DIPLOMARBEIT

Titel der Diplomarbeit

Screening of various valerian root extracts and selected single compounds for anxiolytic activity using the elevated plus maze in mice

angestrebter akademischer Grad

Magistra der Pharmazie (Mag.pharm.)

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Studienkennzahl	A449
Studienrichtung:	Pharmazie
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Wien, im Januar 2011

AKNOWLEDGEMENTS

First and foremost I would like to thank Univ.Prof.Dr. Brigitte Kopp for the supervision of this project at the University of Vienna and for guidance and critical annotations. I appreciate her helping me arranging this project abroad at the University of Florida.

I would like to express the deepest appreciation to Dr. Veronika Butterweck, my main supervisor at the University of Florida. Many thanks for providing all the equipment I needed to produce and complete my thesis and for mentoring me all the way.

I gratefully thank my colleague Franziska Könner who assisted me during the experiments, for advices and encouragement.

I also want to express my gratitude to my friends and colleagues Katharina Dvorak, Regina Schüller and Stefanie Weinöhrl at the University for the years of study and encouragement.

Finally, I thank my boyfriend Wilfried and my family for supporting me throughout my studies at University.

Table of contents

1 ABBREVIATIONS

CNS   -   central nervous system

BZD   -   benzodiazepines

VA   -   valerenic acid

GABA   -   gamma amino butyric acid

ESCOP   -   The European Scientific Cooperative On Phytotherapy

USP   -   US Pharmacopoeia

Eur. Ph.   -   European Pharmacopoeia

i.p.   -   intraperitoneal

p.o.   -   peroral

EPM   -   elevated plus maze

MBT   -   marble burying test

DSM IV TR -   The Diagnostic and Statistical Manual of Mental Disorders Text Revision

2 ABSTRACT

Anxiety affects many people world-wide and has become an important area of research interest in psychopharmacology during this decade. Benzodiazepines are the major class of compounds used in anxiety and they have remained the most commonly prescribed treatment for anxiety. However, the realization that benzodiazepines present a narrow safety margin between the anxiolytic effect and causing unwanted side effects has prompted many researchers to develop new compounds in the hope to find anxiolytic drugs that have less undesirable effects. It has been shown recently that extracts prepared from the roots of *Valeriana officinalis* L. exerted anxiolytic activity in animal models. However, the compounds responsible for this effect are still unknown. It is therefore the goal of the present study to test selected isolated compounds (valerenic acid, apigenin, linarin) from *Valeriana* in order to get information about possible anxiolytic active compounds using the elevated plus maze (EPM) in mice. Mice have been orally treated with these isolated compounds in different concentrations and the anxiolytic behavior was evaluated in the EPM. Valerenic acid showed significant anxiolytic effects at the concentration of 0.5 mg/Kg. A lower dose as well as higher doses did not show an anxiolytic effect in comparison to the used control diazepam. The flavonoids apigenin and linarin did not show any anxiolytic effects in comparison to the control group.

3 Zusammenfassung

Diese Diplomarbeit beschäftigt sich damit, die anxiolytische Wirkung von verschiedenen isolierten Inhaltsstoffen aus *Valeriana officinalis* L. (Valerianaceae) zu untersuchen. Allgemeine Angstzustände sind heute eine weit verbreitete ernstzunehmende Erkrankung welche das Leben der Betroffenen stark beeinträchtigen kann. Benzodiazepine werden in diesen Fällen am häufigsten verschrieben, jedoch weisen diese Arzneistoffe schwerwiegende Nebenwirkungen auf, wie Sedierung oder Beeinträchtigung der kognitiven Leistung. Diese Nebenwirkungen können das Alltagsleben der Patienten ebenfalls beeinträchtigen, daher ist es wichtig Anxiolytika ohne die beschriebenen unerwünschten Wirkungen zu finden.

Baldrian hat sich im traditionellen Gebrauch als wirksames Einschlaf- und Beruhigungsmittel etabliert. Es sind auch zahlreiche Studien zur einschlaffördernden Wirkung des Baldrians durchgeführt worden, jedoch wird die anxiolytische Wirkung noch untersucht. Bisher wurden in einigen *in vitro* Studien nachgewiesen, dass die Wirkung von, der im Baldrian enthaltenen Valerensäure im Zentralnervensystem über $GABA_A$-Kanäle vermittelt wird.

Das Ziel der vorliegenden Arbeit war es, die anxiolytische Wirkung von Valerensäure und anderen Inhaltsstoffen (Apigenin, Linarin) des Baldrians im Tiermodell festzustellen, um Hinweise auf moegliche Wirkstoffe zu erhalten. Als Testsystem wurde der sogenannte Elevated Plus Maze Test (EPM) in Mäusen verwendet. Von den getesteten Substanzen konnte für Valerensäure, in einer Konzentration von 0.5 mg/Kg eine anxiolytische Wirkung festgestellt werden. Eine niedrigere Konzentration zeigte keine Wirkung im Vergleich mit der Kontrollsubstanz Diazepam. Mit steigender Konzentration nahm die anxiolytische Wirkung ebenfalls ab. Für die Flavonoide Apigenin und Linarin konnte im EPM kein anxiolytischer Effekt festgestellt werden.

4 Introduction

Anxiety disorders are a group of mental disorders that become manifest in different forms and may interfere with activities of daily living. Individuals suffering from anxiety disorders (AD) have large social and economic implications, such as loss of workplace productivity. Treatment of anxiety disorders includes psychoanalytic, cognitive and pharmacologic therapies. Benzodiazepines are the most prescribed anti-anxiety drugs though they can have severe sedative and cognitive side effects. Therefore, there is a goal for the pharmaceutical industry to develop new anti-anxiety drugs to reduce undesirable sedative and amnesic side effects. Many patients suffering from AD also seek help outside the realm of psychiatry such as alternative medicine providers. There is a growing market for self-administration of herbal and other dietary supplements having anxiolytic effects. Among anxiolytic herbs kava kava (not used anymore because of hepatotoxicity), valerian root, passion flower and others are considered. The safe use of plant derived medicines requires a detailed understanding of their pharmacologic mechanisms based on in vitro and in vivo test results. [A.A. Roberts; Compl alt appr to Biomedicine]

4.1 Classification of anxiety disorders

The Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR) includes the following conditions under the heading of anxiety disorders: acute stress disorder, post-traumatic stress disorder, social phobia and specific phobia. These disorders vary in their severity and symptomatology, as well as treatment responses. [A.A. Roberts; Compl alt appr to Biomedicine]

There are five different forms of anxiety disorders which are:

- panic disorder (PD),
- obsessive-compulsive disorder (OCD),
- social anxiety disorder (SAD),
- post-traumatic stress disorder (PTSD)
- generalized anxiety disorder (GAD).

(This thesis main focus is on generalized anxiety disorders.)

Due to research it is now understood that anxiety disorders are mediated by autonomic and neuroendocrine systems that are under the control of the central nervous system (CNS). The modulation of normal and pathologic anxiety states is associated with multiple regions of the brain and abnormal function in several neurotransmitter systems, including norepinephrine (NE), gamma-aminobutyric acid (GABA), serotonin (5-HT), corticotrophin-releasing factor (CRF) and cholecystokinin. Therefore, the system of action of anxiety disorders in the central nervous system has been characterized and gives the impulse for research and development of anxiolytic drugs. [A.A. Roberts; Compl alt appr to Biomedicine], [Pharmacotherapy: A Pathophysiologic Approach, Chapter 73…]

In the search for benzodiazepine site ligands with higher therapeutic selectivity and a reduced side effect profile, GABA$_A$-receptor subtypes have long been considered to be promising targets [H. Möhler et al]. Therefore, pharmaceutical research in that area aims to develop selective anxiolytics without a sedative component and the major side effects known of benzodiazepines. Anxiolytic agents are evaluated by behavioral tests using animals that are exposed to environmental stressors in controlled laboratory conditions.

4.2 GABA$_A$-Receptors and Anxiety

The pathophysiologic mechanisms underlying anxiety are not yet completely determined. GABA (γ-aminobutyric acid) is a neurotransmitter in the central nervous system that has a dampening influence on neuronal activity by protecting neurons from toxic overstimulation. This neurotransmitter has shown to be involved in the system of anxiety disorders. The GABA receptors are the point of action for GABA. There are two superfamilies of GABA protein receptors: GABA$_A$ and GABA$_B$. With regards to anxiety disorders the effect of GABA takes place mainly on the type A GABA receptors (GABA$_A$) which are widely located throughout the CNS. They are also the main targets for several classes of anxiolytic and sedative drugs as benzodiazepines (e.g. diazepam), barbiturates or alcohol. These drugs potentiate the activity of GABA$_A$-receptors by allosteric stimulation of the inhibitory effect of GABA on these receptors.

GABA$_A$ receptors are ligand-gated trans-membrane ion channels [fig. 1]. They consist of five trans-membrane protein subunits that surround a central pore which is a chloride-conducting ion channel.

Activation of these receptors leads to an increased chloride influx which results in a hyperpolarized membrane and hence neuronal inhibition occurs. Subunits are 6α, 3β, 3γ, δ, ε, θ and π. GABA$_A$ receptors containing $α_{1-3}β_{2/3}γ_2$ in a 2:2:1 stoichiometry represent the major GABA$_A$ subtype in the mammalian CNS. However, the determining factor of the GABA activity is merely the identity of the α subunit(s). The benzodiazepines binding site is located at the interface of an α and a γ subunit, therefore the identity of both subunits have an influence on the activity of BZD site ligands.

Studies have shown that α2-subunit containing receptors mediate an anxiolytic effect. Anxiolytic, sedative and amnesic properties of diazepam are mediated by distinct subtypes of GABA$_A$ receptors. Therefore, drugs have been developed to be subtype-specific in order to avoid undesirable effects. The focus of research considering new anxiolytic drugs has been put in order to have a maximum of anxiolytic activity while reducing undesirable sedative and amnesic effects. Studies using genetic engineering techniques (e.g. Knock-out, knock-in) have been conducted to determine the GABA$_A$ receptor complex in the pathophysiology of anxiety. Neuroimaging studies in humans with anxiety disorders have reported reductions in GABA levels and GABA$_A$-benzodiazepines receptor binding sites so there is considerable evidence for a connection between GABA$_A$-dysfunction and the pathophysiology of anxiety disorders.
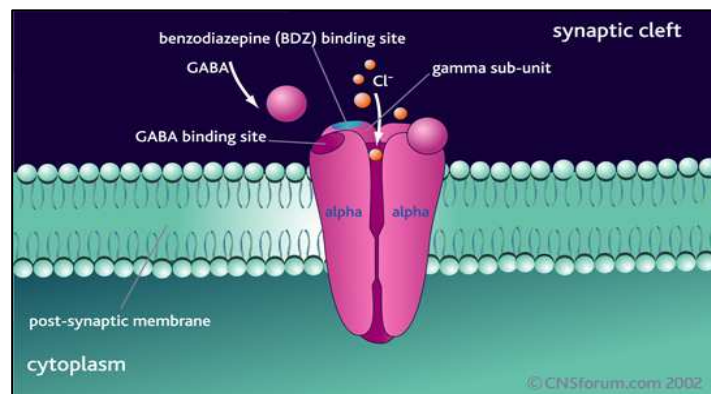

Fig. 1: GABA$_A$ receptor

http://www.cnsforum.com/imagebank/section/Receptor_Gabanergic/default.aspx

10

4.3 Drug Therapy of Anxiety Disorders

The desired outcome of the treatment of anxiety disorders is to reduce the severity and duration of the anxiety symptoms while improving the overall functioning. Therefore, psychotherapy should be considered for all patients. Standard therapy consists of a combination of psychotherapy and drug therapy. Antianxiety medication is indicated in case of functional disability.

Due to comorbid depressive symptoms, antidepressants such as selective-serotonin-reuptake-inhibitors (SSRIs) emerged as the treatment of choice and became first-line drugs. However a therapy with antidepressants shows an optimal response after 6 to 8 weeks or longer.

Second-line drugs and most commonly prescribed drugs are on $GABA_A$-receptor interacting benzodiazepines. They provide rapid relief in acute situations as an effect is shown to the initial treatment after 2 to 4 weeks. Benzodiazepines reduce somatic symptoms but they do not have any effects on psychic symptoms which can be relieved with antidepressants. All benzodiazepines are equally effective anxiolytics but their pharmacokinetic varies. The pharmacologic effect of benzodiazepines is based on an allosteric binding to the $GABA_A$ receptor which leads to conformational changes in the GABA binding site, thereby increasing the affinity of the receptor for GABA. The frequency of chloride channel openings is increased which leads to neuronal hyperpolarization and therefore central nervous depression. Long-term use of benzodiazepines has shown to lead to dependence, withdrawal symptoms and impairment of the memory. Other adverse effects which may occur are sedation and drowsiness.

Additionally benzodiazepines do also have a high abuse potential. Therefore it is necessary to find anxiolytic drugs with less central nervous system related side effects.

Some herbal medicines have shown to possess anxiolytic like effects which lead researchers to conduct numerous studies approaching this topic. Valerian is one of these plants. A lot of studies have been already conducted on Valerian's sleep enhancing but less on its anxiolytic properties.

4.4 Botanical Characterization

Botanical nomenclature: *Valeriana officinalis L., s.l*.
Botanical family: Valerianaceae

The name Valerian is said to be derived either from Valerius, the first who reportedly utilized the plants medicinal properties, or from velere, the Latin term for health or well-being. Valerian has been used medicinally for at least 2000 years. Dioscorides (ca. AD 40-80) already wrote about several Valerian species and Galen reported sedative effects. One of its first utilization was as a treatment for epilepsy in the late 16th century and became routinely used for the treatment of various nervous disorders. In the late 19th and early 20th century Valerian was considered to be sedative and to have an activity as a cerebral stimulant as well as having an analgesic effect. It was used as a treatment of hysteria, epilepsy and menopausal nervous anxiety. [Amer Herb Pharmac - Monograph]

Valeriana officinalis is an herbaceous plant that can reach about one meter in height and is cultivated in many European countries, as well as in Japan and North America. The root, rhizomes and stolons are the pharmaceutically used parts of the plant. Valerian root is known for its characteristic, usually as unpleasant described smell. [Heinrich M et al]
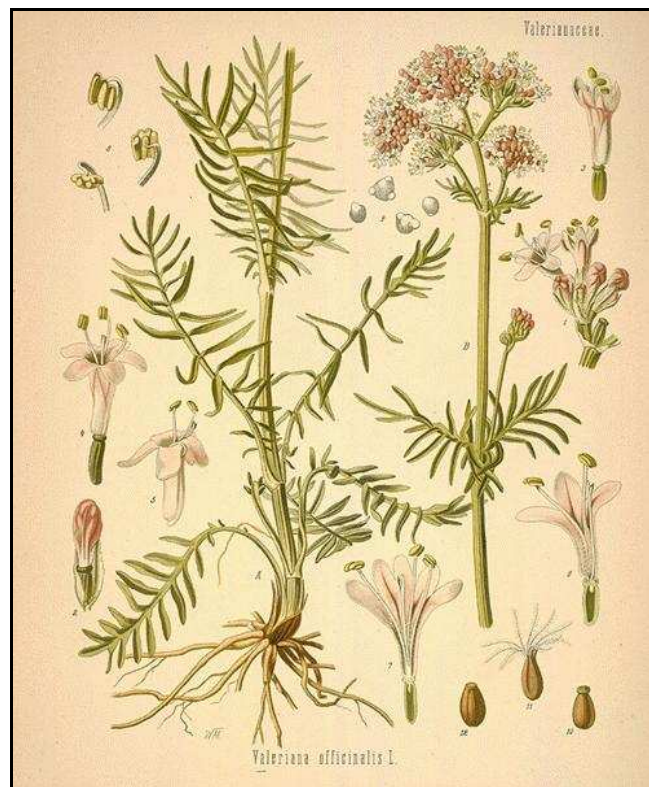

Fig. 2: Valeriana officinalis L.

Fig. 3 Valeriana officinalis L.

http://www.botanikus.de/Heilpflanzen/Baldrian/baldrian.html

4.4.1 Constituents

The roots of Valerian are used for therapeutic purposes. According to divers Pharmacopoeia, Valerian roots are defined as dried underground parts of *Valeriana officinalis* L., s.l. including the rhizome, the roots and stolons [ESCOP Monographs, USP]. The roots contain several compounds some of with demonstrable pharmacological activity as [Am Herb Pharmac]

- essential oils, sesquiterpenes,
- volatile sesquiterpene carboxylic acids (valerenic acid and derivatives)
- flavonoids: 6-methylapigenin, linarin, hesperidine
- amino acids: GABA, glutamine and arginine, tyrosine
- alkaloids

and small amounts of:

> phenolic acids, chlorgenic acid, caffeic acid, choline, ß-sitosterol, fatty acids and various minerals

[Am. Herb. Pharmac], [Hänsel, Sticher]

The Pharmacopoeia Europaea (Ph. Eur.) monograph specifies that valerian root contains not less than 5 ml/Kg of essential oil for the whole drug and not less than 0.17% of sesquiterpenic acids expressed as valerenic acid. Everything was calculated with reference to the dried drug. [ESCOP]

4.4.2   Indication

Therapeutic indications for Valeriana radix are for relief of temporary mild nervous tension and/or difficulty in falling asleep. The drug is used for oral administration e.g. as a tea infusion. One of the main advantages of using Valerian root as a sedative is that there is no dependence nor withdrawal symptoms reported thus no restrictions on the duration of administration. There is also no impairment on vigilance which was shown in several studies. No hangover effect has been shown eight hours after taking the preparation. Neither repeated evening administration for 14 days of 600 mg of valerian root extract (comply with 3 g of drug) showed any impairment of the vigilance the morning after. Furthermore no adverse effects have been confirmed, although there were benign symptoms observed at an overdose of Valerian root according to 20 g like fatigue, abdominal cramp, chest tightness, light headedness, hand tremor and mydriasis. [ESCOP]

4.4.3   Anxiolytic Properties

Valerenic acid (VA) [fig. 4] has been identified as a β-subunit specific GABA$_A$ receptor modulator by a study performed at the University of Vienna on Xenopus laevis oocytes. It is suggested that VA interacts with the binding site of anesthetics such as loreclezol or etomidate [S. Khom et al. 2007, G. Trauner et al. 2008]. Therefore, it is supposed that VA is responsible for Valerian's sedative, sleep-enhancing and anxiolytic properties. However, Valerian extracts might contain additional modulators of GABA$_A$ receptors. Various compounds have been discussed to be also responsible for the CNS-depressant effect. It is still uncertain what the actual active compound might be. Flavonoids such as apigenin [fig. 5] and linarin [fig. 6] did also show sleep-enhancing, sedative and anxiolytic-like properties in animal models. Data of a study published by G. Trauner et al in 2008 suggests that the extent of GABA$_A$ receptor modulation by Valerian extracts is related to the content of VA. It should be considered that many other compounds play an important role in the efficacy of *Valeriana officinalis* L.

In vitro experiments of various valerian root extracts have shown that some interact with GABA chloride channel complex. Additionally the extract inhibits the presynaptic re-uptake and induces the release of radiolabelled GABA. A receptor binding of various constituents on adenosine receptors as well as on serotonin receptor subtypes has been observed which might also have an influence to increased presynaptic neurotransmitter concetration. [Hänsel, Sticher 2010] Valerian extracts interact with GABA$_A$ receptor complexes on a different site than diazepam. [ESCOP] The binding sites of VA and the BZDs like diazepam are independent of each other thus studies proved VA to be β-subtype specific where as benzodiazepine binding site is supposed to be at the interface

between the α and γ subunit. Additive effects have been observed when VA was coapplicated with diazepam. [S. Khom et al 2007].

Diazepam permeates quickly the blood-brain barrier through passive diffusion, therefore an effect can be observed within short time. Whereas VA permeates slowly and its transport systems are still unclear, an effect can be observed only after two to four weeks of therapy. [Hänsel, Sticher 2010]

In vivo experiments of isolated compounds such as valerenic acid, valeranal and valeranone, which were administered by intraperitoneal (i.p.) injection, showed a central depressive and/or muscle relaxant activity in mice. After i.p. administration of valerenic acid to mice of 17 – 25 g body weight 400 mg/Kg caused heavy convulsions leading to the death of 6 out of 7 mice within 24 hours. [Khom S. et al] The mechanism of action and the substances responsible for the already mentioned CNS-effects are like for many other herbal medicines yet not fully determined. In vivo studies need to be conducted to investigate anxiolytic properties of isolated Valerian compounds.

Several studies with natural and synthetic flavones and flavanons have shown that they can modulate GABA-induced chloride current, either positively or negatively. Linarin is reported to have sedative effects therefore it is assumable that it might also have anxiolytic properties.

Camomille tea has been traditionally used as a relaxing tea. Its relaxing properties have been attributed to the flavonoid apigenin, which can also be found in *Valerina officinalis* L. Ethological studies testing apigenin have shown an anxiolytic like effect but no sedative side effects, although in-vitro studies did not prove any anxiolytic properties [Viola H et al 1994]. Apigenin has been shown to be a non-competitive antagonist of $GABA_A$-receptors but its inhibitory effect could not be blocked by benzodiazepine antagonist flumazenil. However, apigenin seems to enhance the actions of diazepam which might be therapeutically interesting, given that the diazepam dose could be reduced. [Campbell EL et al 2004]

Flavonoids are natural compounds that are highly abundant in all higher plants. They are of an immense chemical diversity and have been described to have a wide range of biological activities such as CNS-mediated activities. A clear anxiolytic effect of flavonoid glycosides with no signs of sedation could be observed in the EPM. [Fernandez SP et al, Viola H. et al] Since flavonoids have been used for generations and are found in our diet, it is unlikely that they would have side effects. [Campbell EL et al]
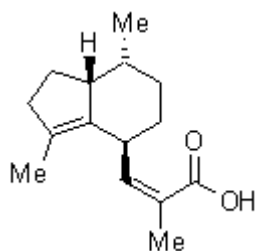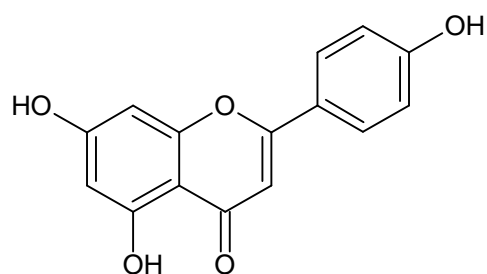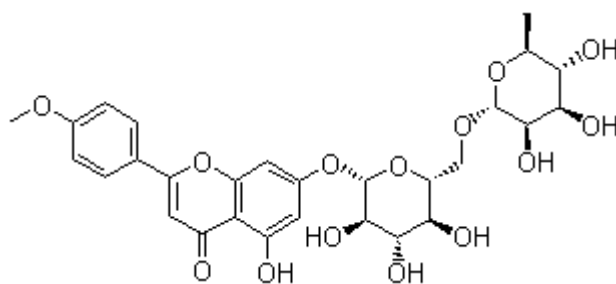
Fig. 4: Valerenic acid

Fig. 5: Apigenin

Fig. 6: Linarin

5 Materials and Methods

The compounds used in this study were diluted in a propylene glycol solution. In most studies, performed to prove the anxiolytic properties of VA in animal models the solutions were administered i.p. In this study, the solutions have been given orally since it is the most common way to administer Valerian drugs.

5.1 Animal Models

Animal models of anxiety examine the natural behavioral pattern of rodents in which animals are exposed to an aversive/threatening environment such as open, elevated arms of the EPM. [Garner M et al] An ideal animal model must fulfill three criteria:

- *Predictive validity: pharmacological treatments known to be effective in humans should induce comparable effects in animals*

- *Face validity: the responses or symptoms observed in patients should be the same in the animal model*

- *Construct validity: both humans and animal models should underlie the same basic principle*

Meeting all three criteria is difficult as there are many forms of pathological anxiety. Therefore, it is recommended to use more than one animal model to assess anxiolytic like properties of drugs. [Ohl F.]

In this thesis two animal models, the elevated plus maze test and the marble burying test, have been chosen to test anxiolytic like effects since both measure different parameters. The elevated plus maze is one of the most frequently used tests for unconditioned anxiety and its reliability has been proven in several studies. This test is based on the observation that rodents tend to avoid elevated and unprotected areas. [Ohl F] Another test performed in this study was the marble (defensive) burying test. Defensive burying can be observed in many rodents in response to an aversive stimulus. The marble burying test was developed to take advantage of this innate behavior to evaluate how many innocuous but novel glass marbles a rodent would bury. [Thomas A et al] Examining the response to previously harmless stimuli have been proven useful in modeling defensive behavior to fear extinction observed in patients with posttraumatic stress disorder (PTSD), specific and social phobias. [Garner M et al]

5.1.1 Elevated Plus Maze

Anxiolytic activity was measured using the elevated plus maze test. The maze consisted of two open (31 cm x 5 cm x 1 cm) and two closed (31 cm x 5 cm x 15 cm) arms, extending from a central platform (5 cm x 5 cm) and elevated to a height of 40 cm above the floor [Fig 7]. On the wooden base frame, the closed arms were made of opaque plastic, while the open arms were made of the same material with a slight elevated edge. Mice were individually placed on the center of the maze facing a closed arm and the number of entries and the time spent in closed and open arms were recorded during a 6 minutes observation period. Arm entries were defined as entry of all four paws into an arm.

The percentage of the time spent on open arms (100 x open/total time) was calculated for each animal. The EPM is claimed to be an ethologically valid animal model of anxiety because it uses natural stimuli that can induce anxiety in humans [Pellow S et al]. It is assumed that the open arms of the maze combine the fear of a novel, brightly-lit open space and the fear of balancing on a narrow, raised platform. The closed arms have high walls forming a narrow alley that offers good protection against predators. When a rat or mouse is allowed to explore the EPM freely, for a certain time (usually 6 minutes), it spends only 20-25% of the time exploring the open arms. The measures of anxiety are the number of open arm entries expressed as a percentage of the total number of arm entries and the amount of time spent on the open arms. In the potentially dangerous situation of the EPM, rodents display a range of behaviors that could be interpreted as the assessment of the potential risk. These include head dipping and stretch-attend postures. These actions can take place in the closed arms (protected) or on the open arms (unprotected). This behavior can be characterized by open arm avoidance with a consistent preference for the closed arms. The rank order preference profile is

closed > centre > open. Together with several other measures of hesitancy and inactivity, these behaviors are known as risk assessment behaviors [Bourin M et al]. Anxiolytic compounds appear to decrease risk assessment behaviors and increase unprotected activity. The simplicity of this test is considered one of the main advantages of the EPM. [Lister RG, 1987]
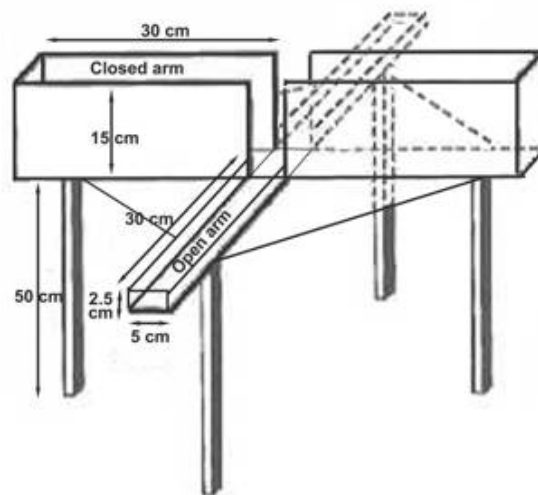

Fig. 7: Elevated Plus Maze Apparatus
http://www.infolizer.com/?title=Elevated+plus+maze

Setting

Two mazes were placed directly side by side so that a closed arm of one component adjoined to a closed arm of the other component. Tape was used to fix the construction. The floor lamps were placed between the open arms on each side of the maze. The lamps are directed towards the ceiling and not at the maze to reduce the formation of shades. During the experiment only the floor lamps are turned on while the ceiling lights are turned off. The video camera is fixed above the maze. The video camera is connected to the computer so if the camera is in the right position can be checked with the software "Directshow Encode", which is used to record the whole experiment.

5.1.2   Marble-burying test

Defensive burying is interpreted as unconditioned, species-specific response towards certain olfactory, tactile and visual stimuli, which will elicit avoidance behavior under appropriate conditions [Treit D et al]. This test consists of a plexiglas cage of 23 x 17 x 14 cm with a smooth lid punctured with small ventilation holes [Fig. 8]. The floor is covered with a 5 cm layer of sawdust and 25 glass marbles are placed in contact with each other in the center of the cage. Various studies about MBT also suggest placing the marbles in different ways [Bourin M et al]. The mouse is placed in the cage for 30 minutes after which it will be removed and the burying response will be quantified by counting the number of marbles that are more than two thirds covered with sawdust. A diminution of the burying reflex reveals a positive anxiolytic-like effect. However, questions regarding the specificity of marble burying as an indicator of anxiety came up due to studies showing a decrease in marble burying after application of antipsychotic drugs. Several studies suggest the marble burying behavior is rather associated with digging behavior than with defensive burying and reflects a more obsessive-compulsive like behavior rather. Therefore, this test has been performed only. It was considered that it was difficult to interpret the results of this test given that marble burying may be simply a by-product of digging behavior.. [Bourin M, 2007, Treit D, 1981].
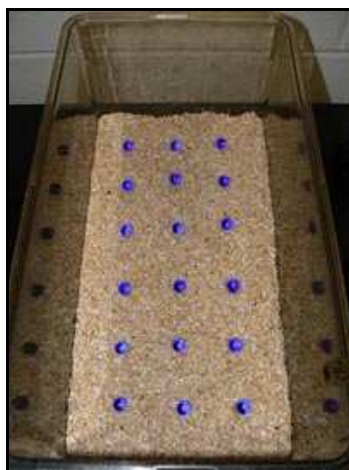
Fig. 8 MBT – cage with aligned marbles

5.2 Animals

Male BL6/C57J mice between 6-12 weeks old and weighing 18-30 g were purchased from Harlan (Indianapolis, IN, USA). Mice were housed in cages of 5 at 20 ± 1°C in a 12-h light/dark cycle. Tap water and standard food pellets were available ad libitum. Groups of 12 to 14 mice were randomly assigned to different treatment groups and tested in a varying order. Animals were tested repeatedly under the same experimental conditions. All experiments were carried out in a quiet room under controlled light conditions between 9:00 a.m. and 2:00 p.m. due to the reversed circadian rhythm and secretion of endogenous cortisol in rodents. All animals were housed and all experiments performed according to the policies and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida, Gainesville, U.S.A.

5.3 Drugs

Diazepam ampoules (5 mg/ml Hoffmann-La Roche, Basel, Switzerland) were used as reference drugs. Deionized water (Millipore quality) containing 0.5% propylene glycol (Fisher Scientific, Inc; Fair Lawn, NJ, USA) was used as control solution. Diazepam was diluted to 1.5 mg in 10ml deionized water containing 0.5% propylene glycol. Four different concentrations (0.25, 0.5, 1, 2 mg/Kg body weight) of the valerenic acid (Institute of Pharmacognosy, University of Vienna) were prepared by in 10 ml deionized water with 0.5% propylene glycol to form a homogenous suspension.

Flavonoids as linarin and apigenin were also tested (Indofine Chemical Company Inc., Hillsborough, NJ, USA. Three concentrations of each flavonoid (linarin; 2.5, 5, 10 mg/Kg and apigenin; 1, 3, 6 mg/Kg) were prepared in 10 ml of the 0.5% propylene glycol solution and put in the sonicator for 10 minutes. Apigenin and linarin were non-soluble in this solution, therefore the flavonoid samples were administered as a suspension. All solutions were prepared freshly on the respective test day and administered orally (p.o.) 60 minutes before testing by a feeding needle in a volume of 0.1 ml/10g body weight of mice. Previous data from our lab has shown that the drug application 60 minutes prior to the tests yields the most reliable and reproducible results with diazepam compared to 30 minutes or 2 hours [Grundmann O. et al].

5.4 Data analysis

The EPM test was videotaped using the high-resolution video camera WV-CP244 (Panasonic, Secaucus, NJ, USA). The computerized analysis of the videos was performed using TopScan, Top View Animal Behavior Analyzing System (version 1.00, Clever Sys Inc. Preston, VA, USA).

Fig. 9: Computer analysis of the EPM - TopScan software, Picture shows the standard setup in our laboratory. Two mice are tested simultaneously.

5.5 Statistics:

Calculation of the percentage of time and number of entries on the open arms with 95% confidence limits and comparisons of the results were performed using GraphPad Prism (version 5.00, GraphPad Software Inc., San Diego, CA, USA). The statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keul's multiple comparison test. In all cases differences were considered significant if $p < 0.05$.

5.6 Experimental design

Group	Treatment	N per group
1	Control (vehicle; 0.5% propylene glycol in deionized water, treatment oral)	12
2	Diazepam 1.5 mg/Kg	12
3	Valerenic acid 0.25 mg/Kg	12
4	Valerenic acid 0.5 mg/Kg	12
5	Valerenic acid 1 mg/Kg	12
6	Valerenic acid 2 mg/Kg	12

Group	Treatment	N per group
1	Control (vehicle; 0.5% propylene glycol in deionized water, treatment oral)	12
2	Diazepam 1.5 mg/Kg	12
3	Apigenin 1 mg/Kg	12
4	Apigenin 3 mg/Kg	12
5	Apigenin 6 mg/Kg	12

Group	Treatment	N per group
1	Control (vehicle; 0.5% propylene glycol in deionized water, treatment oral)	12
2	Diazepam 1.5 mg/Kg	12
3	Linarin 2.5 mg/Kg	12
4	Linarin 5 mg/Kg	12
5	Linarin 7.5 mg/Kg	12

5.7 Experimental Run

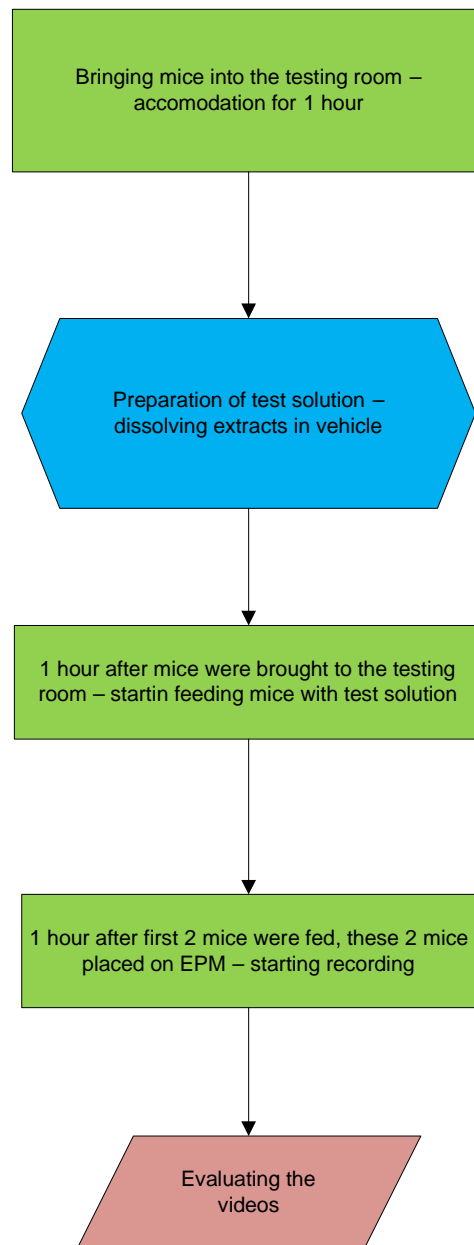The following flow chart shows the experimental run on one day.

Fig. 10: Experimental run

6 Results

One-Way Analysis of Variance revealed that mice treated with valerenic acid at a dose of 0.5 mg/Kg tended to enter more frequently into the open arms. Additionally they spent more time outside the closed arms in comparison to the control group. The number of open arm entries decreases with each higher concentration indicating an inverted-U-shaped dose response curve. There were no significant differences neither in the number of open arm entries nor in the time spent on the open arms at the doses of 1 and 2 mg/Kg to the control group. The lower dose of 0.25 mg/Kg had the lowest number of open arm entries and the least time spent on the open arms. There were no significant differences on the distance set back [Fig 11 - 13].

Diazepam showed a significant increase on open arm entries and on the time spent on the open arms versus the control group.

Apigenin and linarin showed neither an increase of open arm entries nor on time spent on open arms compared to the control group [Fig. 14 - 19].
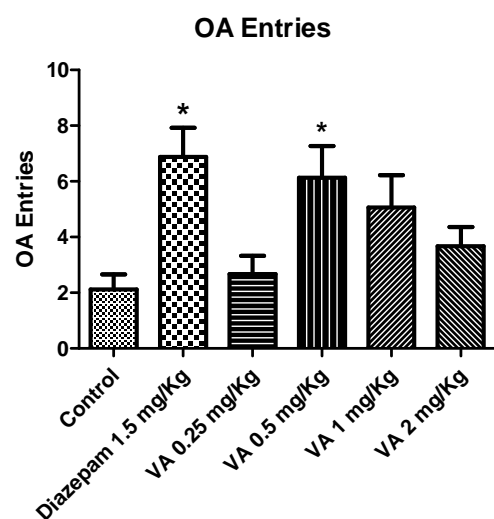
OA Entries

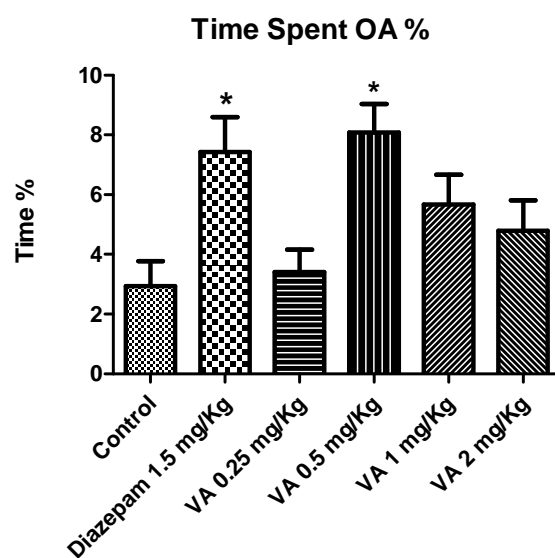Fig. 11: Number of open arm entries after VA application

Time Spent OA %

Fig. 12: Percentage of time spent on OA after VA application
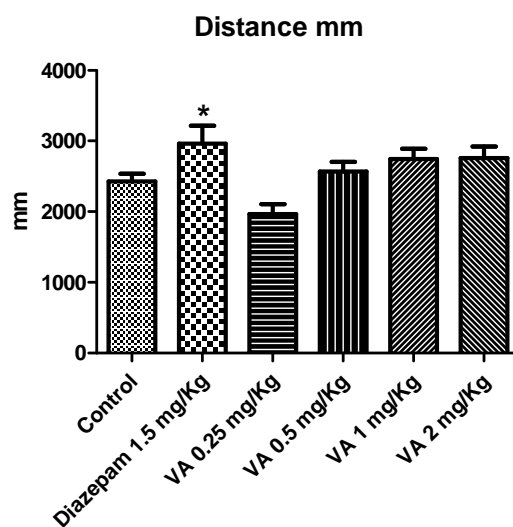
Distance mm

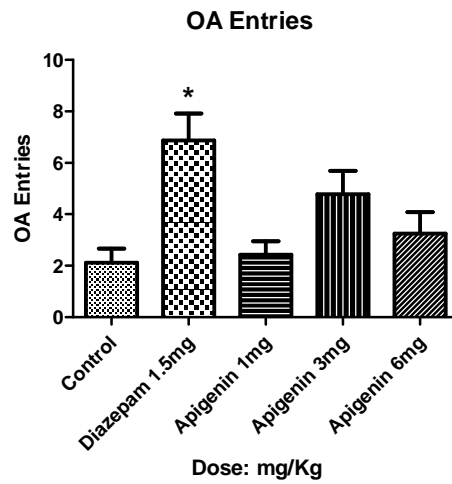Fig 13: Distance in mm set back in 6 min after VA application

31

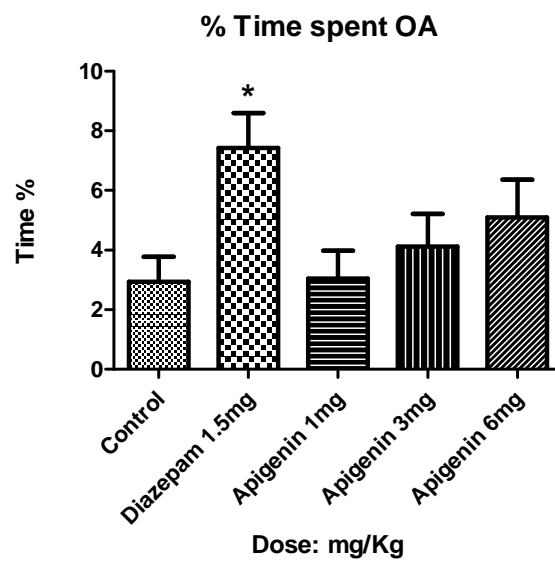Fig. 14: Number of open arm entries after application of apigenin

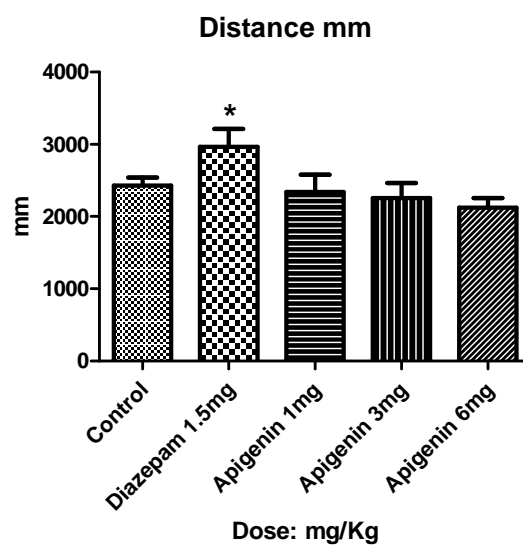Fig. 15: Percentage of time spent on OA after application of apigenin

Fig 16: Distance in mm set back in 6 min after application of apigenin
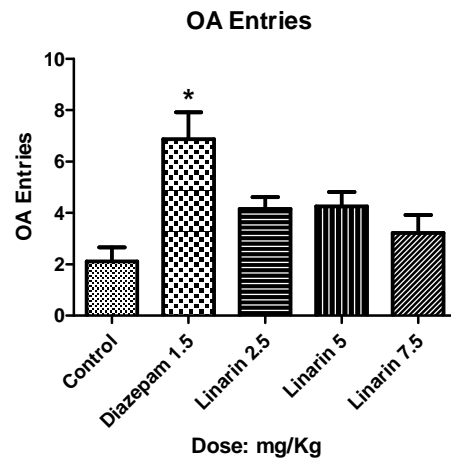
32

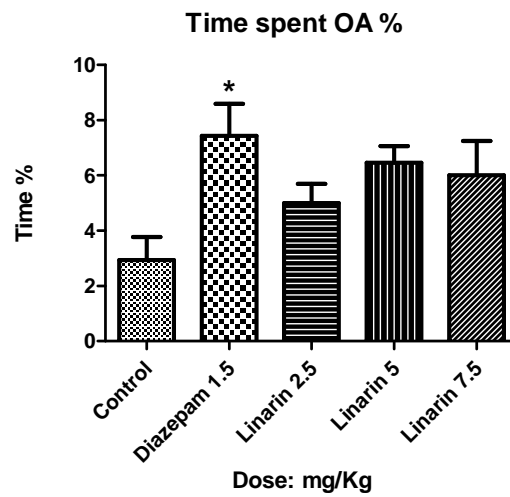Fig. 17: Number of open arm entries after application of linarin

Fig. 18: Percentage of time spent on OA after application of linarin
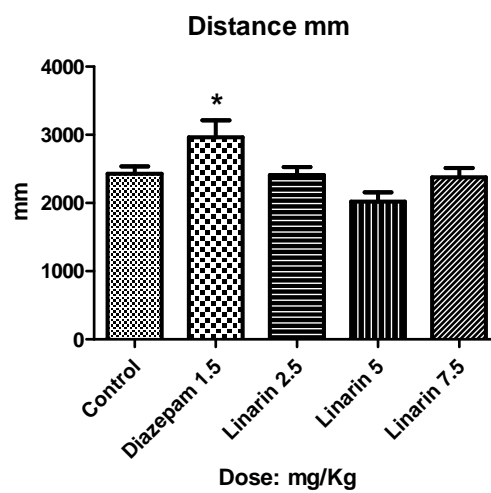

Fig 19: Distance in mm set back in 6 min after application of linarin

7 Discussion and Conclusion

Research of valerian is exclusively focused on its sedative and spasmolytic properties. Furthermore no single constituent has been shown to be responsible for valerian's total action. Initially it was believed that the essential oil was the component responsible for the sedative effect of valerian but nowadays it is known that the essential oil is accounted for only one-third of the sedative activity of the extract. Valepotriates were also excluded as the active component. Therefore, in the present study we focused on other constituents which have already been proven [Khom et al] to have an effect on the central nervous system such as isolated compounds as valerenic acid and different flavonoids. Valerenic acid and acetylvalerenic acid have been reported to inhibit GABA transaminase which as a result prolongs the inhibitory effect of GABA [Am Herb Pharmac]. Benzodiazepines exert their actions via the GABA-ergic system. Valerian extracts contain amino acid GABA in insufficient quantity to account for the activity but GABA does not cross the intact blood-brain barrier. Valerenic acid has been proven to interact with the $GABA_A$-receptor complex but independent to the BZD binding site. [Khom S, 2007] A few other studies focused on the anxiolytic effects of the flavonoids apigenin and linarin which can as well be found in valerian.

Animals cannot model every aspect of human anxiety but studies in animals permit detailed investigations for neurobiological and psychological processes in states of fear. Due to divers mechanisms mediating anxiety there are different animal behavior models which are more appropriate to one type of anxiety. The manner in which animals react to threatening stimuli or situations distinguishes considerably to the human defensive behavior. There are basically two types of animal behavior models used to detect

the anxiolytic effect of drugs. Models can be based on conditioned or unconditioned behavior. Whilst models relying on conditioned behavior need specific trained animals, the model based on unconditioned behavior relies on natural behavioral reactions. Unconditioned models have a higher degree of validity and are easier to conduct given that animals need not to be trained. Potential threats that lead to unconditioned responses are amongst others social interaction, light/dark exploration or defensive burying. The elevated plus maze is an unconditioned model used to study anxiolytic effects of drugs for generalized anxiety disorders. This test permits a rapid screening of anxiety-modulating drugs without training or involvement of complex schedules. [Bourin M, et al] The elevated plus maze is considered to be an etiologically valid animal model of anxiety. An anxiolytic agent increases the frequency of entries into the open arms and increases the time spent on open arms of the EPM. [A.A. Roberts]. The validity of the marble burying test is controversial since it shows no selectivity for anxiolytics. An alteration on marble burying could be observed for anxiolytics but also for antipsychotics. Studies have been conducted to improve the predictive validity of the marble burying test; by measuring locomotor activity using a video tracking system or by using specific mouse strains. Some strains show more burying activity than others. It cannot be excluded that strain-specific pharmacodynamic and/or pharmacokinetic properties influence behavior in MBT. [Nicolas LB et al]

In the present study different concentrations of the isolated compounds valerenic acid, apigenin and linarin were orally administered to mice. An anxiolytic effect could be observed for valerenic acid at the dose of 0.5 mg/kg since the frequency of the open arm entries was increased to the control group. An anxiolytic effect could be observed neither at a lower dose nor on higher doses. Interestingly the number of open arm entries decreases with increasing dose. It is assumed that an open channel block due to a higher dose of valerenic acid might be the reason. [S. Khom, et al.]. The flavonoids apigenin and linarin did not show an anxiolytic effect at any dose in the EPM. It is still uncertain which component of valerian induces the anxiolytic effect. Valerenic acid shows an anxiolytic effect, though studies suggest a possible synergism between multiple active compounds. A co-application of VA and linarin showed a potentiating effect [S. Khom, AA Roberts]. Therefore, further studies on synergism between valerian compounds could offer promising results.

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9 Figure legend

Fig. 1: GABA$_A$ receptor

http://www.cnsforum.com/imagebank/section/Receptor_Gabanergic/default.aspx

Fig. 2: *Valeriana officinalis L.*

http://www.botanical.com/botanical/mgmh/v/valeri01-l.jpg

Fig. 3: *Valeriana officinalis L.*

http://www.botanikus.de/Heilpflanzen/Baldrian/baldrian.html

Fig. 4: Valerenic acid

http://www.organische-chemie.ch/chemie/2007jun/valerensaeure.shtm

Fig. 5: Apigenin, drew with ChemSketch

Fig. 6: Linarin

http://www.chemblink.com/products/480-36-4.htm

Fig. 7: Elevated Plus Maze Apparatus

http://www.infolizer.com/?title=Elevated+plus+maze

Fig. 8: MBT – cage with aligned marbles

http://www.cincinnatichildrens.org/research/cores/abc/marble.htm

Fig. 9: Screenshot of TopScan software, standard setup in our laboratory

Fig. 10: Flow chart: experimental run made with MS Visio

Fig. 11 – 19: Results: Graphs on the results exported from GraphPad Prism 5

10 Curriculum vitae

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