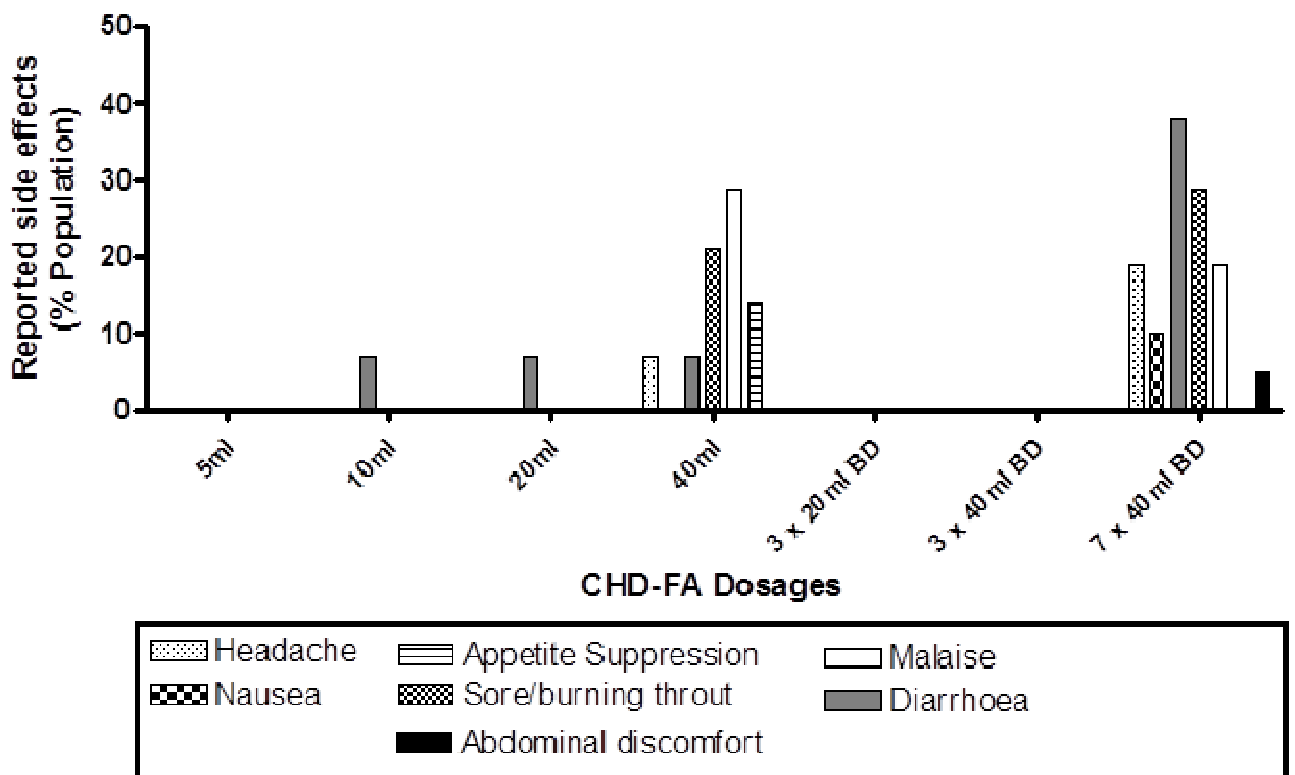


Figure 3.1: Summary of side effects of CHD-FA. (n=21)

3.13 DISCUSSION

Safety

In a preclinical toxicity study in rats treated daily with an oral dosage of potassium humate, a humic substance, at 1g/kg for 1 month, it was found that the dosage had no effect on the safety parameters nor did a dosage of 500 mg/kg body weight have any effect on the pups when it was administered to pregnant female rats (Van Rens burg *et al.*, 2007).

Extensive safety testing was performed that resulted in fairly regular blood samples being drawn for safety haematological and biochemistry markers, especially that of the potassium and ECG's. This was due to an earlier trial where the investigation was temporarily halted due to an unexpected fluctuation in the potassium levels of the initial participants. The fluctuation was attributed to the formulation which was rectified and the protocol was subsequently resubmitted for ethics approval which was accepted provided more extensive and thorough plasma potassium levels were monitored at regular intervals.

Following the change in the formulation, it can be stated with confidence that the biochemical marker parameters were not affected in any significant way, proving that the maximal dose of CHD-FA tested correlated well with the safety aspects reported for the preclinical animal studies, where a 6 month daily dosing with CHD-FA revealed no significant fluctuations in safety parameters.

This clinical data confirms the safety data collected in preclinical animal studies with dosages up to 15 mg/kg body mass, demonstrated no toxic effects or abnormal blood biochemistry after topical application or oral administration (Snyman *et al.*, 2002).

Efficacy

In previous efficacy studies, potassium humate, a humic substance, at an oral dosage of 60 mg/kg, inhibited a delayed type hypersensitivity reaction in rats immunised with sheep red blood cells, a carrageenan-induced oedema and a graft-versus-host reaction in rat models (Van Rensburg *et al.*, 2010), as well as a contact hypersensitivity reaction in rats sensitised with dinitrofluorobenzene (Van Rensburg *et al.*, 2007).

In these studies, potassium humate compared favourably with indomethacin and prednisolone. Furthermore, a chemical complex was prepared by Anwer *et al.* (2010) with humic acid and aspirin by lyophilization. This complex enhanced the anti-inflammatory activity of aspirin alone in the rat paw oedema model (Anwer *et al.*, 2010).

When comparing the wheal size after a skin prick challenge with a known allergen, eliciting an immediate inflammatory response due to mast cell degranulation and histamine release (Prussin *et al.* 2003), it was demonstrated that there was a significant difference between the CHD-FA versus the placebo (5% decrease) group, with a significant reduction (25%) in the wheal measurements when adjusting for baseline and after CHD-FA administration. This confirmed that CHD-FA may be used orally as an anti-inflammatory agent for the treatment of atopic dermatitis and probably other acute hypersensitivity reactions such as pollen allergy.

Side effects

The side effects that were noteworthy was that of diarrhoea, headache and a burning sore throat after administration, which may all probably be attributed to the low pH of the CHD-FA administered orally, (for dosing purposes the manufacturer recommends dilution in juice/water).

The majority of these side effects occurred after the ingestion of the higher dosage of CHD-FA, namely 40 mL twice a day. These side effects were not significant, considering the number of dosages of CHD-FA ingested and the number of participants taking part in the trial. Most of these side effects subsided a short period later or after the ingestion of food.

3.14 CONCLUSION

In this study there were no severe adverse events reported, demonstrating CHD-FA to be safe at the dosages tested. It also demonstrated that the product is systemically available and effective as an anti-inflammatory agent during the acute atopic reaction as demonstrated by the significant decrease in wheal formation in the skin prick test, which implies that CHD-FA acts as an inhibitor of the immediate hypersensitivity reaction.

With these recent findings and all the prior data from previous toxicity studies by Van Rensburg *et al* it can be stated with confidence that a daily dose of 15 mL CHD-FA diluted in water/juice, as recommended by the manufacturer is well tolerated. Thus CHD-FA 3.8% can be used safely for at least 2 weeks using an undiluted volume of 40 mL without experiencing any severe side effects whilst experiencing the potential anti-inflammatory effects.

Although well tolerated it is worth noting the potential limitations of the study. This was a relatively small yet significant trial with only 21 of the initial 30 male atopic volunteers aged between 21 and 30 completing the study. This does not allow the scope or heterogeneity as may be required in order register a particular drug for treatment of a disease or for use for prevention. Although this was mainly a safety and proof of concept trial, all the planned outcomes were reached and completed. It is noteworthy that upon completion the trial was audited by the South African Department of Health with only minor findings and irregularities found which were addressed and accepted. This trial has also been subsequently published and thus allows for the greater scientific community to learn and use this information and data going forward. CHD-FA proved to be safe when and efficacious ingested orally.

CHAPTER 4: TOPICAL EFFICACY OF CHD-FA AS AN ANTI- INFAMMATORY AGENT

4.1 INTRODUCTION AND BRIEF STUDY OUTLINE

The anti-inflammatory activity of topically applied oxifulvic acid, a fulvic-acid product derived from bituminous coal, was compared with a 1% preparation of both diclofenac sodium and betamethasone in a murine model of contact hypersensitivity. In this experiment, mice were sensitised to dinitrofluorobenzene and then challenged with dinitrofluorobenzene on the dorsal surface of one ear. The mice's inflamed ears were treated with a topic application of either a placebo cream or a formulation containing oxifulvic acid, diclofenac sodium or betamethasone. The thickness of their ears was measured on a daily basis. Oxifulvic acid, as well as the betamethasone and diclofenac sodium formulations, reduced the cutaneous inflammatory response (Van Rensburg *et al.*, 2001). Thus demonstrating the potential efficacy of humic substances as anti-inflammatory agents when applied topically.

The purpose of this clinical trial was to evaluate the efficacy and safety of a topically applied, synthetic, carbohydrate derived fulvic acid (CHD-FA) formulation in the treatment of atopic dermatitis in patients aged two years and older. Atopic dermatitis is often a recurrent and difficult condition to treat, with topical glucocorticoids used to treat flares and normal emollients used for maintenance therapy. In chronic conditions, topical calcineurin antagonists (eg, pimecrolimus or tacrolimus) are used or may even be supplemented with systemic immunosuppressive therapy in severe cases.

The importance of pH is also recognized in skin diseases. Some diseases or stages are associated with different pH levels. An increase in skin pH may be due to atopic and seborrhoeic dermatitis, especially acute lesions that are erythematous with exudates and crusts, while chronic atopic dermatitis lesions with lichenification and scaling are only slightly more alkaline than normal skin (Knor *et al.*, 2011). In many of these diseases, the pathogenesis of alkalization is not clear, but the higher pH likely predisposes the skin to secondary infections (Chikakane and Takahashi, 1995).

Fulvic acid, with its reported anti-inflammatory properties and safety profile, has the added benefit of being acidic. Reducing the pH of the skin also relieves the itch during atopic dermatitis. In this study, a 3.5% CHD-FA in an emollient (buffered to pH 4.8) was compared with an acidic (pH 4.8) emollient to establish the anti-inflammatory properties of CHD-FA in patients with atopic dermatitis.

This study involved a second pilot clinical trial which took the form of a small single-centre, double-blind, placebo-controlled, parallel-group comparative Phase II study. A total of 36 volunteers with known atopic dermatitis were randomly assigned to receive either the study drug or a placebo emollient applied twice daily for four weeks. Treatment period observations and measurements included improvement in a self-assessed visual analogue scale score rating the extent of their respective local itch and an investigator global assessment.

Blood samples were taken at baseline and again at the end of the study, and included a full blood count, and liver and kidney function tests. Clinical examination, electrocardiogram, documentation of adverse events, and laboratory investigations were also performed to confirm safety.

4.2 HYPOTHESIS

Null-hypothesis: CHD-FA has the same efficacy as the placebo in treating atopic dermatitis.

Alternative: CHD-FA is superior to placebo in treating atopic dermatitis.

4.3 STUDY OBJECTIVE

To confirm the safety and the therapeutic efficacy of CHD-FA in reducing the early and/or late-phase cutaneous hypersensitivity reactions in atopic dermatitis patients of 2 years and older.

4.4 STUDY DESIGN AND EVALUATION

Study Design

This study involved a limited participant, single-centre, clinical trial which took the form of a double-blind, placebo-controlled, parallel-group comparative Phase II study. A total of 36 participants with confirmed atopic dermatitis were randomly assigned to receive either the study drug in a topical emollient or an equivalent placebo emollient which was applied twice daily to affected areas for a period of four weeks.

Study objective

The main study outcome was to confirm the safety and the therapeutic efficacy of CHD-FA in reducing the early and/or late-phase cutaneous hypersensitivity reactions in atopic dermatitis patients 2 years and older when applied topically as an emollient containing 3.5% CHD-FA.

Study population

Healthy males and females over the age of two years were eligible for inclusion in this study. Female patients were required to use reliable contraception if they were of childbearing age. A confirmed diagnosis of atopic dermatitis was required. Patients were required to give their or their legal guardians written informed consent to participate. Exclusion criteria included renal impairment, liver disease, abnormal liver function tests, haematological abnormalities, any autoimmune disease other than atopic dermatitis, pregnancy, lactation, participation in any other clinical trial within the previous month, clinical signs of infection, use of concomitant medication, systemic corticosteroids, phototherapy/immune suppression/ antihistamines, topical tacrolimus/pimecrolimus within the previous four weeks, or topical corticosteroids within the previous four days.

Ethical Aspects

This study was approved by the University of Pretoria (South Africa) Research Ethics Committee (125/2007) and 40 patients above two years were recruited from the Pretoria (South Africa) area after approval.

- This study was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria (South Africa).

- This study was conducted according to best Good Clinical Practice Guidelines and the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects (as amended: Edinburgh Scotland October 2000).
- Informed Consent and Information document was discussed and signed by all recruited patients.

Study drug

A 3.5% CHD-FA or placebo (pH 4.8) gel (blue sticker) was applied twice daily to affected areas as well as an emollient was given to be used as needed. A 9% oxifulvic acid had already been proven to be safe and effective in adult volunteers with intradermally elicited hypersensitivity reactions (Snyman *et al.* 2002). The emollient used was Epizone A® (red sticker) (Van Dyk Pharmaceutical Products, 63 Banfield Rd, Industria North, Johannesburg, South Africa, 1709).

If the condition of the atopic dermatitis deteriorated or if the study drug had no effect on the atopic dermatitis the clinician could prescribe a rescue medication in the form of a topical corticosteroid of the participants choice, but instructed the participant that it should be used in addition to the study drug, sequentially, not simultaneously, allowing at least an hour between the application of topical corticosteroid and the study drug.

Outcomes

Volunteers entered a 4 week treatment period. During the treatment period safety and efficacy was assessed at randomisation and after 4 weeks of treatment. Treatment period observations and measurements included improvement of itch (VAS), and Investigators Global Assessment.

The investigator assessed the following: severity, erythema, vesiculation, fissuring and scaling. A standard severity grading scale was used. (0 = absent, 1 = mild, 2 = moderate, 3 = moderately severe, 4 = severe)

The investigator also assessed global response to treatment using the following scale: 0 = completely clear except for possible residual hyperpigmentation; 1 = almost clear, very significant clearance (about 90%); 2 = marked improvement, significant improvement

(about 75%); 3 = moderate improvement (< “marked” and > “severe”),(about 50%); 4 = slight improvement, some improvement (about 25%), but significant disease remaining; 5 = no change (moderate to severe disease); 6 = worse.

Safety was assessed through analysis of the documentation recording adverse events.

A screening period of 2 weeks allowed the washout of prohibited medication if patients were otherwise eligible.

Throughout this study, the whole-body Investigators Global Assessment (IGA) score was based on all lesions that exist at baseline. Newly occurring lesions after baseline screening were not included in this score, although they were treated with the same medication as the rest of the lesions.

Blood samples were taken at baseline and again at the end of the study and included a full blood count, liver and kidney function tests for assessing any changes that could indicate safety concerns. A clinical examination and ECG were also done at baseline and at the end of the study to confirm safety.

The participants were required to apply the test CHD-FA (blue sticker) or the placebo twice per day but also received an emollient (red sticker) to be used as required.

Inclusion criteria

Otherwise healthy males or females aged >2 years

Female patients using reliable contraception if of childbearing potential.

Previous confirmed diagnosis of mild to moderate atopic dermatitis

Exclusion criteria

Renal impairment

Liver disease or abnormal liver functions

Haematological abnormalities

Any disease that will limit lifespan to less than a year

Use of concomitant medication

Any autoimmune disease other than atopic dermatitis

Malignancies

Pregnant or lactating females

Patient taking part in any other clinical trial within the last month

Clinical signs of any infection at atopic areas

Concomitant medication or therapy

Excluded:

- Systemic corticosteroids
- Phototherapy / immune suppression / antihistamines
- Topical tacrolimus / pimecrolimus within last 4 weeks
- Topical therapy: corticosteroids within last 4 days

Statistical analysis

The study enrolled at least 20 patients per arm to have a 90% power of detecting 20% difference in regimens. A biostatistician was consulted and the results for the blood parameters, VAS and Investigators Assessment of Severity of Disease were analysed using A Wilcoxon rank test for each of the parameters at 95% confidence level. The Investigator's Assessments of Global Response to Treatment were analysed using Mann-Whitney test for non-parametric data. All analysis was done on Graphpad Prism 4.0.

Study outline

Visit 1: Screening

Discussions and signing of informed consent.

Assessment of disease status and confirmation of diagnosis.

Assessing inclusion and exclusion criteria.

Assessing concomitant medication use.

Completion of medical history.

Visit 2: Randomization

- Reassess inclusion and exclusion criteria.
- Assess adverse events.
- Physical examination.
- Baseline blood parameter assessment: Urea, uric acid, creatinine, Cl⁻, K⁺, Na⁺, γGT, Alkaline phosphatase, AST and ALT and a full blood count (differential and morphology).

- ECG
- Investigators Assessment of Severity of Disease (as described above)
- Patient completion of VAS
- Dispensing and application demonstration of medication

Visit 3: End of study: return after 4 weeks of treatment

- Assess adverse events
- Physical examination
- Study endpoint blood parameter assessment: Urea, uric acid, creatinine, Cl⁻, K⁺, Na⁺, γ GT, Alkaline phosphatase, AST and ALT and a full blood count (differential and morphology).
- ECG
- Investigators assessment of severity of disease (as described above)
- Patient completion of VAS
- Collecting of unused medication

4.5 RESULTS

Patients

36 patients completed the trial with a relatively even distribution with regards to male/female age and disease severity; two patients used concomitant medication during the trial and were excluded from the results.

Safety

Kidney function tests

No significant differences were observed in either of the groups. (Table 4.1)

Table 4.1: Mean kidney function results for both baseline and final of placebo vs CHD-FA respectively.

	Placebo				CHD-FA			
	Base	±SD	Final	±SD	Base	±SD	Final	±SD
Sodium (mmol/l)	139.94	1.34	140.33	1.78	139.79	2.51	139.28	2.56
Potassium (mmol/l)	4.37	0.41	4.57	0.36	4.26	0.24	4.42	0.49
Chloride (mmol/l)	104.24	2.88	104.72	2.27	105.11	3.02	105.17	2.26
Urea (mmol/l)	4.84	0.07	5.36	1.60	4.55	1.18	4.49	2.26
Creatinine (µmol/l)	80.06	13.84	84.94	15.60	74.37	18.75	73.61	19.25
Urate (mmol/l)	0.30	0.07	0.32	0.06	0.32	0.11	0.33	0.12

Liver function test

With statistical analysis, significant differences were observed for AST. The rest of the parameters remained within baseline limits. (Table 4.2)

Table 4.2: Mean liver function results for both baseline and final of placebo vs CHD-FA respectively.

	Placebo				CHD-FA			
	Base	±SD	Final	±SD	Base	±SD	Final	±SD
Alkaline Phosphatase (U/l)	68.18	18.09	61.79	25.40	76.63	35.59	81.50	45.90
Gamma GT (U/l)	30.41	29.94	29.44	23.15	22.37	16.06	22.94	15.28
ALT (U/l)	24.59	25.44	26.72	21.64	18.79	9.41	23.94	18.45
AST (U/l)	29.18	26.42	20.61	7.04	20.58	5.19	*24.67	9.04

*Indicating statistical significance ($p < 0.05$)

Full blood count

All safety parameters remained within normal limits. There were no significant differences in either group. (Table 4.3)

Table 4.3: Mean full blood count results for both baseline and final of placebo vs CHD-FA respectively.

	Placebo				CHD-FA			
	Base	±SD	Final	±SD	Base	±SD	Final	±SD
Haemoglobin (g/dl)	14.36	1.40	14.41	1.44	14.50	2.20	14.29	2.15
Red cell count ($10^{12}/l$)	4.86	0.47	4.89	0.55	5.09	0.61	4.99	0.63
Haematocrit (%)	42.21	3.74	42.29	4.38	43.24	5.67	42.74	5.81
White cell count ($10^9/l$)	6.80	2.72	6.47	1.47	6.60	1.45	6.83	1.82
Neutrophils Abs ($10^9/l$)	4.41	2.60	4.02	1.18	3.80	1.23	3.98	1.67
Lymphocytes Abs ($10^9/l$)	1.58	0.52	1.68	0.62	2.03	0.69	2.01	0.55
Monocytes Abs ($10^9/l$)	0.42	0.12	0.40	0.12	0.41	0.14	0.46	0.27
Eosinophils Abs ($10^9/l$)	0.22	0.16	0.23	0.17	0.20	0.16	0.23	0.15
Basophils Abs ($10^9/l$)	0.53	0.25	0.44	0.26	0.39	0.22	0.42	0.28
LUC Abs ($10^9/l$)	0.14	0.07	0.12	0.04	0.14	0.06	0.14	0.04
Platelet ($10^9/l$)	263.12	73.81	244.18	84.51	283.42	61.49	285.11	72.11

Side effects

The only side effect that was reported was a short burning sensation after the application of the treatment. All blood parameters remained within acceptable ranges.

Efficacy

Investigators assessment of severity of disease

Severity, erythema, vesiculation, fissuring and scaling were examined by the investigators. Significant differences were observed for both severity and erythema in both placebo as well as CHD-FA treated groups. A significant difference was observed in the scaling observed in the placebo treated group. The rest of the parameters remained within baseline limits. (Table 4.4)

Table 4.4: Mean Investigators Assessment of Severity of Disease results for both baseline and final of placebo vs CHD-FA respectively.

	Placebo (n=18)				CHD-FA (n=18)			
	Base	±SD	Final	±SD	Base	±SD	Final	±SD
Severity	2.278	0.826	*1.222	0.732	1.833	0.514	¹ 0.833	0.707
Erythema	2.056	0.998	**1.056	0.873	1.176	0.728	² 0.235	0.437
Vesiculation	0.333	0.686	0.056	0.236	0.353	0.493	0.000	0.000
Fissuring	0.056	0.236	0.056	0.236	0.000	0.000	0.000	0.000
Scaling	0.500	0.707	***0.056	0.236	0.176	0.529	0.059	0.243

(0 = absent, 1 = mild, 2 = moderate, 3 = moderately severe, 4 = severe)

* p=0.0009 (Wilcoxon signed rank test)

** p=0.0059 (Wilcoxon signed rank test)

*** p=0.0313 (Wilcoxon signed rank test)

1 p<0.0001 (Wilcoxon signed rank test)

2 p=0.0002 (Wilcoxon signed rank test)

Investigators Assessment of Global Response to Treatment

Investigator assessment of global response to treatment used the following 7 point scale: 0 = completely clear, 1 = almost clear (about 90%), 2 = marked improvement (75%), 3 = moderate improvement (50%), 4 = slight improvement (25%), 5 = no change (moderate to severe disease) and 6 = worst. (Barbier *et al.* 2004) A significant improvement was observed in the CHD-FA group when compared to the placebo group. (Table 4.5)

Table 4.5: Mean Investigators assessment of global response to treatment results for both baseline and final of placebo vs CHD-FA respectively.

	Scale response (0-6)	
	Average	±SD
Placebo (n=18)	2.94	1.11
CHD-FA (n=18)	*1.77	1.00

*Indicating statistical significance (p < 0.05)

0 = completely clear except for possible residual hyperpigmentation

1 = almost clear, very significant clearance (about 90%)

2 = marked improvement, significant improvement (about 75%)

3 = moderate improvement (about 50%)

4 = slight improvement (about 25%), but significant disease remaining

5 = no change (moderate to severe disease)

6 = worse

Visual Analogue Scale (VAS)

With statistical analysis, a significant decrease was observed in both groups, when comparing the baseline with the final results. One patient in the CHD-FA group failed to complete the VAS at baseline and thus no comparator to be completed. (Table 4.6)

Table 4.6: Mean Visual analogue Scale (VAS) results for both baseline and final of placebo vs CHD-FA respectively.

	% Scale response (0-100mm)			
	Base	±SD	Final	±SD
Placebo (n=18)	62.81	20.89	*34.72	26.81
CHD-FA (n=17)	69.50	14.65	**29.60	19.71

CHD-FA- Carbohydrate derived Fulvic Acid

* p=0.003 (Wilcoxon signed rank test)

** p=0.0005 (Wilcoxon signed rank test)

4.6 DISCUSSION

Safety

All blood parameters remained within acceptable ranges and were close to the baseline levels in all cases. The differences observed in the AST levels in the CHD-FA group may be due to the natural variation between and within patients but could be due to the relatively small sample size. They still remain well within normal limits/ranges.

With atopic dermatitis the skin is already exposed, sensitive and in an inflammatory state thus the burning sensation that was reported could be due to the relatively low pH of the treatment being applied to this already irritated site.

Extensive clinical safety parameters have already been established for systemically administered CHD-FA (Gandy *et al.*, 2012). This study has thus further established that changing the route of administration from systemic to topical where the CHD-FA is applied directly to the area of inflammation did not affect the safety profile of CHD-FA tested.

Efficacy

Investigators Assessment of Severity of Disease

A standard 5 point severity grading scale (0 = absent to 4 = severe) was used to evaluate five characteristics of atopic dermatitis; Severity, Erythema, Vesiculation, Fissuring, Scaling (Faghihi *et al.*, 2008). Both groups showing significant improvements in some of the characteristics tested which is a characteristic of atopic dermatitis where application of an emollient or a pH adjusted substance (4.8) may improve the condition.

Investigators Assessment of Global Response to Treatment

With the investigators assessment of global response to treatment, the group treated with CHD-FA showed a significant improvement when compared to the placebo treated group, thus demonstrating the anti-inflammatory properties of CHD-FA, leading to a significant overall improvement of the disease.

Visual analogue Scale (VAS)

The severity of itching was evaluated via a visual analogue scale. VAS was defined on a 10cm line in that point 0 refers to clear allergic dermatitis and point 10 refers to the most severe allergic dermatitis (Faghihi *et al.*, 2008).

A significant decrease was observed for both groups, thus indicating that both treatments alleviated the patients' perception of their itching which is an indication of disease severity. However a greater and more significant alleviation of symptoms experienced was observed by the CHD-FA group, thus indicating this to be more effective treatment.

These findings tie in well with two separate studies on two different fulvic-acid products, one derived from bituminous coal and the other from a carbohydrate source (CHD-FA), it was found that fulvic acid is safe and effective in the reduction of a contact hypersensitivity reaction in rats when applied topically (Van Rensburg *et al.*, 2001; Sabi *et al.*, 2011).

4.7 CONCLUSION

In reviewing the currently available highest level of evidence it appears that a good understanding of the use of non-pharmacologic and pharmacologic topical therapies for AD is in place but there is still much to be learned. Significant gaps in research have been identified, including controlled studies to define the frequency, duration, and the effects of bathing and use of bath emollients; well-designed, large trials to better test the effects of topical antimicrobial agents other combinations; and studies to determine long-term safety and efficacy of some treatments that show promise but use potent immune suppressant drugs. This recognises that there are gaps in research on how to further optimize the use of topical therapeutic options.

This study highlighted that in most cases participants condition improved significantly irrespective of which treatment group they were assigned to, which is a previously reported characteristic observed in many atopic dermatitis related trials. Certain parameters showed significant or more significant improvements in the group treated with the CHD-FA when compared to the placebo, thus proving that CHD-FA, when applied topically can elicit anti-inflammatory effects.

Although the mechanism of action of the CHD-FA is unknown this study has provided evidence that there are improvements in the status of AD which is notoriously difficult to treat. This provides some support that further targeted investigations should be undertaken to compare CHD-FA as an alternative to the current standard therapies that tend to treat the symptoms only and not the underlying cause, which has proved to be costly, both financially and emotionally to those that suffer from AD.

CHD-FA proved to be safe and efficacious alternative therapeutic option when used topically in the treatment of atopic dermatitis.

CHAPTER 5: LC-MS/MS INVESTIGATION OF CHD-FA BIOMARKERS IN PLASMA

5.1 INTRODUCTION

CHD-FA is a complex mixture consisting mainly of acidic organic compounds derived from a defined carbohydrate origin, having mostly an acidic and supramolecular character. Although CHD-FA has been suggested as a nutritional supplement and even a medication there is currently no data available with regards to the systemic kinetics of CHD-FA when ingested orally. The observed physiological or therapeutic effects of CHD-FA could be due to either a direct binding to a target zone or indirect by changing some control point that results in a knock-on effect.

The current hypothesis is that due to the acidic and ionic nature of CHD-FA, it is likely to be highly protein bound after oral absorption. This study was conducted in order to determine whether or not an unbound CHD-FA derived plasma soluble analyte exists that could be used to determine the *in vivo* kinetics of orally administered CHD-FA.

LC-MS/MS was used in order to conduct comparative analyses, where initially the parent masses of ionisable compounds in stock solutions of CHD-FA were identified. To confirm the applicability of the identified compounds, multiple reaction monitoring (MRM) was performed, which is a mass spectrometer based technique where specifically selected precursor mass compounds were fragmented within the MS/MS and any uniquely charged product fragments monitored to characterise the precursor compound.

This type of assay is widely used in the pharmaceutical industry in order to detect very low concentrations of drugs and their metabolites (Kitteringham *et al.* 2009) in complex matrices due to the elimination of most of the background interferences. This technique was used in order to obtain the maximum sensitivity combined with selectivity for detection of any unique marker compounds in the CHD-FA and in combinations of CHD-FA and plasma from healthy volunteers.

5.2 MATERIALS AND METHODS

Chemicals and reagents

CHD-FA was obtained from Fulhold, Stellenbosch, as a 3.8% solution.

Human plasma from healthy unexposed volunteers

Baseline and 1 hour post CHD-FA oral ingestion human plasma from a clinical trial where safety and efficacy of CHD-FA was established.

The following reagents were of HPLC or MS grade solvents or reagents obtained from Rathburn, Sigma Aldrich or Merck:

Methanol.

Formic acid.

Ammonium hydroxide.

Double deionised H₂O.

Acetonitrile.

Acetone

Preparation of stock solution

3.8% CHD-FA solution as supplied from manufacturer was diluted in methanol, H₂O and plasma on the day of analysis, in order to prepare three separate working stock solutions. These dilutions were prepared in a range of ratios ranging from 5:1 to 1:2, CHD-FA to respective diluent.

Sample preparation

On day of analysis, each working stock solution was prepared; vortex mixed for 1 minute, and allowed to stand for 10 minutes in order to permit any precipitation reaction to take place. Subsequently each sample was centrifuged at 16 000 times gravity for 10 minutes at room temperature. The resultant supernatant was transferred to tapered glass autosampler vial inserts for LC-MS/MS analysis.

LC-MS/MS conditions

Equipment

For LC separation a triple quadrupole LC-MS/MS system consisting of Agilent 1100 series autosampler, binary pump, degasser and column oven, was coupled to a AB Sciex 4000 QTrap mass spectrometer with a Turbo “V” electrospray ionisation (ESI) (Applied Biosystems/MDS Sciex, Concord, Canada). The complete system was centrally controlled and data collected and analysed using Analyst Software, version 1.5.2 (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada).

HPLC conditions

Samples (10 µl injection volume) were separated on a Discovery ZR-PBD column (Supelco), with dimensions of 150 x 4.6 mm, with 5 µm particle size, with a guard ZR-PBD guard cartridge with dimensions 4 x 3 mm, with a 100 µm particle size (Supelco). The gradient mobile phase consisted of (A) 0.1% formic acid in H₂O and (B) 54% acetonitrile, 40% methanol and 6% aqueous 0.1% ammonium formate buffer, adjusted to a pH of 7.5. Flow-rate was 1.0 mL/min. The gradient started at 100% A for the first 0.5 minutes after which B was increased to 100% over 7.5 minutes, held at 100% B for 7 minutes and returned to 100% A over 2 minutes. A three minute re-equilibration time was allowed before the next injection. The eluted analytes were directly infused into the ESI source of the mass spectrometer where they were assayed under the optimal conditions predetermined during initial infusion experiments without chromatographic separation. The autosampler tray was set to 10°C to ensure on-autosampler sample stability. The gradient is summarised in Table 5.1.

Table 5.1: Mobile phase gradient for separation of CHD-FA by HPLC.

Total Time (min)	Flow Rate (µl/min)	A(%)	B(%)
0.00	1000	100.0	0.0
0.50	1000	100.0	0.0
8.00	1000	0.0	100.0
15.00	1000	0.0	100.0
17.00	1000	100.0	0.00
20.00	1000	100.0	0.00

Mass spectrometric conditions

In order to operate optimally, tuning was initially performed to select the best combination of ionisation parameters as well as precursor and product ion combinations for the fragmentation products. A solution of roughly 1 µg/mL CHD-FA was directly infused into the ESI source at a flow rate of 10 µl/min using a Harvard syringe drive pump. Experiments were performed in both positive and negative ionisation mode to determine the most abundant and unique ions. The resulting mass spectra were acquired in full scan mode from m/z 70 to 1000 and detected at a rate of 1 scan every 600 msec.

A drying gas temperature of 450 °C, ion spray voltage of +4500V for positive mode and -4200V for negative mode, a curtain gas setting of 23.0 (for both pos/neg) and an entrance potential of (pos) 10.0 Volts was used for optimization. The most abundant parent ions were selected from Q_1 scans, and the optimal declustering potential (DP) and collision energy (CE) determined for each of these ions. Fragmentation parameters to form daughter ions were optimised using peak intensities of the daughter ions at Q_3 during product ion assay.

Due to the complexity of the sample, an alternative method to characterise major components of the CHD-FA was attempted. This technique made use of mass/time “contour plots” of the chromatographic separations that were continuously analysed to determine the retention time of unique and abundant ions and were performed in both the positive and negative ionisation modes. The mass range scanned during these analyses was from 70 – 800 m/z .

Crude fractionation

Crude fractionation was achieved by building a silica column using a glass Pasteur pipette and loading it with dry silica, using cotton wool stoppers at either end as plugs. The silica was then conditioned using acetone.

An aliquot of 1 mL of 1/50 solvent diluted CHD-FA was then applied to the column and eluted using 2 mL of the following pure solvent respectively and in a sequential fashion, namely, acetone, acetonitrile, methanol, methanol/water 50:50 and ammonium hydroxide. Each of these eluted fractions was transferred to tapered glass autosampler vial inserts for LC-MS/MS analysis, which was performed in both positive and negative ionisation modes.

5.3 RESULTS

The data was collected as precursor total ion chromatograms (TIC) from which the m/z data was extracted to give extracted ion chromatograms (XIC) at different times or converted to a contour plot mode where all ions at all elution times were plotted onto a two dimensional matrix with intensity shown in varying colours relative to ion intensity.

This was done in both positive and negative ionisation modes and representative TICs with selected extraction data are illustrated in the following figures. Figures 5.1, 5.2 and 5.3 illustrate TICs with selected extracted ion chromatograms (XIC) at different times where ion intensities were high. Figures 5.4 and 5.5 are of the same sample taken from an un-treated patient shown as a TIC (Figure 5.4) and an averaged XIC (Figure 5.5) over the full chromatographic time period to identify the most abundant ions eluting at any time during the chromatographic separation.

Figures 5.6 and 5.7 are the same combination of graphs as in Figures 5.4 and 5.5 except the sample in this case was the diluted CHD-FA alone. Figure 5.8 is a typical contour plot of the same sample used in Figures 5.6 and 5.7. Figures 5.9 and 5.10 are equivalent to Figures 5.4 and 5.5 except that the sample is from a treated CHD-FA patient taken 1 hour after treatment. Figure 5.11 is the equivalent contour plot.

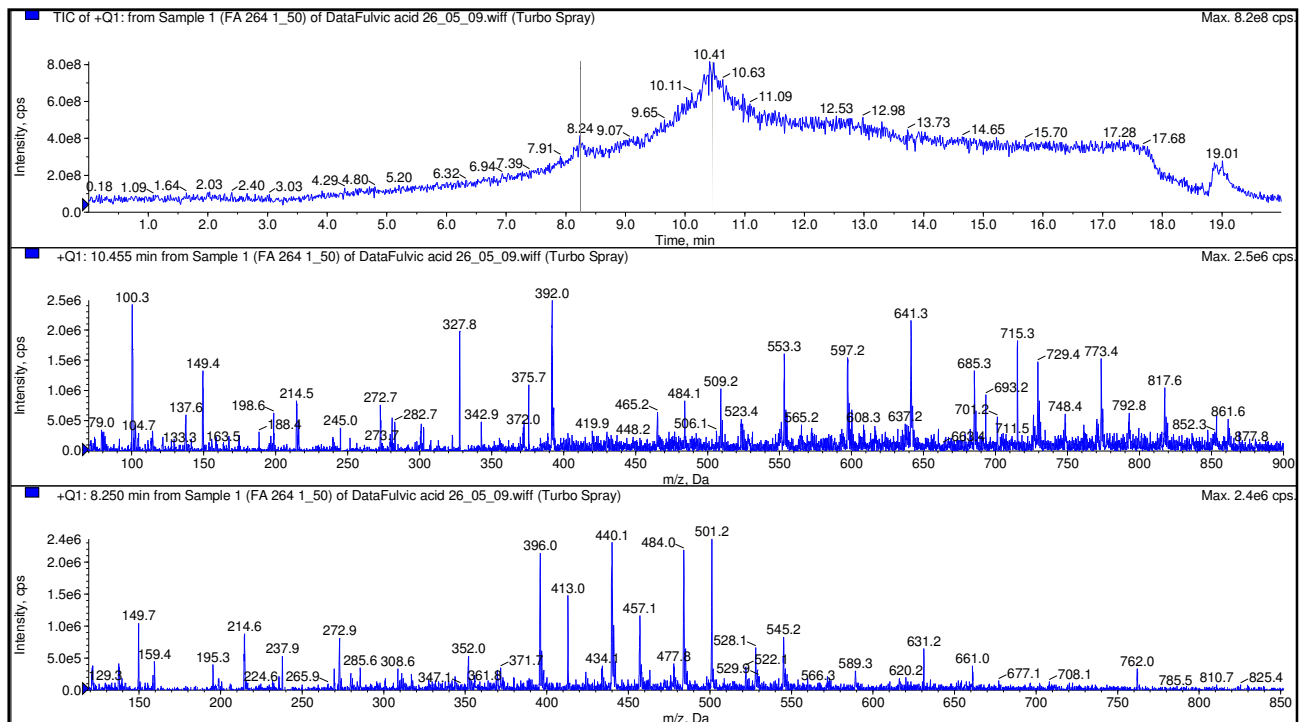


Figure 5.1: Combined TIC with XIC LC-MS/MS analysis of a 1:500 dilution of pure CHD-FA in water analysed in positive ionisation mode.

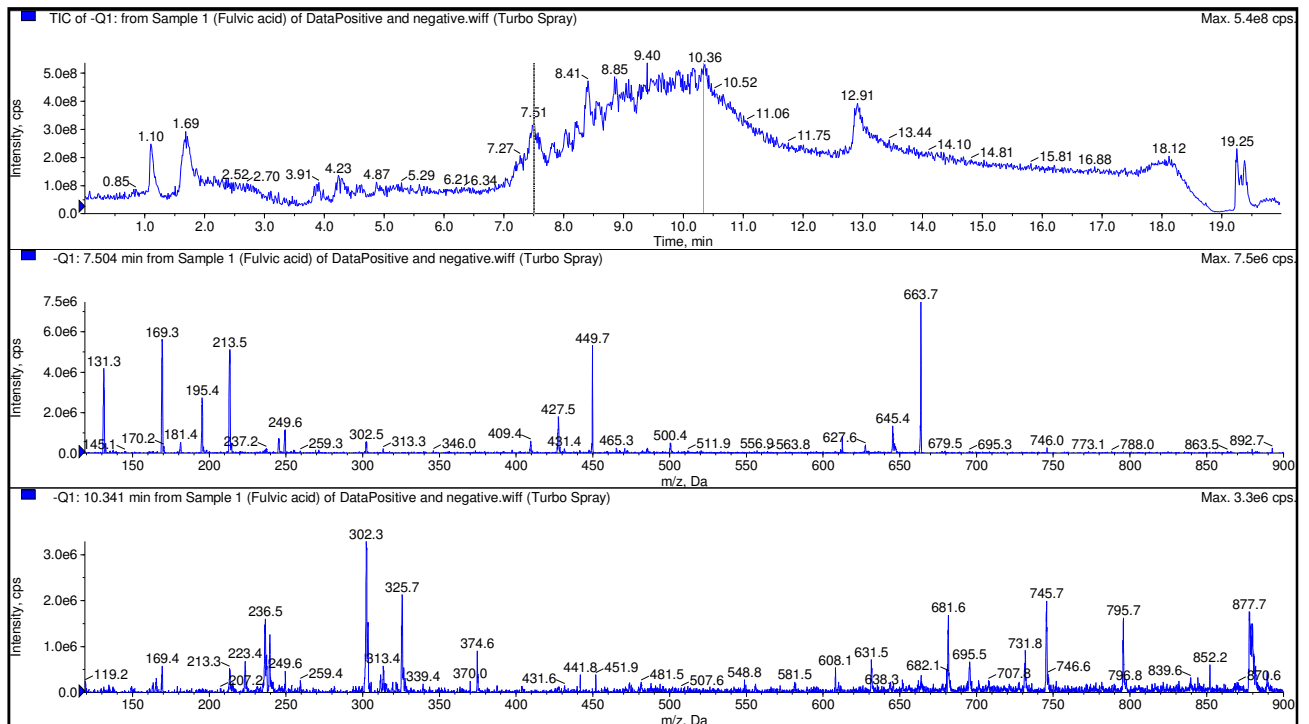


Figure 5.2: Combined TIC with XIC LC-MS/MS analysis of a 1:500 dilution of pure CHD-FA analysed in negative ionisation mode.

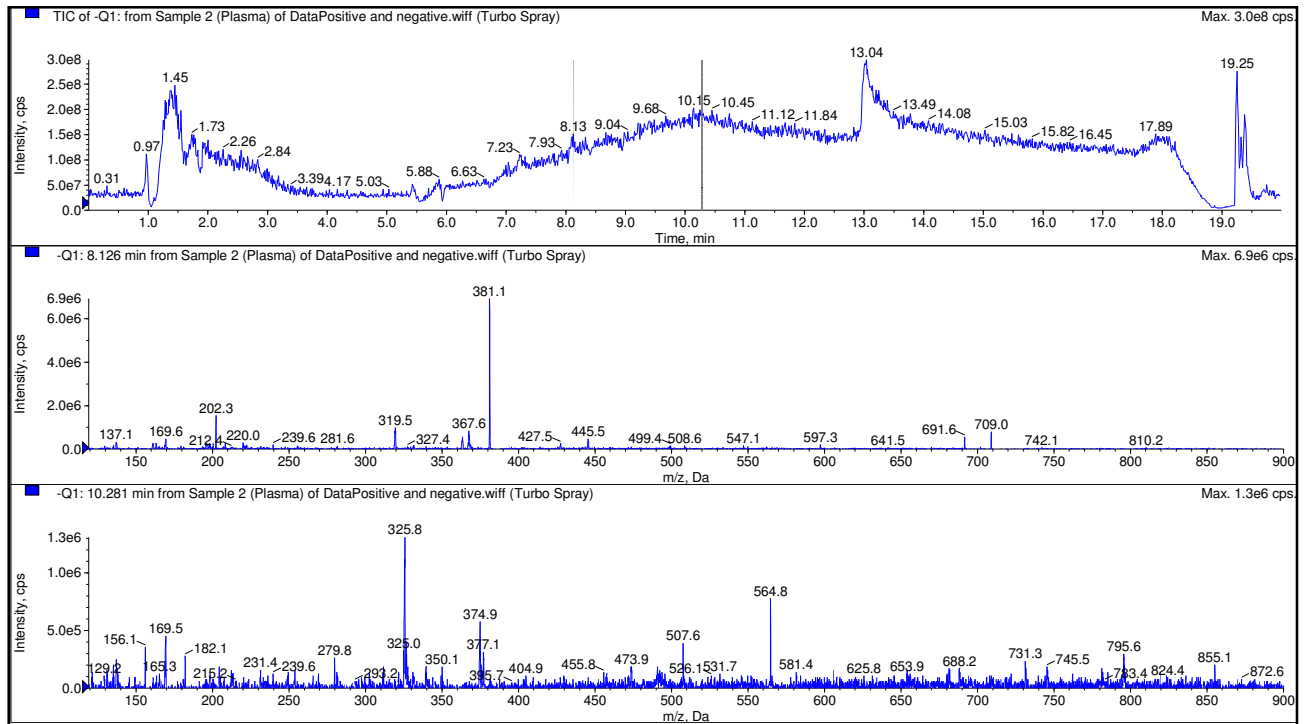


Figure 5.3: Combined TIC with XIC LC-MS/MS analysis of a 1/5000 dilution of human plasma analysed in negative ionisation mode.

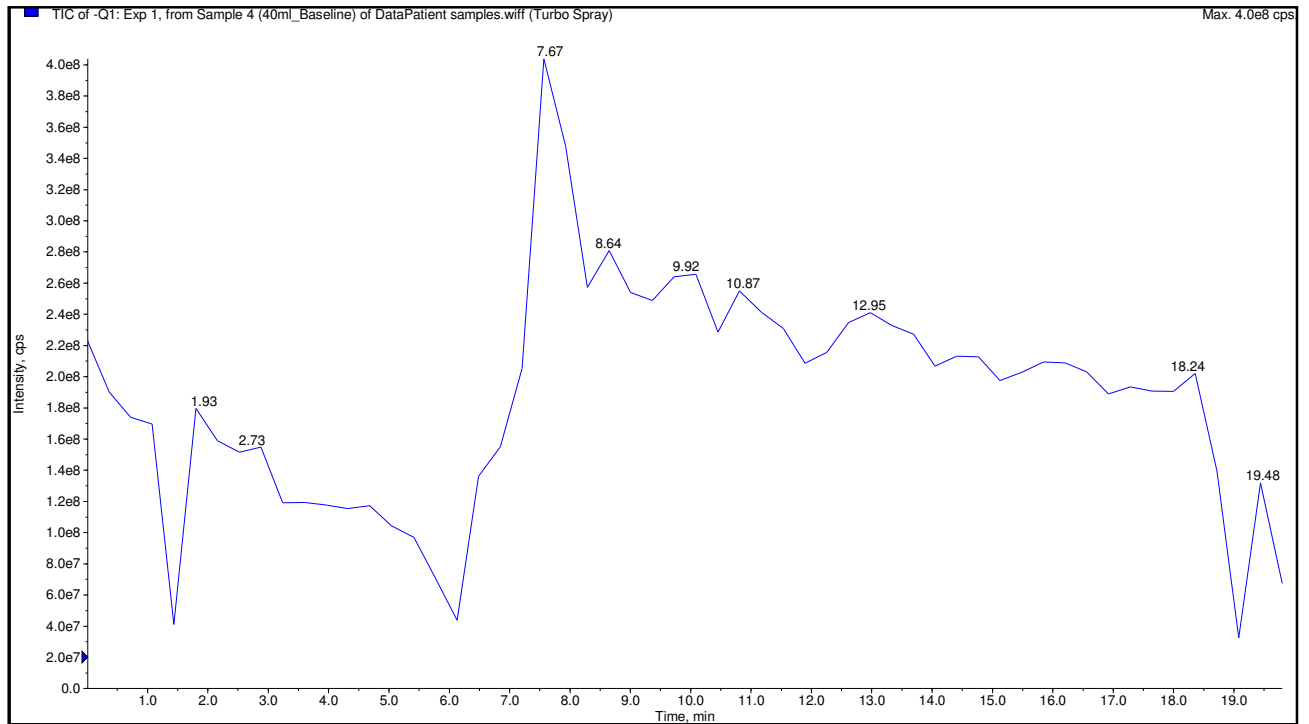


Figure 5.4: TIC LC-MS/MS analysis of diluted patient plasma taken at baseline (pre CHD-FA treatment) analysed in negative ionisation mode.

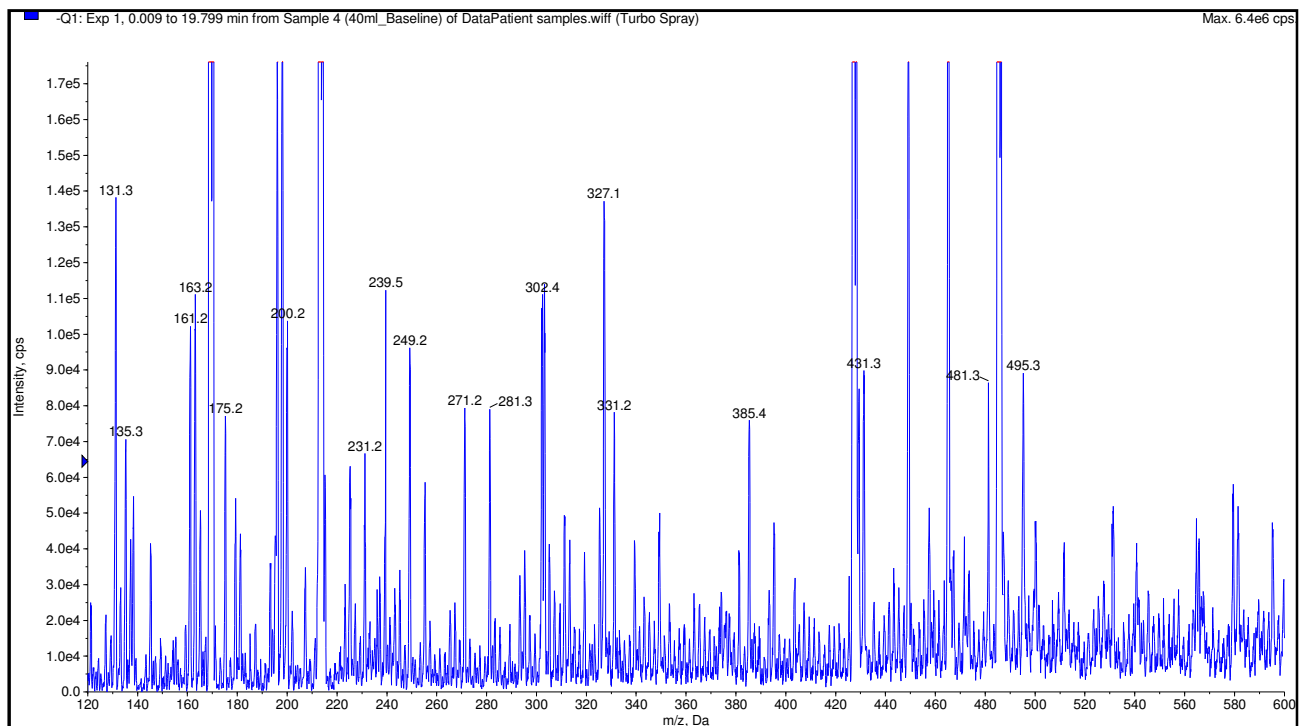


Figure 5. 5: XIC LC-MS/MS analysis of diluted patient plasma taken at baseline run in negative ionisation mode. The 169.2 and 212.2 m/z ions were related to contaminants in the water used to make up the samples.

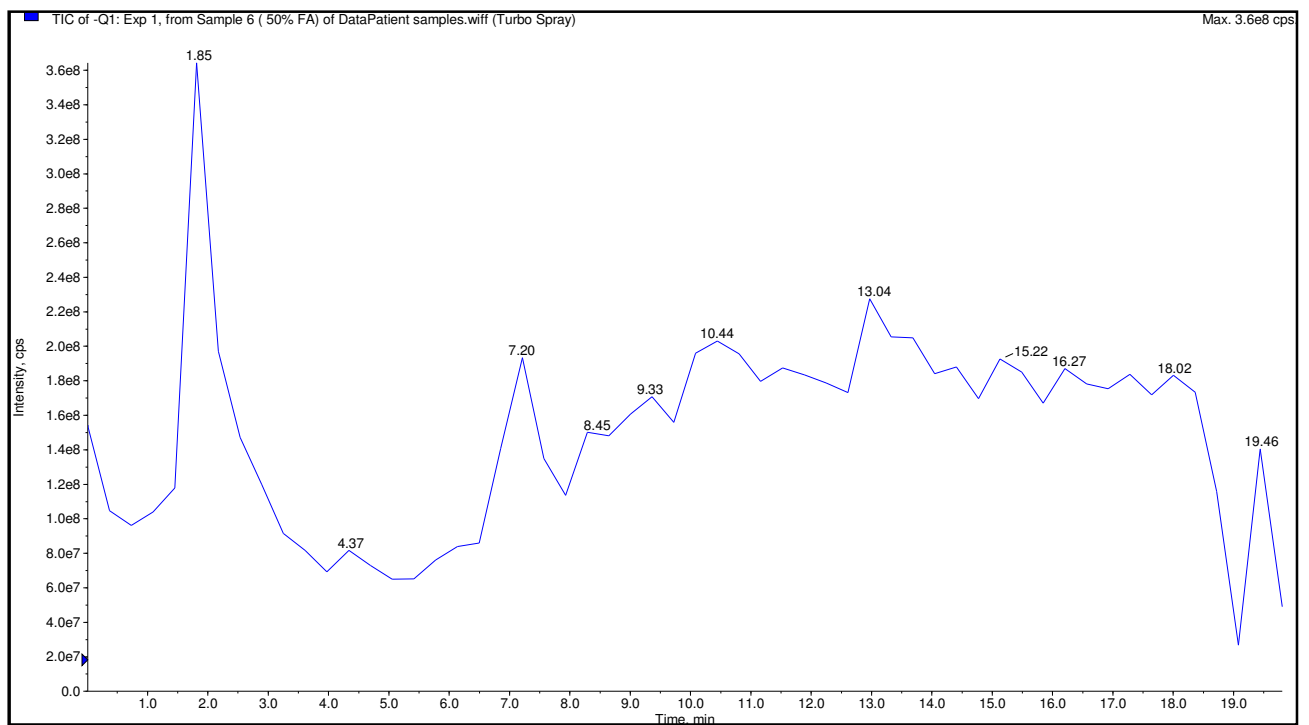


Figure 5.6: TIC LC-MS/MS analysis of 2:1 dilution of CHD-FA in water used in the clinical trial run in negative ionisation mode.

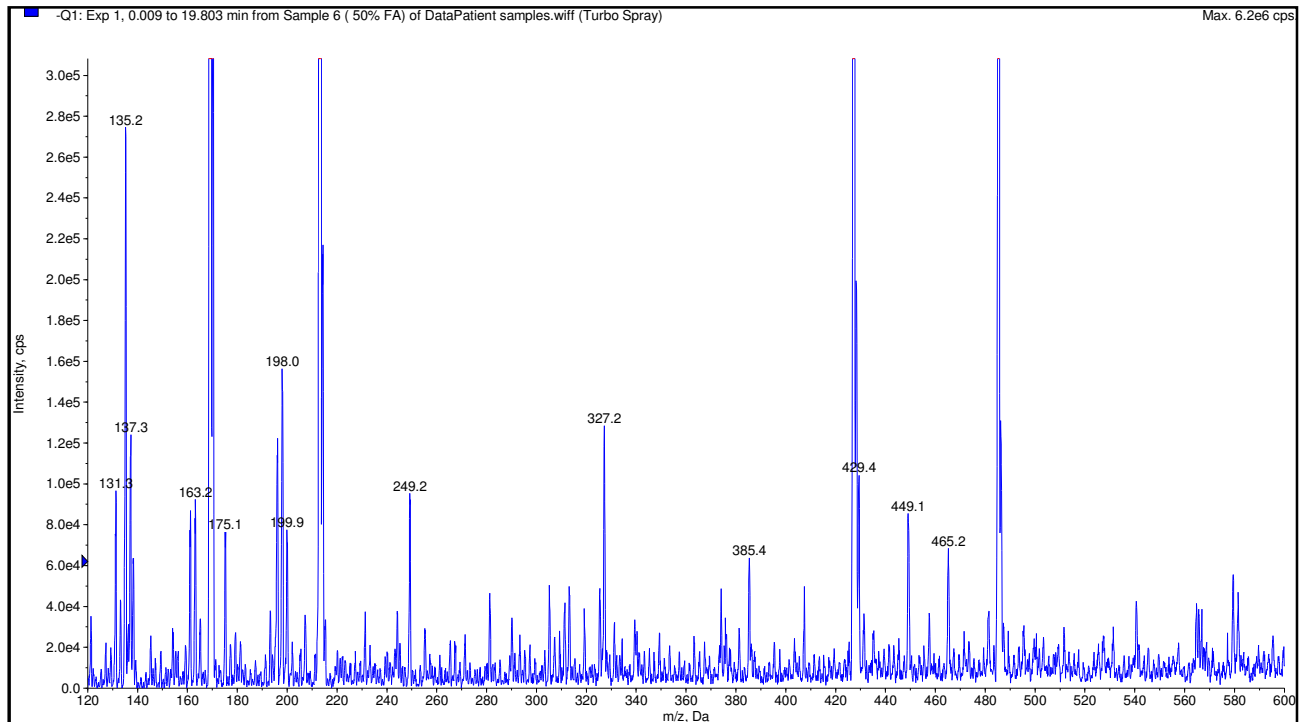


Figure 5.7: XIC LC-MS/MS analysis of 2:1 dilution in water of the CHD-FA used in the clinical trial analysed in negative ionisation mode. The 169.2 and 212.2 m/z ions were related to contaminants in the water used to make up the samples.

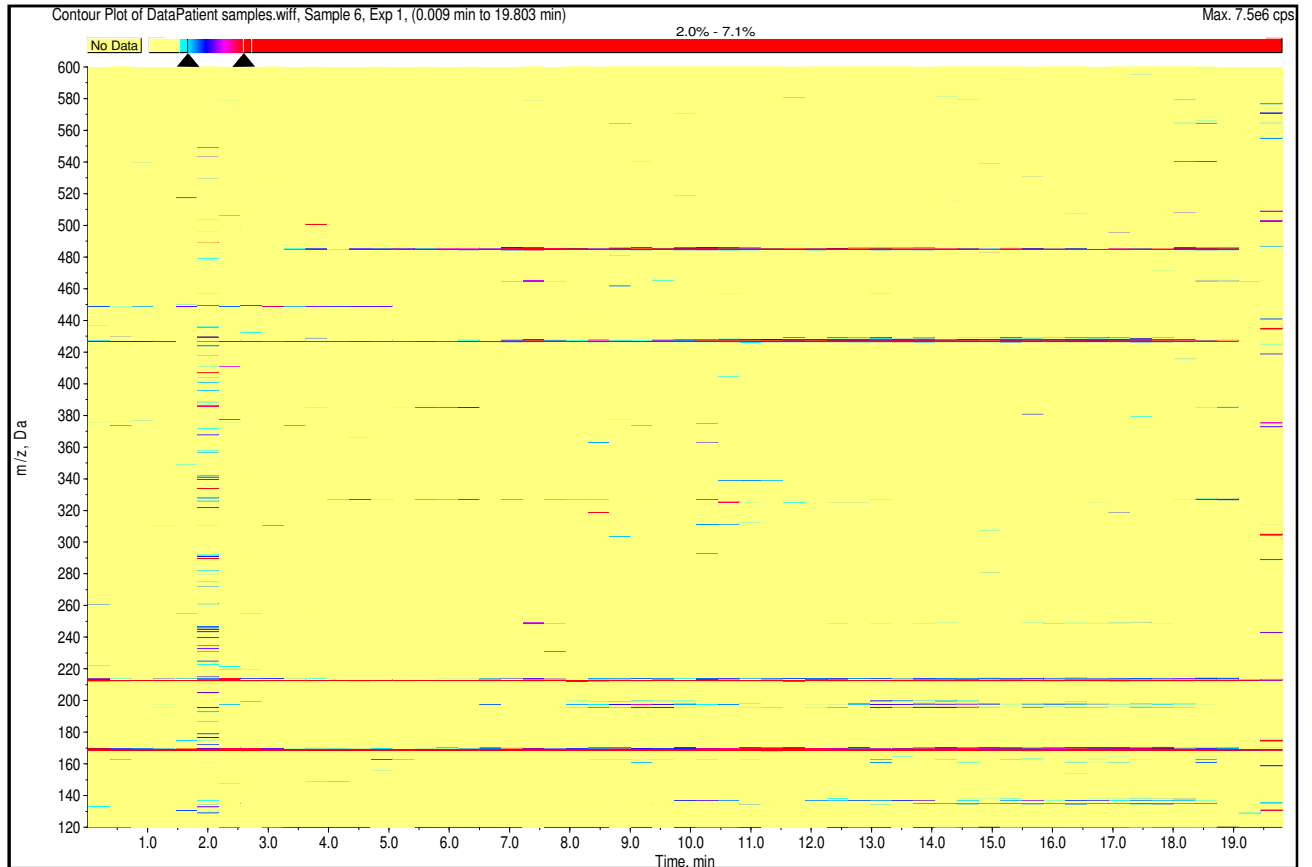


Figure 5.8: A contour plot of LC-MS/MS analysis of 2:1 dilution in water of CHD-FA used in the clinical trial analysed in negative ionisation mode. The red lines at m/z at 212 and 425.6 (the dimeric form) is due to a plasticiser found to be originating from the aqueous phase used for the sample.

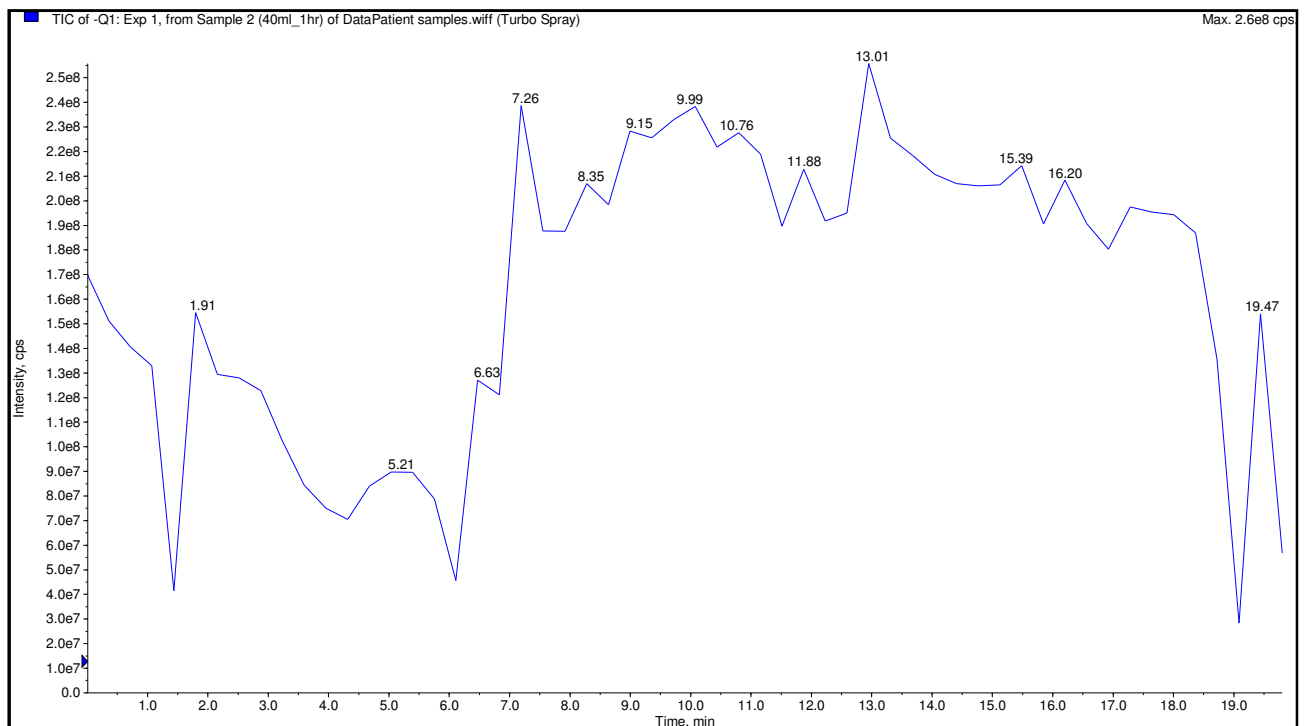


Figure 5.9: TIC LC-MS/MS analysis of patient plasma one hour post CHD-FA dosing from clinical trial analysed in negative ionisation mode.

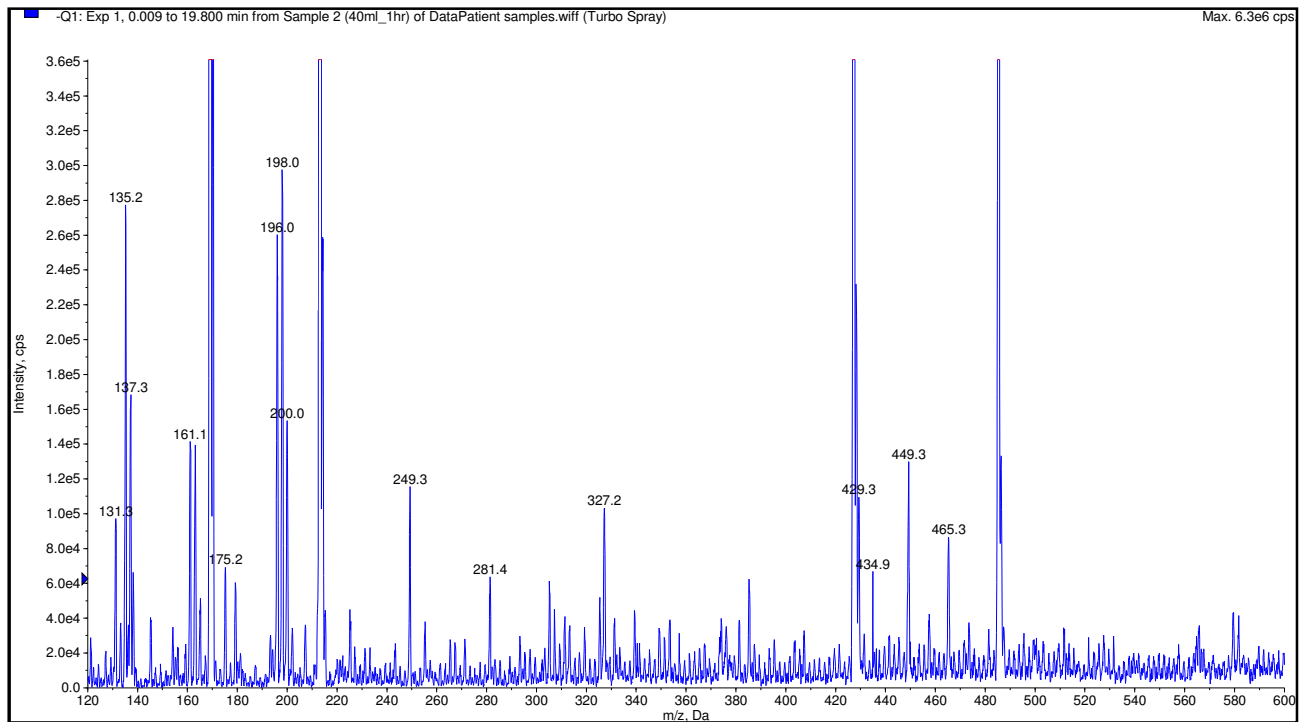


Figure 5.10: XIC LC-MS/MS analysis of patient plasma one hour post CHD-FA dosing from clinical trial analysed in negative ionisation mode. The 169.2 and 212.2 m/z ions were related to contaminants in the water used to make up the samples.

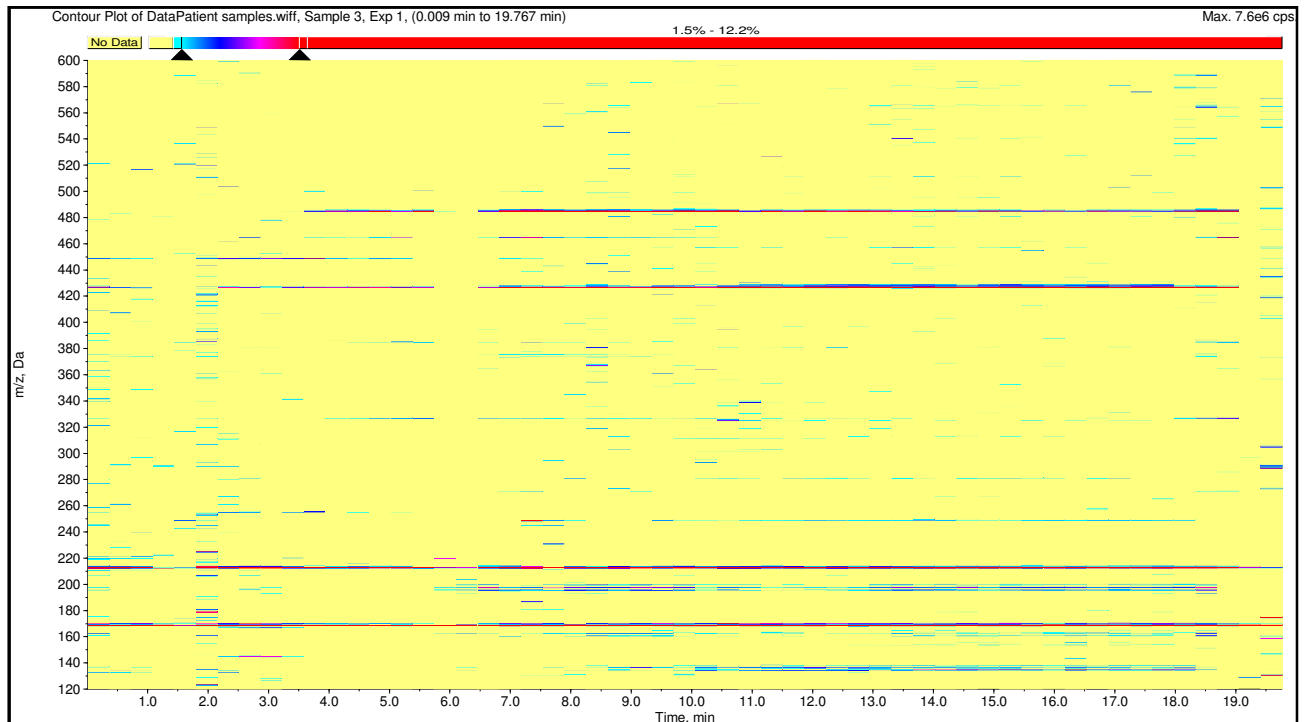


Figure 5.11: A contour plot of LC-MS/MS analysis of patient plasma one hour post CHD-FA dosing during a clinical trial and analysed by LC-MS/MS in negative ionisation mode. The ion at continuously eluting during the assay at 169.2 and 212.2 were found to be from contaminants in the water used during the sample preparation.

Figures 5.12 through 5.22 are the averaged XIC of the eluents from the short silica columns with different solvents that have been analysed using direct infusions with negative and positive ionisation mode respectively. From this data it can also be seen that the 214.4 in positive mode and the 212.2 and 169.2 peaks in negative mode are present in all samples due to the water used for dilution of the samples and tracked down to the presence of a contaminating plasticiser in the water. In some samples a dimeric form of the plasticiser was observed.

What is clearly evident is the complexity of the samples and the differences between the different solvent eluents. There appears to be an abundance of low molecular mass compounds ($< 450 m/z$) but this is unlikely and is probably due to multi-charged ions of larger compounds. In several cases where there was limited overlap of compound masses there was evidence of 2- and 3- ions due to the isotope distribution but in general the number of compounds with close mass to charge ratios overlapped which made isotope distribution shift unusable. None of the abundant masses in the samples were detected in the plasma samples from treated patients.

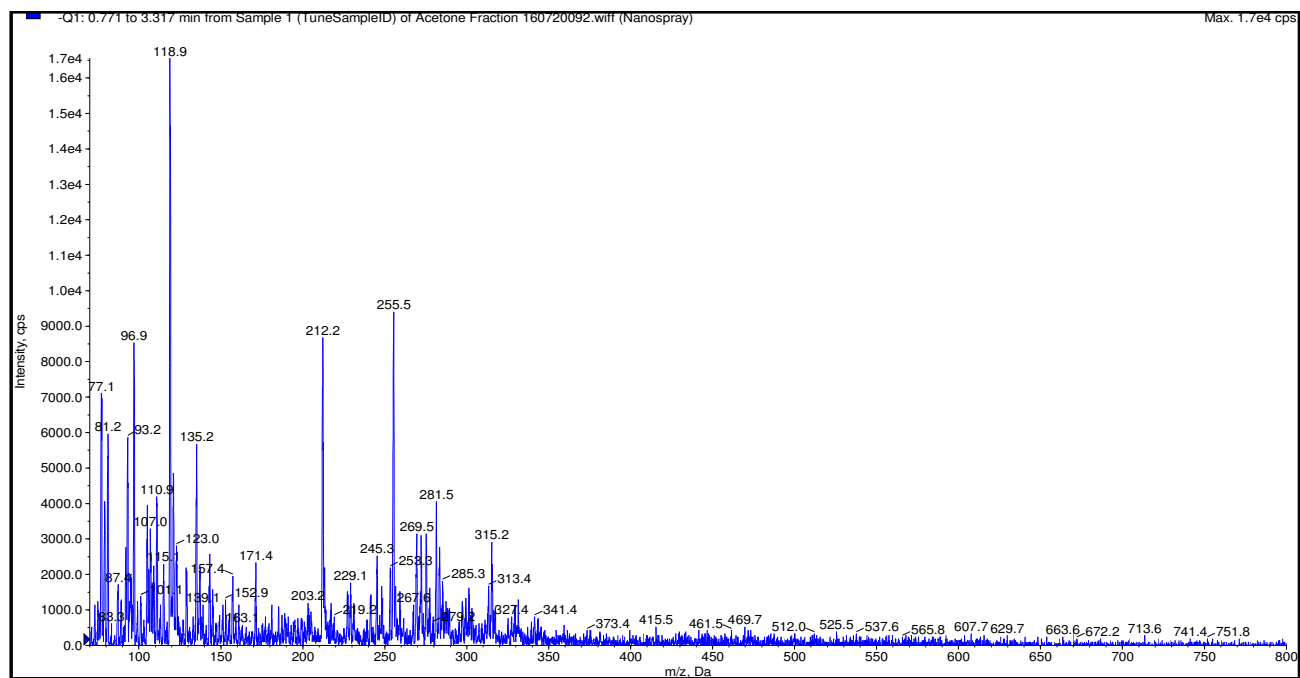


Figure 5.12: LC-MS/MS analysis of the CHD-FA crude fraction using acetone as elution solvent run in negative ionisation mode. The negative ion at 212.2 was found to be a plasticiser from the water used during the sample preparation.

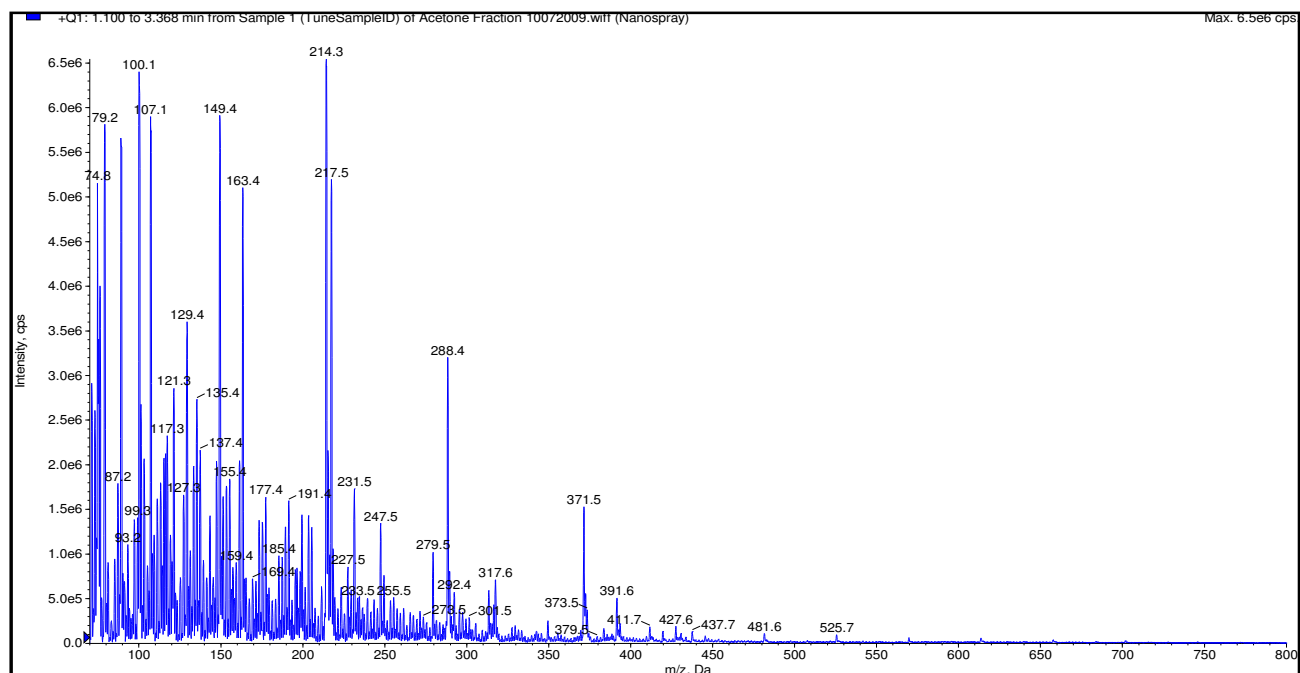


Figure 5.13: LC-MS/MS analysis of the CHD-FA crude fraction using acetone as elution solvent run in positive ionisation mode. The positive ion at 214.4 was found to be a plasticiser from the water used during the sample preparation. This is the same peak seen in negative mode ionisation at 212.4 m/z as seen in Figure 5.12.

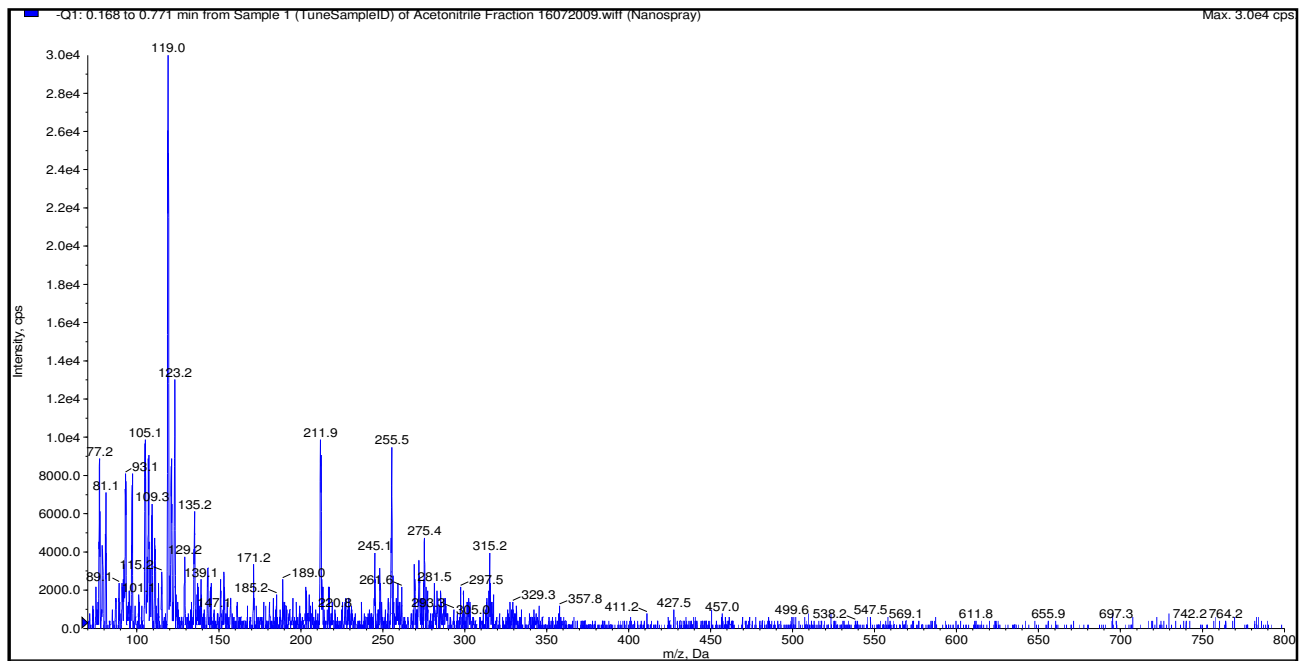


Figure 5.14: LC-MS/MS analysis of the CHD-FA first crude fraction using acetonitrile as elution solvent run in negative ionisation mode.

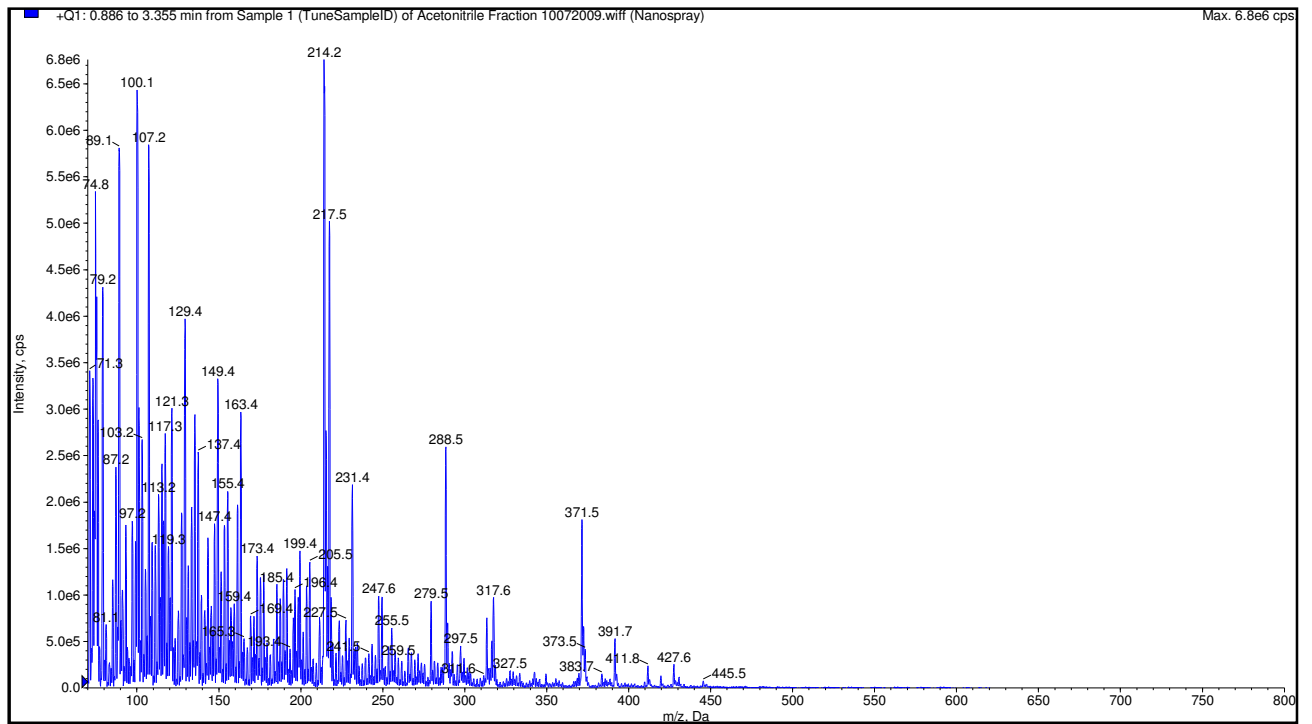


Figure 5.15: LC-MS/MS analysis of the CHD-FA first crude fraction using acetonitrile as elution solvent in positive ionisation mode. The ion at 214.4 was found to be a plasticiser from the water used during the sample preparation.

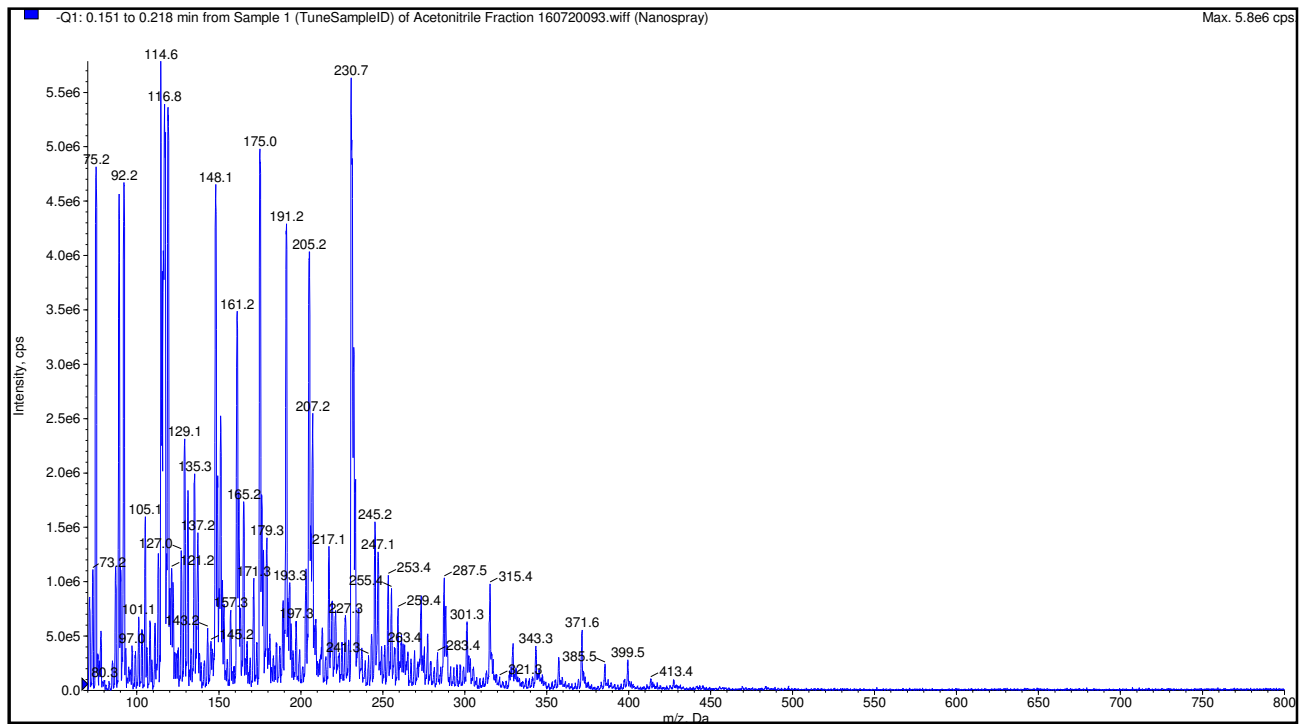


Figure 5.16: LC-MS/MS analysis of the CHD-FA second crude fraction using acetonitrile as elution solvent run in negative ionisation mode.

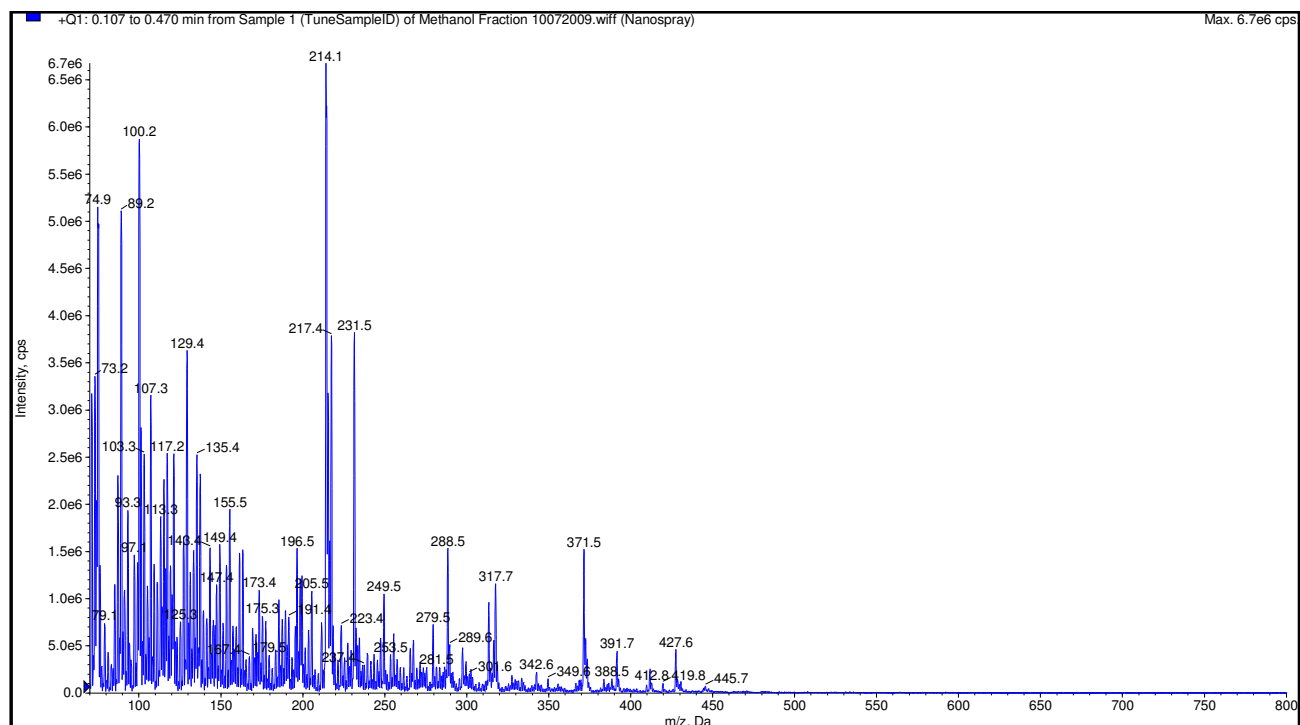


Figure 5.17: LC-MS/MS analysis of the CHD-FA crude fraction using methanol as elution solvent run in positive ionisation mode. The ion at 214.4 was found to be a plasticiser from the water used during the sample preparation.

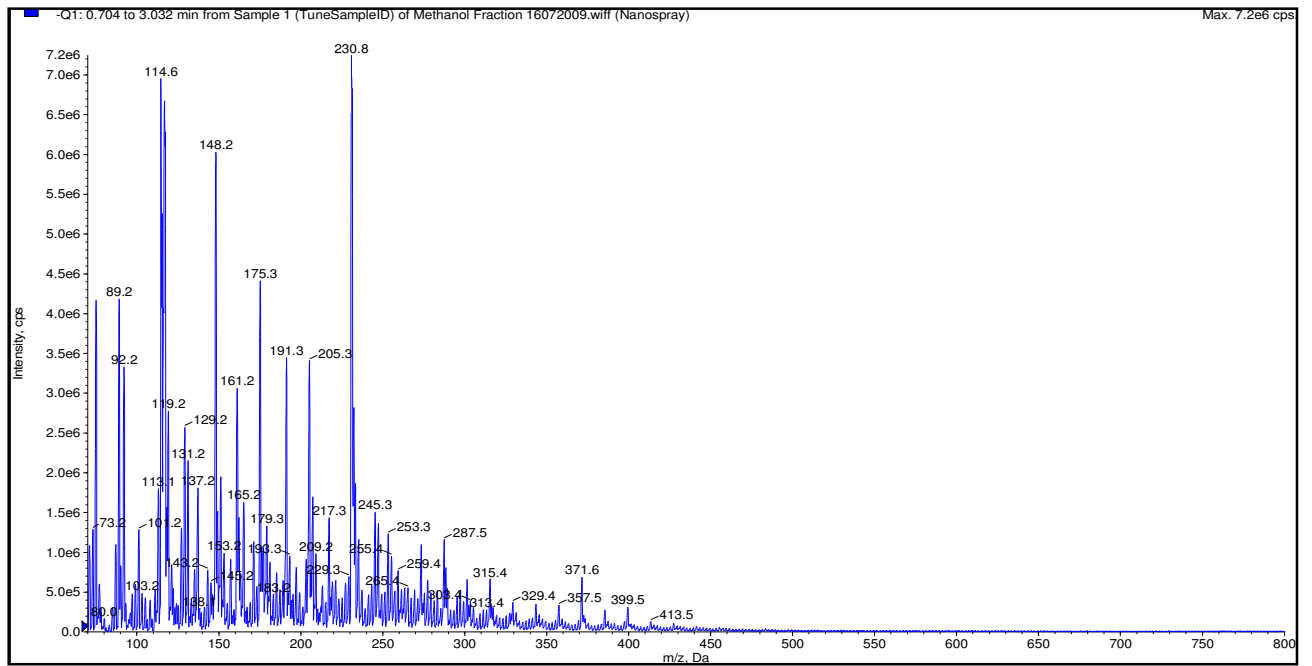


Figure 5.18: LC-MS/MS analysis of the CHD-FA crude fraction using methanol as elution solvent run in negative ionisation mode.

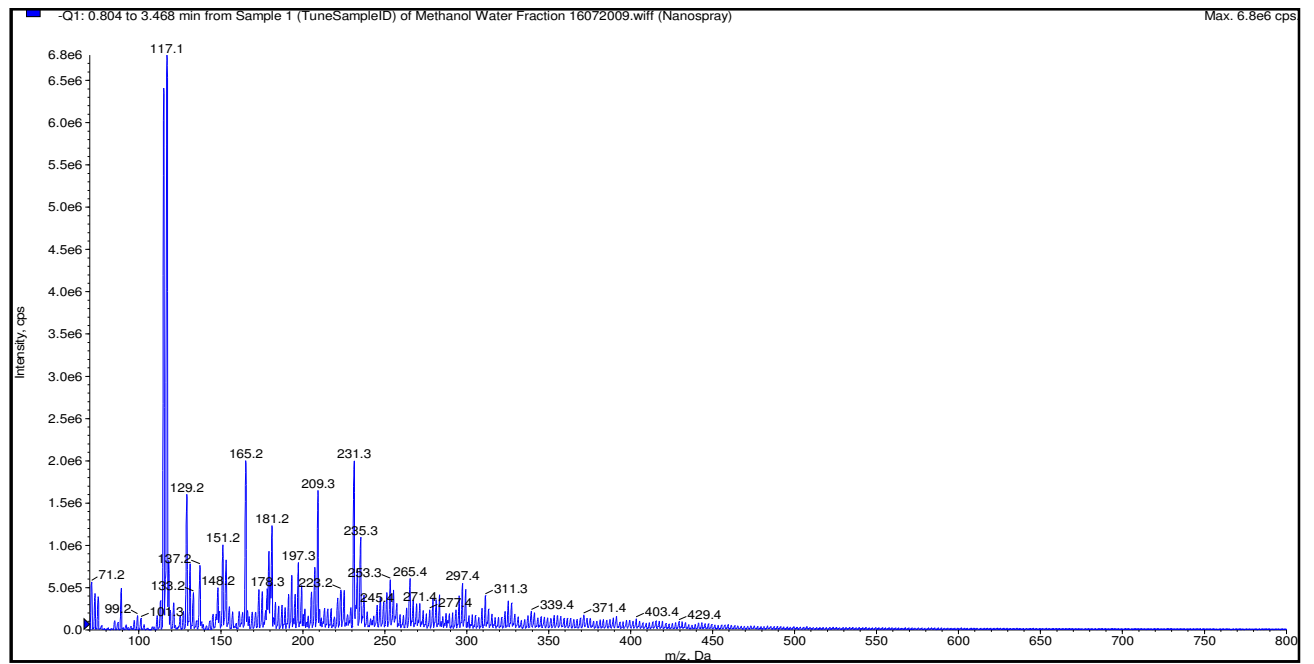


Figure 5.19: LC-MS/MS analysis of the CHD-FA crude fraction using methanol and water 50:50 as elution solvent run in negative ionisation mode.

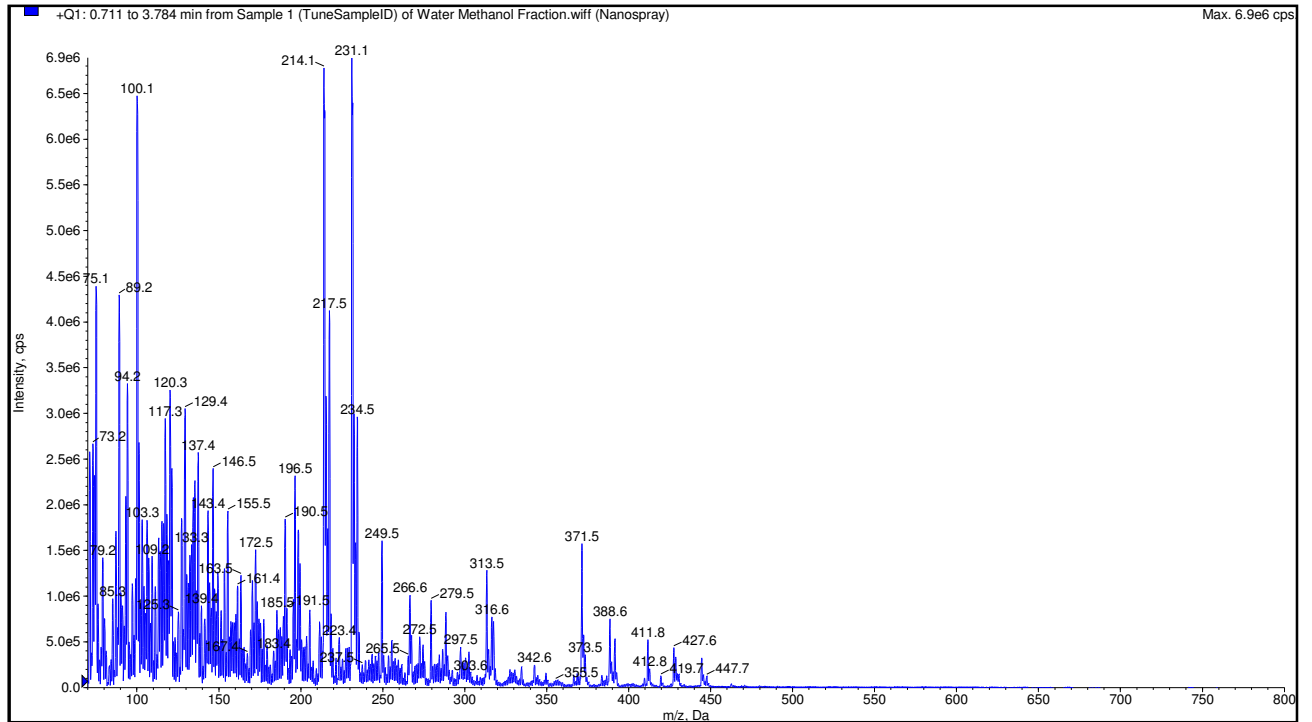


Figure 5.20: LC-MS/MS analysis of the CHD-FA crude fraction using methanol and water 50:50 as elution solvent run in positive ionisation mode.

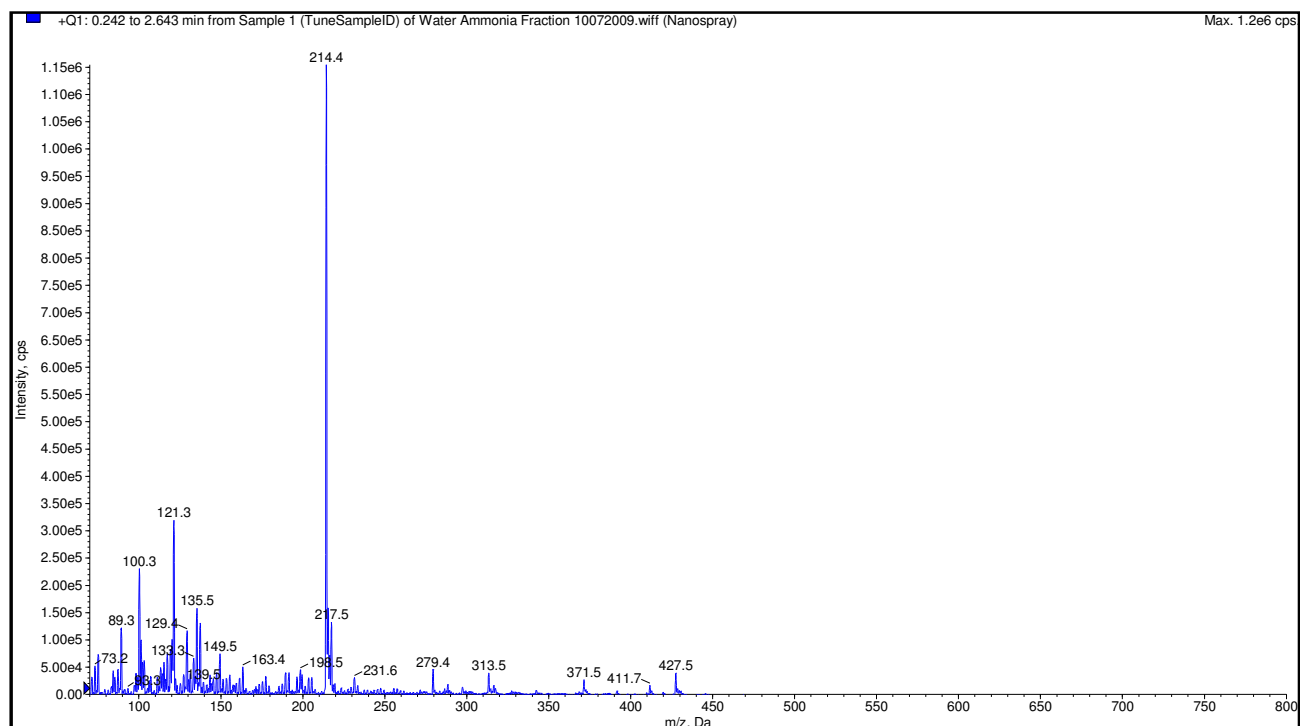


Figure 5.21: LC-MS/MS analysis of the CHD-FA crude fraction using ammonium hydroxide as elution solvent run in positive ionisation mode. The ion at 214.4 was found to be a plasticiser from the water used during the sample preparation.

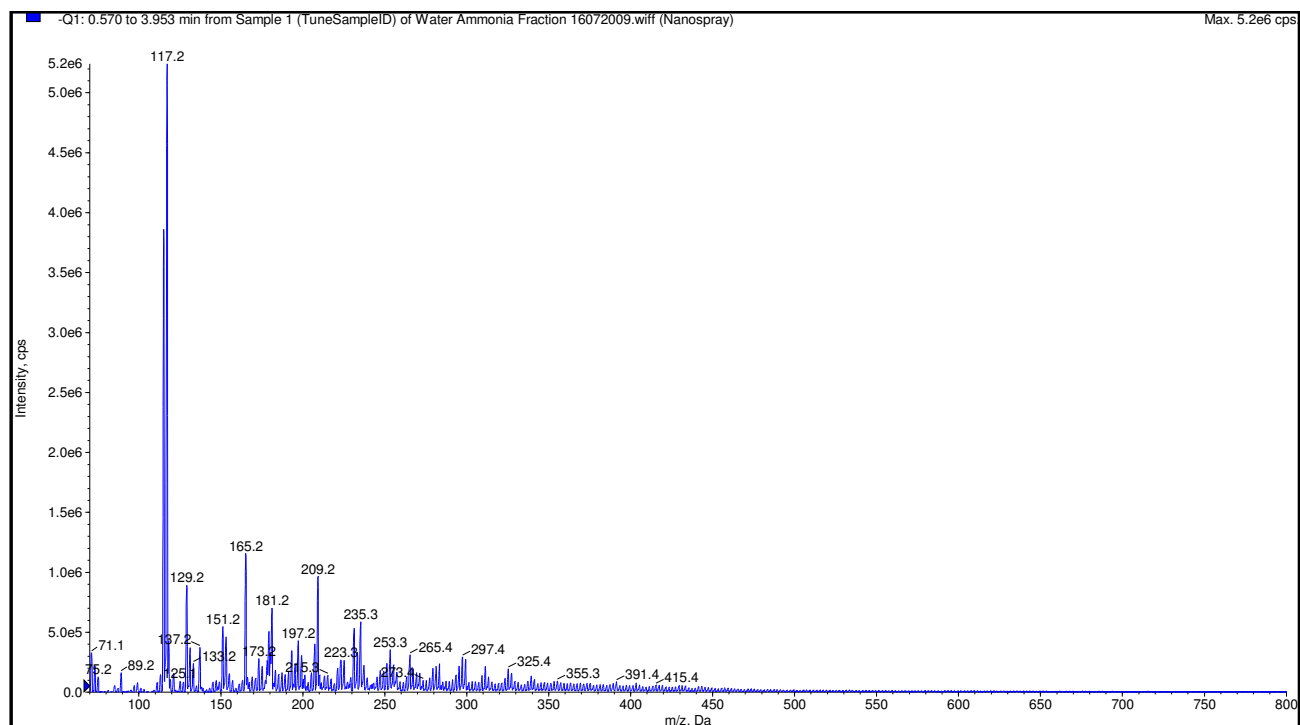


Figure 5.22: LC-MS/MS analysis of the CHD-FA crude fraction using ammonium hydroxide as elution solvent run in negative ionisation mode.

5.4 DISCUSSION

The initial LC-MS/MS testing was conducted in order to determine whether or not any unique or dominant ionic analytes in either the positive or negative ionisation mode could be found in the complete CHD-FA. The reason for assaying by the negative ionisation mode is the fact that many compounds in the fulvic acid already carry a negative charge. These assays used the CHD-FA diluted in double deionised water from an in-house water purification system or HPLC grade methanol.

In this early stage the various dilutions in solvents alone were analysed before and after background subtraction to identify any ions that were unique or abundant, several potential ions were detected for use as marker ions for the CHD-FA, but upon further investigation these analytes proved to be background analytes due to column bleed of earlier injections that had been retained or plasticisers from the laboratory consumables and a compound that is derived from the water purification unit.

An attempt to further separate and characterize the CHD-FA was then undertaken using a simplified chromatographic technique involving various elution solvents in order to simplify the complexity of the CHD-FA combination of compounds, followed again by the LC-MS/MS assessment for any unique ionic compounds. Although there were distinct differences in the profiles of each of the different solvent elution profiles, no dominant marker could be identified in these eluents that were also seen in the treated patient samples and this approach to try find a marker ion also proved to be unsuccessful.

In addition, none of the major compounds seen in the different solvent eluted fractions were found to be consistently present in any of the samples where the CHD-FA was combined with plasma protein. This could be due to a very high binding efficiency of the CHD-FA to the protein or a very high concentration of binding sites on the plasma protein. Although not assessed at the time, irreversible retention of some of the CHD-FA compounds on the short silica columns was also a possibility, but this would be consistent for all analytical methods using silica based chromatographic stationary phases.

As mentioned above, due to the acidic and ionic nature of CHD-FA, it is likely to be highly protein bound after absorption from the GIT. The protein which may act as the carrier for the CHD-FA compounds has not yet been identified but due to the character of the CHD-FA it could be assumed that albumin would play a major role due to its characteristic binding of acidic molecules. It is possible that CHD-FA saturated protein could release the CHD-FA in the order of weakest to strongest binding affinity to determine if a usable marker analyte can be found.

It was also noted during the experiments that when the CHD-FA was diluted with either methanol or plasma, even at low dilution ratios of CHD-FA, that a precipitate formed. Due to the low percentage CHD-FA in these solutions it cannot be attributed to a drop in pH as the cause of this precipitation effect. Further experimentation would be required to assess this aspect in more detail, this would however be costly and would have to encompass a greater knowledge of the chemistry and binding affinities of different fulvic acids as well as the development and validation of techniques with greater sensitivity.

5.5 CONCLUSION

Both plasma and CHD-FA are complex matrixes that show high variability depending on the patient, sample preparation procedures and potentially their relevant concentrations. Despite the availability of LCMS/MS technology that is highly selective and sensitive, the complexity and nature of the fulvic acid making up the CHD-FA is still too complex and dynamic to enable the identification of select CHD-FA ions that could be used as markers to assess the ADME and protein binding of fulvic acids after absorption from the GIT.

Using the techniques presently available to the study it was not possible to identify a unique marker for the CHD-FA which was the aim of the LCMSMS assays performed during this study. Without a suitable marker/s that can be identified against the background matrix it is not possible to assess the bioavailability or the pharmacokinetics of CHD-FA in human plasma. It is possible that a suitable marker could be found using more complex CHD-FA verses plasma saturation studies and comparative mass spectral analytical software as used in metabolomic analysis but that is beyond the scope of this study.

Unfortunately a suitable marker for CHD-FA could not be established, but hopefully with the advances in analysis techniques, equipment and knowledge this will be discovered in the not too distant future.

THESIS SUMMARY AND CONCLUSION

Fulvic and humic acids have a rich and impressive history, not only in research at the University of Pretoria (South Africa), but over the whole world. It only requires a short search in Google to find the thousands of medicinal claims being made, from miracle cures to food supplements. In certain earlier reports humic and fulvic acids are reputed to cause various ailments, while other claims make out they are the saving grace for almost every ailment imaginable.

The University of Pretoria's (South Africa) Pharmacology Department has researched these compounds for several years, in various forms and in multiple settings. From powders, to capsules and even liquid forms, varying from sources in the outback of Australia to locally manufactured synthetic heavy metal free versions that prove to be less toxic and more systemically friendly.

Through all of these studies and with all the experimental data at hand and online claims, it is the duty of the scientist to sift through this information and find some clarity amongst the unwarranted claims and statements of random uninformed individuals. Researchers have the obligation not only to investigate the data and claims, but also have an obligation to find potential new treatments or treatment option for those suffering from ailments that are yet to have safe, effective and cost-effective options.

The aims of the study were to test the safety and some efficacy parameters of the CHD-FA and these aspects as set out in the aims were achieved. It was found that there is negligible genotoxicity measured by a validated technique after treatment of lymphocytes with the CHD-FA which confirms previously collected data by the Pharmacology Department using the well-known Ames assays. Two pilot clinical trials were completed during which positive anti-inflammatory results were measured for both oral administrations of the CHD-FA, as well as topical applications. Both these studies demonstrated that the CHD-FA had anti-inflammatory effects and both showed efficacy against conditions know to be difficult to treat.

The clinical trials were carried out on limited participant numbers but it could be stated that there were no severe adverse effects although there were some minor adverse effects. None of these adverse effects would cause safety concerns and it should be taken into account that the safety data collected did include a wide ranging group of aspects including cardiac, hepatic and renal toxicity assessment. In general the oral dosing was well tolerated but there were some complaints about the acidity which resulted in a burning throat in some of the participants. This and diarrhoea appeared to be the most common complaint. The fact that there was a clear improvement in the wheal size of the skin prick challenge after treatment with CHD-FA proves that the compound is active as an anti-inflammatory agent, but also confirms that the active components within the complex are bioavailable after administration via the oral route.

The identification of a marker that could be used to determine the bioavailability or other pharmacokinetic parameters was however not successful and can be due to a number of different factors but the complexity of the CHD-FA and the plasma as a matrix makes it almost impossible to track the molecular masses of the different components in the complex biological matrix of plasma. It is possible that the compounds bind strongly to the protein or other macromolecules but it could also be that the compounds are masked within the plasma. In the samples collected from the participants there is a further potential that the CHD-FA components are rapidly absorbed or excreted due to their acid nature but they may also be metabolised. It was however optimistically hoped that there would be at least one clear component that could be used as a marker of the presence and concentration of the CHD-FA in the plasma to enable pharmacokinetic studies to be performed. This part of the study however did not deliver what was hoped and further more complex experiments will need to be designed and better mass spectral information collected and analysed using advanced metabolomics based software used to identify any potential CHD-FA markers.

Any attempt at providing data that can be used to support the registration of new drug entities, or to register new indications for existing drugs requires sound and valid data which in turn requires well-planned and executed experiments collecting usable data and research findings. Well planned and defined and reachable goals and outcomes are required, but above all this, unambiguous indisputable research is of the utmost necessity.

In the drug development arena, this can only be achieved when partnerships are established; between universities and government, universities and private companies, private companies and the government. Until a unified goal is decided upon, science will not be able to provide the answers or way forward to benefit our country, nation and human kind as a whole.

The research carried out during this study is to a degree a combination of the three partnerships as it included a research institution, a private company and the initial start-up and final watchful eye of the government. From molecules that were discovered decades ago in joint ventures with government and academic institutions only to be shelved due to political and research disconnects. Then having the private sector picking up the potential lead compounds again, and searching for clues and answers that could not be answered in the past, whilst having unsubstantiated claims and visions of potential grandeur.

Amongst all this, there are patients in need of new treatments who are suffering while waiting for treatments they can afford, treatments they will have access to, treatments with limited side effects, and treatments that address diseases as soon as we can identify them.

The different aspects investigated in this study did not prove to be the next golden standard in treatment or cure for what are currently debilitating diseases. Diseases that require constant, chronic approaches to alleviate symptoms, diseases that require ongoing investigations and investment, as they affect many of us on a daily basis, despite race, colour or socio-economic class.

What this research did provide is new insights, into the safety of a new carbohydrate-derived fulvic acid, not only in a genotoxic/mutagenic aspect, but also in a topical and systemic aspect, in both acute and chronic usage. The CHD-FA used in this study has undergone several pre-clinical investigations during *in vitro* research processes, from cell culture effects through acute and chronic toxicity assays in rats and mice, efficacy studies using several immune response models and thus it was warranted to test the safety and efficacy profile in humans.

This study included experiments and clinical assessment to provide proof of systemic uptake and efficacy in the control of a rapid atopic inflammatory response as well as efficacy when topically administered, which in both cases proved that there is indeed a degree of efficacy which warrants further investigation.

However, with the complexity of the CHD-FA compound, reported to contain many different organic and inorganic molecules, the specific active ingredient(s) is yet to be identified, which makes patenting such a molecule virtually impossible, as funding for further research becomes almost unattainable.

In the study an attempt was made to venture one step further by trying to identify a biomarker, or analyte that would be unique to this CHD-FA. If this would be possible, this too would warrant more research, more specific and in-depth research into what makes fulvic acid effective and how to amplify the results further, into other disease states and populations.

Unfortunately due to the complexity and limitations of the methods available to attempt this, a unique marker could not be found or determined for various reasons that are now, in hindsight evident. Searching for the proverbial needle in a haystack, but the haystack has not even been identified, and with the number of options available in research and complexity of fulvic acid, a lifetime could be dedicated to achieve this. Thus what was learnt will be carried forward to the next vibrant scientist to try and discover.

In summary this research added to the body of evidence around fulvic acid and in particular CHD-FA. Though many aspects were investigated, there are yet many more concepts that would need to be investigated. It is however acknowledged that certain gems of knowledge and science were discovered along the way and that these will hopefully entice not only academic institutions but funders and scientists alike in order to one day hopefully find new clinical usages and treatments for diseases that still weigh heavily on individuals, families and economies.

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RESEARCH OUTPUTS

Gandy JJ, Snyman JR, van Rensburg CEJ. An observational trial: Patient profile of users of Secomet V®. *South African Family Practice* 2010 165 Vol 52 No 2.

Gandy JJ., Meeding JP., Snyman JR., van Rensburg CEJ. Phase 1 clinical trial, acute, sub-acute safety and proof of concept efficacy of carbohydrate derived fulvic acid. *Clinical Pharmacology: Advances and Applications Clinical Pharmacology*. 2012; 4: 7–11.

Gandy JJ, Snyman JR, van Rensburg CEJ. Randomized, parallel-group, double-blind, controlled study to evaluate the efficacy and safety of carbohydrate-derived fulvic acid in topical treatment of eczema. *Clinical, Cosmetic and Investigational Dermatology*.
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Gandy, J.J., Meeding, J.P., Snyman, J.R. (2008) A phase 1 acute and sub acute safety clinical trial and proof of concept efficacy of carbohydrate derived fulvic acid (CHD-FA). Posters were presented at both the University of Pretoria Faculty Day September 2008 as well as at the SASBCP Annual Congress held in Grahamstown on 2-5 October 2008.

Gandy JJ, Meeding JP, Snyman JR, van Rensburg CEJ. (2010) The clinical efficacy of potassium humate in the treatment of allergic rhinitis: a double blind placebo controlled trial. *Drug Development Research* 71: 358–363.

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Gandy, J.J., Joone, G., Gelderblom, W. C. A. (2008) Evaluation of the possible genotoxic effects of a carbohydrate derived fulvic acid (CHD-FA) using a micronucleus assay and Ames test. Poster was presented at both the University of Pretoria Faculty Day September 2008 as well as at the SASBCP Annual Congress held in Grahamstown on 2-5 October 2008.

JJ Gandy, JP Meeding, JR Snyman, and CEJ van Rensburg . A Phase 1 clinical trial on the acute and sub-acute safety and proof of concept efficacy of carbohydrate derived fulvic acid. Poster presented at WAC 2009 in Buenos Aires, Argentina from 6 - 10 December 2009.

SUPPLEMENTARY DOCUMENTATION

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

STUDY NUMBER: FULV002

STUDY TITLE: A phase 1 clinical trial on the acute and sub acute safety and proof of concept efficacy of fulvic acid (CHD-FA).

SPONSOR: Dept of Pharmacology
Faculty of Health Sciences
University of Pretoria
and
Fulvimed (Pty) Ltd

INVESTIGATOR: Dr JP Meeding

INSTITUTION: University of Pretoria, Department of Pharmacology.

DAYTIME AND AFTER HOURS

TELEPHONE NUMBER: 082 821 9186

PATIENT NAME:

PATIENT FILE NO:



To the Participant: *This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand. You may take home **an unsigned copy of this consent** form to think about or discuss with family or friends **before making your decision.***

INTRODUCTION:

Fulvic acid is a natural occurring derivate of coal or brown coal and has been used traditionally to treat various diseases, including skin conditions. The anti-inflammatory properties of fulvic acid have been demonstrated in both animal and human models of inflammation. The safety of fulvic acid has been extensively investigated in animal and human models and it was found to have no known side-effects or toxicity at 1000 times the normal dose when applied topically.

So with this significant animal data it can therefore be confirmed that fulvic acid is safe and effective as an anti-inflammatory agent at the dosage tested, for topical or oral usage as indicated in the animal trials, and has to now be taken over to humans in order to test the safety of this product *in vivo*.

You are invited to consider participating in a research study to examine the safety of fulvic acid and its potential efficacy as an anti-inflammatory agent. Your participation in this study is entirely voluntary.

1. Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as the alternative procedures that are available to you, and your right to withdraw from the study at any time.
This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.
2. If you have any questions, do not hesitate to ask me.
3. You should not agree to take part unless you are satisfied about all the procedures involved.
4. You may not participate in another investigational medicine research study, nor take any other investigational medicine during your participation in this study.
5. You should not have participated in an investigational medicine research study within the past month and need to have a months washout period if on any medication.
6. Please be completely truthful with me regarding your health history, since you may otherwise harm yourself by participating in this study.
7. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.
8. If you have a personal doctor, please discuss with or inform him/her of your possible participation in this study. If you wish, I can also notify your personal doctor in this regard.

9. LENGTH OF THE STUDY AND NUMBER OF PARTICIPANTS:

- The study will be performed in Pretoria.
- Approximately 30 participants will participate in this study.
The participants will be over the age of 18.
- The total amount of time required for your participation in this study will be a maximum of 43 days.
- You will be asked to visit me 48 times during the study.

10. PROCEDURES:

If you agree to take part in this study, you will first be asked questions and examined to see if you qualify for this study.

There will be 48 visits at the investigators' site:

Visit 1: investigator must obtain written informed consent from the patient. Demography of patient: initials, date of birth, sex, weight, height. Habits: smoking, illicit drugs, alcohol. Medical history. Participants' perception of general health questionnaire, General physical examination plus ECG. Exclusion criteria for anatomical abnormalities. Skin prick test. Laboratory tests. Urea, uric acid, creatinine, Cl^- , K^+ , Na^+ , γ GT, Alkaline phosphatase, AST and ALT. Full blood count (differential and morphology). Verify eligibility of the patient – inclusion and exclusion criteria. Randomization into either group A or group B.

Commence part 1: Acute safety (See Annexure B)

Visit 2: clinical exam. Ingestion of a once off oral dosage of either 5mL (3.3%) FA for group A or 10 mL (3.3%) FA for group B.

Visit 3: 30 min after ingestion of FA the participant will undergo a physical exam, ECG and blood drawn to test the biochemistry markers.

- Visit 4: 1 hour after ingestion of fulvic acid the participant will undergo a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 5: 8 hours after ingestion of FA the participant will undergo a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 6: 24 hours after investigational product ingestion participant will undergo a clinical exam, ECG and drawing blood for biochemistry markers.
- Visit 7: 72 hours after investigational product ingestion participant will undergo a clinical exam, ECG and drawing blood for biochemistry markers.
- Visit 8: 7 days after product ingestion participant will undergo a clinical exam, ECG, participants' perception of general health questionnaire, drawing blood for haematology and biochemistry markers. Ingestion of a once off oral dosage of either 20mL (3.3%) FA for group A or 40 mL (3.3%) FA for group B.
- Visit 9: 30 min after ingestion of FA the participant will undergo a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 10: 1 hour after ingestion of fulvic acid the participant will undergo a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 11: 8 hours after ingestion of FA the participant will undergo a physical exam, ECG and blood drawn to test the biochemistry markers.
- Visit 12: 24 hours after investigational product ingestion participant will undergo a clinical exam, ECG and drawing blood for biochemistry markers.
- Visit 13: 72 hours after investigational product ingestion participant will undergo a clinical exam, ECG and drawing blood for biochemistry markers.
- Visit 14: 7 days after product ingestion participant will undergo a clinical exam, ECG, participants' perception of general health questionnaire, drawing blood for haematology and biochemistry markers.

Commence part 2: Sub acute safety and proof of concept (See Annexure C)

- Visit 14: 14 days after initial product ingestion, 20mL (3.3%) FA administered to group A and 40mL (3.3%) FA administered to group B.
- Visit 15: 6-8 Hours after previous ingestion of investigational product, 20mL (3.3%) FA administered to group A and 40mL (3.3%) FA administered to group B.
- Visit 16: 15 days after initial product ingestion, physical exam, ECG and 20mL (3.3%) FA administered to group A and 40mL (3.3%) FA administered to group B.
- Visit 17: 6-8 Hours after previous ingestion of investigational product, drawing blood for biochemistry markers, 20mL (3.3%) FA administered to group A and 40mL (3.3%) FA administered to group B.
- Visit 18: 16 days after initial product ingestion, physical exam, 20mL (3.3%) FA administered to group A and 40mL (3.3%) FA administered to group B.
- Visit 19: 6-8 Hours after previous ingestion of investigational product, participant will undergo a clinical exam, ECG, participants' perception of general health questionnaire, drawing blood for haematology and biochemistry markers, 20mL (3.3%) FA administered to group A and 40mL (3.3%) FA administered to group B.

Commence part 3: Proof of concept:

- Visit 20: 23 days after initial product ingestion, physical exam, ECG, participants' perception of general health questionnaire, drawing blood for haematology and biochemistry markers, skin prick test and 40mL (3.3%) FA/placebo administered.
- Visit 21: 6-8 Hours after previous ingestion of investigational product, 40mL (3.3%) FA/placebo administered.

- Visit 22: 24 days after initial product ingestion, 40mL (3.3%) FA/placebo administered.
- Visit 23: 6-8 Hours after previous ingestion of investigational product, 40mL (3.3%) FA/placebo administered.
- Visit 24: 25 days after initial product ingestion, 40mL (3.3%) FA/placebo administered.
- Visit 25: 6-8 Hours after previous ingestion of investigational product, 40mL (3.3%) FA/placebo administered.
- Visit 26: 26 days after initial product ingestion, 40mL (3.3%) FA/placebo administered.
- Visit 27: 6-8 Hours after previous ingestion of investigational product, physical exam, ECG, participants' perception of general health questionnaire, drawing blood for haematology and biochemistry markers and 40mL (3.3%) FA/placebo administered.
- Visit 28: 27 days after initial product ingestion, 40mL (3.3%) FA/placebo administered.
- Visit 29: 6-8 Hours after previous ingestion of investigational product, 40mL (3.3%) FA/placebo administered.
- Visit 30: 28 days after initial product ingestion, 40mL (3.3%) FA/placebo administered.
- Visit 31: 6-8 Hours after previous ingestion of investigational product, 40mL (3.3%) FA/placebo administered.
- Visit 32: 29 days after initial product ingestion, 40mL (3.3%) FA/placebo administered.
- Visit 33: 6-8 Hours after previous ingestion of investigational product, physical exam, ECG, participants' perception of general health questionnaire, drawing blood for haematology and biochemistry markers, skin prick test and 40mL (3.3%) FA/placebo administered.

7 day washout period then commencement of cross-over phase of proof of concept, where the groups receiving the placebo will now receive the fulvic acid and visa versa.

Additional visits will be arranged with patients as dictated by clinical need.

11. WILL ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

- Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions. A total of 252mL of blood will be collected over the course of the entire study.
- Skin prick test will illicit an allergic response which will lead to inflammation on the site where the test was carried out. It could also lead to irritation and bronchoconstriction.
- The fulvic acid, when given in high doses could lead to a loose or watery stool.
- May cause hypokalaemia at high doses.
- Volunteers will have to provide their own, safe transport to and back from the testing facility, the investigators and sponsor take no responsibility with this regard.

12. RISKS OF THE STUDY MEDICINE:

This is a natural product that has been commercially available and used as a food supplement for at least the past 2 years, the only reported side effect is that when taken at high dosages patients may experience a loose or watery stool. In a recent study it caused a hypokalaemia at high doses

13. UNFORSEEN RISKS:

- The study medicine is investigational and there may be other risks or side effects which are unforeseen or unknown. You should immediately contact me if any side effects occur throughout your participation in this study.

14. BENEFITS:

- You may not benefit from this study.
- Your participation in this study will contribute to medical knowledge on the safety and efficacy of this product that may lead to further investigation into this products development.

BENEFITS AND RISKS OF STANDARD INTERACTIONS:

- It is important that you let me know of any medicines (both prescriptions and over-the-counter medicines), alcohol or other recreational substances that you are currently taking.

RIGHTS AS A PARTICIPANT IN THIS STUDY:

15. and 17 - Voluntary:

Your participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. Your withdrawal will not affect your access to other medical care.

- Discontinuation of study treatment.
You must inform me if you wish to stop taking your study medicine.
I will supervise any discontinuation with your health as first priority.

16. Withdrawal:

- I retain the right to withdraw you from the study if it is considered to be in your best interest. If your participation is ended early, you may be asked to return for study-ending tests and procedures for your safety
- If you did not give an accurate history or did not follow the guidelines of the study and the regulations of the study facility, you may be withdrawn from the study at any time.

ARRANGEMENTS:

- Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology will provide payment for all study procedures and reasonable medical expenses that you may incur as a direct result of this study as determined by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology and me.
- Neither you nor your medical scheme will be expected to pay for any study medication, study related visit or study procedures.

19. REIMBURSEMENT FOR STUDY PARTICIPATION:

REIMBURSEMENT:

You will receive R150-00 per active visit as compensation for taking part in the trial. There are 22 active visits, thus on completion of the trial you will receive R3300-00. If for any reason you do not complete the trial, or you are withdrawn, you will be paid for the attended active visits.

INSURANCE:

Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology has obtained insurance for you and me in the event of study related injury or illness. A study-related injury or illness is one that occurs as a direct result of the administration of the study medicine or of study-specific procedures.

20. ABPI STATEMENT ON COMPENSATION:

Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology will provide compensation for reasonable medical expenses incurred as a result of study-related injury or illness, determined according to the guidelines laid down by the Association of the British Pharmaceutical Industry (ABPI Guidelines), and Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa.

- You must notify me immediately of any complications, side effects and/or injuries during the study and the nature of the expenses to be covered.
- If a research related injury occurs, you have not waived any of the legal rights which you otherwise would have as a participant in this study by signing this form.

Further detailed information on the payment of medical treatment and compensation due to injury can be obtained from me. I have a copy of the ABPI Guidelines and the Insurance Certificate, should you wish to review them.

The insurance does not cover and Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology will not pay for:

- Medical treatment of other injuries or illnesses
- Injury caused by non-observance of the protocol

I am indemnified by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology conditional upon:

- My compliance with the applicable requirements of the study protocol

- My compliance with the regulations of the Medicines Control Council and the University of the Pretoria Research Ethics Committee.
- The handling and administration of the study medication in accordance with instructions and guidelines provided in the protocol, subsequent amendments and related documents
- The indemnification is not intended to be and is not a substitute for my personal malpractice insurance.

Please note that if you have a life insurance policy you should enquire whether your insurance company requires notification of your intention to participate in a clinical study. Information to date is that it should not affect any life insurance policy taken out. Nevertheless, you are strongly advised to clarify it with the company concerned.

21. ETHICAL APPROVAL:

- This clinical study protocol has been submitted to the University of the Pretoria Research Ethics Committee and written approval has been granted by that committee.
- The study has been structured in accordance with the Declaration of Helsinki (last updated: October 2000), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from me should you wish to review it.

This study is sponsored by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology. I do not have any financial or personal interests with this organisation that may bias my actions.

22. SOURCE OF ADDITIONAL INFORMATION:

- For the duration of the study, you will be under the care of Dr JP Meeding.
- If at any time between your visits, you feel that any of your symptoms are causing you any problems, or you have any questions during the study, please do not hesitate to contact me.
- The 24-hour telephone number, through which you can reach me or another authorised person, is 082 821 9186.

23. CONFIDENTIALITY:

- All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be

reported in scientific journals will not include any information that identifies you as a participant in this study.

This information will be reviewed by authorised representatives Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology.

- These records will be utilised by them only in connection with carrying out their obligations relating to this clinical study.
- Any information uncovered regarding your test results or state of health as a result of your participation in this study will be held in strict confidence. You will be informed of any finding of importance to your health or continued participation in this study but this information will not be disclosed to any third party in addition to the ones mentioned above without your written permission. The only exception to this rule will be cases of communicable diseases where a legal duty of notification of the Department of Health exists. In this case, you will be informed of my intent to disclose such information to the authorised state agency.

INFORMED CONSENT:

- I hereby confirm that I have been informed by the study doctor, Dr JP Meeding about the nature, conduct, benefits and risks of clinical study FULV002 A phase 1 clinical trial on the acute and sub acute safety and proof of concept efficacy of fulvic acid (CHD-FA).
- I have also received, read and understood the above written information (Participant Information Leaflet and Informed Consent) regarding the clinical study.
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology on their behalf.
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.

PARTICIPANT:

Printed Name

Signature

Date and Time

I, Dr JP Meeding, herewith confirm that the above participant has been fully informed about the nature, conduct and risks of the above study.

STUDY DOCTOR:

Printed Name

Signature

Date and Time

WITNESS (If applicable):

Printed Name

Signature

Date and Time



Universiteit van Pretoria
University of Pretoria

Faculty of Health Sciences Research Ethics Committee
University of Pretoria
HW Snyman Building, (South) Private Bag
X169
Level 2-34 Pretoria

Date: 2/10/2007

AMENDMENT PROTOCOL NO.	Patient Information Leaflet & Protocol 52/2007
PROTOCOL TITLE	A Phase 1 Clinical Trial On The Acute And Sub Acute Safety And Proof Of Concept Efficacy Of Fulvic Acid (CHD-FA)
INVESTIGATOR	Dr J P Meeding
DEPARTMENT	Pharmacology, University of Pretoria
STUDY DEGREE	PhD
SUPERVISORS	Prof JR Snyman and Prof CE Medlen
CONTACT DEATAILS	T: 012-3192243 F: 012-3192411 E: jjgandy@webmail.co.za C: 0828219186
VAT NO.	None.
ORDER / CONTRCT NUMBER	FULV002
MEETING DATE	25/04/2007

This Amendment has been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 26/09/2007 and found to be acceptable.

*Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datametrics (UNISA)
Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); Ph.Dd. (Leuven)
Dr N K Likibi	MB.BCh.; Med.Adviser (Gauteng Dept.of Health)
Snr Sr J. Phatoli	(female) BCur (Et.Ai) Senior Nursing-Sister
*Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
*Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
*Dr A P van Der Walt	BChD, DGA (Pret) Director: Clinical Services of the Pretoria
Academic Hospital	
*Prof C W van Staden	MBChB; Mmed (Psych); MD; FTCL; UPLM; Dept of Psychiatry

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.
SECRETARIAT of the Faculty of Health Sciences Research Ethics Committee - University of Pretoria

PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

STUDY NUMBER: FULV003

STUDY TITLE: A 4 week, randomized, parallel-group, double-blind, controlled study to evaluate the efficacy and safety of a carbohydrate derived fulvic acid (CHD-FA) in the topical treatment of atopic dermatitis (eczema).

SPONSOR: Dept of Pharmacology
Faculty of Health Sciences
University of Pretoria
and
Fulvimed (Pty) Ltd

INVESTIGATOR: Dr P Soma

INSTITUTION: University of Pretoria, Department of Pharmacology.

DAYTIME AND AFTER HOURS

TELEPHONE NUMBER: 012 319 2166

PATIENT NAME:

PATIENT FILE NO:

To the Participant: *This consent form may contain words that you do not understand. Please ask the study doctor or the study staff to explain any words or information that you do not clearly understand. You may take home an unsigned copy of this consent form to think about or discuss with family or friends before making your decision.*

INTRODUCTION:

Eczema is an inflammatory disorder and is often a recurrent and difficult disease to treat. Topical glucocorticoids are used to treat flares and normal emollients for maintenance therapy. Some diseases are associated with an increase in skin pH.

Fulvic acid is a natural occurring derivate of coal or brown coal and has been used traditionally to treat various diseases, including skin conditions. The anti-inflammatory properties of fulvic acid have been demonstrated in both animal and human models of inflammation. The safety of fulvic acid has been extensively investigated in animal and human models and it was found to have no known side-effects or toxicity at 1000 times the normal dose.

You as the parent/guardian of the affected child are invited to consider the participation of your child in a research study to examine the safety of fulvic acid and its potential efficacy in the topical treatment of eczema. You and your child's participation in this study is entirely voluntary.

9. Before agreeing to participate, it is important that you read and understand the following explanation of the purpose of the study, the study procedures, benefits, risks, discomforts, and precautions as well as the alternative procedures that are available to you, and your right to withdraw from the study at any time. This information leaflet is to help you to decide if you would like to participate. You should fully understand what is involved before you agree to take part in this study.
10. If you have any questions, do not hesitate to ask me.
11. You should not agree to take part unless you are satisfied about all the procedures involved.
12. You may not participate in another investigational medicine research study, nor take any other investigational medicine during your participation in this study.
13. You should not have participated in an investigational medicine research study within the past month and need to have a months washout period if on any medication.
14. Please be completely truthful with me regarding your health history, since you may otherwise harm yourself by participating in this study.

15. If you decide to take part in this study, you will be asked to sign this document to confirm that you understand the study. You will be given a copy to keep.

16. If you have a personal doctor, please discuss with or inform him/her of your possible participation in this study. If you wish, I can also notify your personal doctor in this regard.

9. LENGTH OF THE STUDY AND NUMBER OF PARTICIPANTS:

- The study will be performed in Pretoria.
- Approximately 50 participants will participate in this study.
- The participants will be over the age of 2.
- The total amount of time required for your participation in this study will be a maximum of 28 days.
- You will be asked to visit me 3 times during the study.

10. PROCEDURES:

If you agree to take part in this study, you will first be asked questions and you or your child will be examined to see if you qualify for this study. If you or your child have qualified to take part in the study, you will receive 2 containers, one with a blue sticker and one with a red sticker. The gel with the blue sticker may or may not contain fulvic acid and you will have to put it to the affected areas at least 2 times per day. We will also give you another normal cream with a red sticker that you can use as much as you like but you must not use it for 1 hour before and 1 hour after you have used the special treated cream (blue sticker).

At each following visit you will undergo:

Visit 1: Screening

- Signing of informed consent.
- Assessment of disease and confirmation of diagnosis.
- Assessing inclusion and exclusion criteria
- Assessing use of concomitant medication.
- Completion of medical history.

Visit 2: Randomization

- Reassess inclusion and exclusion criteria
- Assess adverse events
- Physical examination
- Safety bloods (Full blood count, Liver and Kidney function)
- ECG (Heart function)
- Investigators assessment of severity of disease
- Dispensing and application of medication

Visit 3: End of study: return after 4 weeks of treatment

- Assess adverse events
- Physical examination
- Safety bloods (Liver and Kidney function)
- ECG (Heart function)
- Investigators assessment of severity of disease (as described above)
- Collecting of unused medication

Additional visits will be arranged with patients as dictated by clinical need.

11. WILL ANY OF THESE STUDY PROCEDURES RESULT IN DISCOMFORT OR INCONVENIENCE?

- Venipunctures (i.e. drawing blood) are normally done as part of routine medical care and present a slight risk of discomfort. Drawing blood may result in faintness, inflammation of the vein, pain, bruising or bleeding at the puncture site. There is also a slight possibility of infection. Your protection is that experienced personnel perform the procedures under sterile conditions, as it is done in normal care.

12. RISKS OF THE STUDY MEDICINE:

This is a natural product that has been commercially available and used as a food supplement for at least the past 2 years. The only reported side effect when taken by mouth is a loose or watery stool and in a recent trial an increase in potassium levels in the blood was experienced when swallowing the fulvic acid at a high dose.

13. UNFORSEEN RISKS:

- The study medicine is investigational and there may be other risks or side effects which are unforeseen or unknown. You should immediately contact me if any side effects occur throughout your or your child's participation in this study.

14. BENEFITS:

- You or your child may not benefit from this study.
- You or your child's participation in this study will contribute to medical knowledge on the safety and efficacy of this product that may lead to further investigation into this product's development.
- If the eczema gets worse or doesn't improve in the first week, the doctor will prescribe a topical corticosteroid for you or your child that you must use an hour before or after you've applied the study drug.

BENEFITS AND RISKS OF STANDARD INTERACTIONS:

- It is important that you let me know of any medicines (both prescriptions and over-the-counter medicines) that either you or your child are taking.

16. RIGHTS AS A PARTICIPANT IN THIS STUDY

You or your child's participation in this study is entirely voluntary and you can decline to participate, or stop at any time, without stating any reason. You or your child's withdrawal will not affect your access to other medical care.

- Discontinuation of study treatment.

You must inform me if you wish to stop using the study medicine.

I will supervise any discontinuation with your health as first priority.

17 WITHDRAWAL

- I retain the right to withdraw you or your child from the study if it is considered to be in you or your child's best interest. If you or your child's participation is ended early, you may be asked to return for study-ending tests and procedures for your safety
- If you did not give an accurate history or did not follow the guidelines of the study and the regulations of the study facility, you or your child may be withdrawn from the study at any time.

ARRANGEMENTS:

- Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology will provide payment for all study procedures and reasonable medical expenses that you may incur as a direct result of this study as determined by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology and I.
- Neither you nor your medical scheme will be expected to pay for any study medication, study related visit or study procedures.

18. REIMBURSEMENT FOR STUDY

PARTICIPATION:

REIMBURSEMENT:

Travel expenses will be reimbursed (paid back) after negotiating with the trial co-ordinator depending on expenses incurred and geographical location (where you live).

INSURANCE:

Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology has obtained insurance for you or your child and I in the event of study related injury or illness. A study-related injury or illness is one that occurs as a direct result of the application of the study medicine or of study-specific procedures.

19. ABPI STATEMENT ON COMPENSATION:

Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology will provide compensation for reasonable medical expenses incurred as a result of study-related injury or illness, determined according to the guidelines laid down by the Association of the British Pharmaceutical Industry (ABPI Guidelines), and Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa.

- You must notify me immediately of any complications, side effects and/or injuries during the study and the nature of the expenses to be covered.
- If a research related injury occurs, you have not waived any of the legal rights which you otherwise would have as a participant in this study by signing this form.

Further detailed information on the payment of medical treatment and compensation due to injury can be obtained from me. I have a copy of the ABPI Guidelines and the Insurance Certificate, should you wish to review them.

The insurance does not cover and Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology will not pay for:

- Medical treatment of other injuries or illnesses
- Injury caused by non-observance of the protocol

20. ETHICS COMMITTEE APPROVAL:

- This clinical study protocol has been submitted to the University of the Pretoria Research Ethics Committee and written approval has been granted by that committee.
- The study has been structured in accordance with the Declaration of Helsinki (last updated: October 2000), which deals with the recommendations guiding doctors in biomedical research involving human participants. A copy may be obtained from me should you wish to review it.
- This study is sponsored by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology. I do not have any financial or personal interests with this organisation that may bias my actions.

21. SOURCE OF ADDITIONAL INFORMATION:

- For the duration of the study, you or your child will be under the care of Dr P Soma
- If at any time between your visits, you feel that any of your symptoms are causing you or your child any problems, or you have any questions during the study, please do not hesitate to contact me.
- The 24-hour telephone number, through which you can reach me or another authorised person, is 012 319 2166.

22. CONFIDENTIALITY:

- All information obtained during the course of this study, including hospital records, personal data and research data will be kept strictly confidential. Data that may be reported in scientific journals will not include any information that identifies you as a participant in this study.
- This information will be reviewed by authorised representatives of Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology.
- These records will be utilised by them only in connection with carrying out their obligations relating to this clinical study.
- Any information uncovered regarding your test results or state of health as a result of you or your child's participation in this study will be held in strict confidence. You will be informed of any finding of importance to you or your child's health or

continued participation in this study but this information will not be disclosed to any third party in addition to the ones mentioned above without your written permission. The only exception to this rule will be cases of communicable diseases where a legal duty of notification of the Department of Health exists. In this case, you will be informed of my intent to disclose such information to the authorised state agency.

INFORMED CONSENT:

- I hereby confirm that I have been informed by the study doctor, Dr P Soma about the nature, conduct, benefits and risks of clinical study FULV003 A 4 week, randomized, parallel-group, double-blinded, controlled study to evaluate the efficacy and safety of a carbohydrate derived fulvic acid (CHD-FA) in the topical treatment of eczema.
- I have also received, read and understood or been read the above written information (Participant Information Leaflet and Informed Consent) regarding the clinical study.
- I am aware that the results of the study, including personal details regarding me or my child's sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report.
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerised system by Fulvimed (Pty) Ltd and University of Pretoria Department of Pharmacology on their behalf.
- I may, at any stage, without prejudice, withdraw my consent and me or my child's participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself or my child prepared to participate in the study.

PATIENT/PARENT/GUARDIAN:

Printed Name

Signature

Date and Time

I, Dr P Soma, herewith confirm that the above patients' parent/guardian has been fully informed about the nature, conduct and risks of the above study.

STUDY DOCTOR:

Printed Name	Signature	Date and Time
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WITNESS (That the patients' parent/guardian has understood and agreed to participate in the trial):

Printed Name	Signature	Date and Time
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Universiteit van Pretoria
University of Pretoria

Faculty of Health Sciences Research Ethics Committee
University of Pretoria
HW Snyman Building, (South) Private Bag
X169
Level 2-34 Pretoria

Date: 2/10/2007

PROTOCOL NO.	125/2007
ATTACHEMENTS	<ul style="list-style-type: none"> • Cover Letter ✓ • Curriculum Vitae of Principal ✓ Co-✓ and Sub-Investigators. • Declaration of Helsinki. ✓ • Full protocol. ✓ • Post Graduate Form ✓ • Letter of Intent. ✓ • NHREC number. ✓ • Patient Information Leaflet Informed Consent forms. ✓
PROTOCOL TITLE	A 4 week, randomized, parallel-group, double-blinded, controlled study to evaluate the efficacy and safety of a carbohydrate derived fulvic acid (CHD-FA) in the topical treatment of atopic dermatitis.
INVESTIGATOR	Person: Justin John Gandy Phone: 012-3192663 Fax: 012-3192411 E-Mail: jjgandy@webmail.co.za Cell: 0824459335
DEPARTMENT	Pharmacology; University of Pretoria; Pretoria
STUDY DEGREE	PhD Pharmacology
SUPERVISOR	Prof J R Snyman
SPONSOR	None.
MEETING DATE	26/09/2007

This Protocol and Informed Consent and all the attachments have been considered by the Faculty of Health Sciences Research Ethics Committee, University of Pretoria on 26/09/2007 and found to be acceptable.

*Advocate AG Nienaber	(female)BA(Hons) (Wits); LLB; LLM (UP); Dipl.Datometrics (UNISA)
Prof V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
Prof M Kruger	(female) MB.ChB.(Pret); Mmed.Paed.(Pret); PhDd. (Leuven)
Dr N K Likibi	MB.BCh.; Med. Adviser (Gauteng Dept.of Health)
Snr Sr J. Phatoli	(female) BCur (Et.Al) Senior Nursing-Sister
*Dr L Schoeman	(female) Bpharm, BA Hons (Psy), PhD
*Dr R Sommers	(female) MBChB; M.Med (Int); MPhar.Med;
Prof TJP Swart	BChD, MSc (Odont), MChD (Oral Path) Senior Specialist; Oral Pathology
*Dr A P van Der Walt	BChD, DGA (Pret) Director: Clinical Services of the Pretoria
Academic Hospital	
*Prof C W van Staden	MBChB; Mmed (Psych); MD; FTCL; UPLM; Dept of Psychiatry

DR R SOMMERS; MBChB; M.Med (Int); MPhar.Med.
SECRETARIAT of the Faculty of Health Sciences Research Ethics Committee - University of Pretoria