Research Paper

Brain Targeted Transcranial Administration of Diazepam and Shortening of Sleep Latency in Healthy Human Volunteers

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Application of medicated oils on scalp had been practiced for centuries in the *Ayurvedic* system of medicine in diseases associated with the central nervous system. It is possible that the effectiveness of the therapy may be a result of targeted delivery of active compounds to the brain transcranially. Evidence also comes from two previous studies with positive results on brain targeted transcranial delivery of methadone base and diazepam on rat models. Possibility of transcranial drug delivery was investigated in healthy human volunteers using electroencephalography techniques by assessing the ability of transcranially administered diazepam in bringing about β activity in the electroencephalographic wave patterns and shortening of the sleep latency period. Non polar drug molecules dissolved in a non-aqueous sesame oil based vehicle is a significant feature in the transcranial dosage design. The study was under taken in two phases. In the Phase-I study scalp application of a single dose of 2 mg/3 ml of the oil was employed and in the Phase-II study repeat application of three doses 24 h apart were employed. Sleep latency changes were monitored with Multiple Sleep Latency Tests with 5 naps employing the standard electroencephalography, electroocculography and electromyography electrodes. Sleep onset was identified with the first epoch of any sleep stage non rapid eye movement 1, 2, 3, 4 or rapid eye movement using electroencephalography, electroocculography and electromyography criteria. In both phases of the study there was significant reduction in the sleep latencies. It was much more pronounced in the Phase-II study. None of the subjects however displayed beta activity in the electroencephalography. Sleep latency reduction following scalp application in both the phases are suggestive of transcranial migration of diazepam molecules to the receptor sites of the nerve tissue of the brain eliciting its pharmacological effect of sedation. Transcranial brain targeted dosage design is therefore feasible.

Key words: Brain targeted, electroencephalography, emissary veins, diazepam, sesame oil, sleep latency, transcranial

The transcranial administration of drugs in the *Ayurvedic* system of medicine has been practiced for centuries and over the years it has been further specialized into a number of techniques known as *Shirodara, Shiropichu, Shiroabyanga, Shirovasthi,* and *Shiropralepa*[1]. The term *'shira'* or *'sheersha'* in *Sanskrit* means the head or the cranium in man and hence the term 'transcranial' was adopted for the new route of brain targeted drug administration.

In order that the medicinal oils have pharmacological effects when tanscranially targeted at the brain, the compounds should have actions based on the CNS and they should be absorbed from the skin of the scalp directly into the brain across the cranial bones. The absorbed drug molecules must reach the receptor sites in the brain. The lipophilic sebum secreted by the sebaceous glands consisting of triglycerides, waxes, cholesterol and their esters is a particularly attractive medium[2] for the oil solution of a drug to form a continuous lipid medium straight into the gland invaginations of the skin. The continuous fatty medium facilitates the migration of oil soluble compounds such as diazepam towards the gland and hair follicle epithelium for absorption. Once absorbed through the skin of the scalp the non polar drug molecules could enter the blood vessels, in particular the emissary veins that drain blood directly into the brain from

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the extracranial sites of the scalp. There are thirteen pairs of emissary veins that pierce the cranial bones through a series of foramina connecting extracranial sites of the scalp with the seven intracranial sinuses of the brain that anastamose among each other[3]. In one of the studies the transcranial blood flow across the bones of the scalp had been demonstrated on two fresh cadavers where the calvaria was removed with scalp adhering. Massaging of the scalp produced abundant drops of blood on the inner surface of the bone demonstrating that cutaneous blood can flow inward through the bone[4]. The drug molecules could also diffuse across the flat cranial bones along with the diploe due to the presence of vascular haversian canals that criss-cross the bones imparting porosity to the bone structure[5]. Ability of drugs to diffuse through the substance of the bone has been supported in a study in which diazepam has been detected in femoral bone tissue following the administration of the drug in rats by intraperitonial route[6]. Diffusion is also possible through the cranial bone sutures. On reaching the intracranial sinuses the drug molecules could diffuse into the nerve tissue of the brain. The blood flow is particularly sluggish in the sinuses providing ample time for the diffusion of drug molecules into the brain tissue[7]. Emissary veins are present in all higher animals starting with the aves[8].

Two centrally acting drugs using three pharmacological screening methods yielded positive results in rat model brain targeted transcranial drug delivery studies. They included diazepam in sesame oil where centrally mediated muscle relaxant effects were measured with rota rod tests[9] and methadone base dissolved in sesame oil where antinociceptive effects were measured with the hot plate and tail flick tests[10].

In the present study electroencephalography (EEG) studies were used for assessing the pharmacological effects of diazepam in humans. The basis for selecting EEG studies is the ability of diazepam to bring about diffuse β activity and to reduce the sleep latency[11,12]. Such tests provide standard, instrumentally determined and quantifiable results of the pharmacological effects of diazepam on human brain. More over it is a non invasive method of determining the effects of diazepam on the nerve tissue of the brain. As the transcranial delivery of diazepam is targeted to the brain it is unlikely for the drug to achieve any possible detectable concentration in the systemic blood circulation by the routine methods of analysis and therefore blood level detection was not attempted in the present study. An important aspect of the transcranial drug delivery is the employment of a non aqueous oil based drug solution prepared by dissolving non polar diazepam drug molecules in sesame oil. This is in contrast to the polar water soluble salt forms of drugs dissolved in aqueous media popular with the modern formulations.

The main objective of the study is to establish the novel route of brain targeted drug delivery in human subjects through transcranial administration of diazepam by demonstrating the sedative effect of the drug on the brain. This study also serves to assess the feasibility of using diazepam in sesame oil by transcranial route and the ability to evaluate the effects by EEG techniques.

MATERIALS AND METHODS

Application of the transcranial medicated oil was carried out in a quantified manner. The important aspects of the application and the EEG monitoring at various stages are described in the following sections.

Selection of subjects:

Ethics clearance for the study was obtained from the Ethics Review Committee of the Faculty of Medicine, University of Colombo, Sri Lanka (reference number EC/07/001). The project was registered with the International Clinical Trials Registry under No: SLCTR/2007/003 maintained at the Sri Lanka Medical Association, Colombo 07, Sri Lanka.

Healthy male volunteers between ages 30–45 years were invited to participate in the study. They were explained about the tests involved and written informed consent was obtained prior to enrollment to the study. Drug dependants, smokers, alcoholics and those under treatment with any other medications were not considered. All the volunteers were screened for common illnesses with special emphasis on CNS, cardiovascular and respiratory systems and also for excessive daytime sleepiness and nocturnal sleep disturbances. They underwent history taking, clinical examination and evaluation with Epworth sleepiness score with standard questionnaires[13]. Participants were required to pass biochemical and hematological evaluation with blood tests including electrolytes, glucose, lipid profile, liver function, renal function and urine analysis prior to the experiments.

Control studies on normal untreated healthy volunteers:

Those who had normal findings in the above screening process numbering five were selected for the study. A baseline EEG followed by Multiple Sleep Latency Tests (MSLT) were performed during this stage. The head was thoroughly washed in the morning on the day of the experiment with unblended shampoo detergent base, sodium laureth sulfate. This would facilitate the removal of debris, old sebum and sweat in order to expose the pore structures of the appendages of the skin of the scalp. Proper cleaning is important for recording of the EEG as well as for diazepam in sesame oil application to get absorbed effectively during the next stage of the test. The scalp has to be thoroughly dry at the time of EEG electrode application as well as at the time of application of the oil on the scalp.

The subjects arrived at 07:30 h to the sleep lab for preparation and application of electrodes. A baseline 30 min EEG was performed commencing at 08:00 h. Standard montages under 10-20 system and simultaneous video monitoring was used. This was to ensure that the baseline EEG has no β activity and there is well defined α activity in the posterior background of the EEG at rest. Subsequently a Multiple Sleep Latency Test (MSLT) was carried out starting at 09:00 h. A montage with C4-A1, C3-A2, O2-A1, O1-A2, F4-A1, F3-A2 scalp electrodes were used. Monitoring of one channel electrooculography (EOG) with diagonally placed electrodes above and lateral to outer canthus on one side and below and lateral to outer canthus on the other side, surface electromyography (EMG) using submental electrodes, lead II electrocardiogram (ECG) and synchronized video monitoring were combined. Sleep was scored in 30 s epochs according Rechtshaffen and Kales guidelines[14]. Standard technique with 5 naps with a 2 h interval between the naps was employed[14]. Sleep onset was identified as the first epoch of any stage of sleep NREM 1, 2, 3, 4 or REM. Volunteers were required to follow standard instructions for MSLT[15]. Those who had α activity in the baseline EEG and normal mean sleep latency on MSLT were selected for the next stage which was fixed for a later date.

The volunteers were then subjected to the Phase I study procedure with a single scalp application of 2 mg of diazepam in 3 ml of the oil in the morning of the test day. A wash out period of over three weeks was allowed before Phase II since diazepam is known to have effects on the EEG for up to two weeks[16]. In the Phase II the application of one dose each of 2 mg of diazepam in 3 ml oil was carried out on the scalp on three successive nights at 21:00 h in addition to the usual single application on the day of the test.

Choice of sesame oil as the solvent for transcranial diazepam solution:

The edible oils popularly employed in pharmaceuticals such as arachis, coconut, olive, soya and sesame oils were reviewed for the purpose. However among *Ayurvedic* medicated oils castor, coconut, mustard and sesame oils were found to be popular and a large majority contained sesame oil in the oil base[17]. Unlike most oils which consist of triglycerides of straight chain fatty acids, sesame oil is unique as it also contains other non fatty acid constituents, sesamin and sesamolin former being a complex cyclic ether and the latter a glycoside[18]. The presence of these heterocyclic organic ring structures in sesame oil is a unique feature. It may facilitate the dissolution and possibly the penetration of medicinal substances through the skin. Castor oil too has a special long chain fatty acid structure in the ricinolic acid triglyceride due to the presence of a hydroxyl group at C_{12} position in the fatty acid chain. Castor oil was however not considered for the present study due to its medicinal properties. The unique chemical structure of castor oil may however enhance the solubility of certain drugs. Considering the above and the actual solubility studies carried out, sesame oil was chosen as the non aqueous solvent for diazepam.

Preparation of transcranial diazepam in sesame oil stock solution 2 mg/ml:

As a safety measure the experiments were performed with the probable effective and safe transcranial diazepam dose of 2 mg in 3 ml of oil per application. A stock solution was prepared by dissolving 50 mg of diazepam B. P. 300 mesh powder in 25 ml of sesame oil to yield a 2 mg/ml solution. A quantity of 1 ml was appropriately diluted with 2 ml of plain sesame oil at the time of experiments to contain the 2 mg dose to be administered in a volume of 3 ml of the oil. This is the reasonable volume that could be applied on human scalp with out undue spillage. Stock solutions were prepared on the day before the test. Diazepam B. P. raw material as such takes a long time to

dissolve in sesame oil. It could be dissolved in half an h when reduced to 300 mesh size.

Analysis of diazepam in sesame oil stock solution 2 mg/ml:

Assay for the diazepam in sesame oil stock solution was developed based on an extraction procedure, since the presence of sesame oil traces interferes with a direct dilution procedure. A volume of 5 ml of diazepam in sesame oil stock solution 2 mg/ml equivalent to 10 mg of diazepam was extracted four times by shaking with 5 ml portions of 0.1M sulfuric acid solution. The combined aqueous phase was neutralized with 0.1M solution of NaOH. The resulting diazepam precipitate was extracted thrice with 10 ml portions of chloroform. Combined extracts were made up to 100 ml with chloroform. A 10 ml portion of the resulting solution was evaporated to dryness. Diazepam crystals obtained were dissolved in 25 ml of 0.05M methanolic-sulfuric acid. A blank was prepared with non medicated oil. Absorbance was measured at 368 nm and the content was calculated taking 151 as the value for A (1%, 1 cm) using Varian Cary 50Bio UV Visible computerized Spectrophotometer provided with Cary Win UV Software. The parameters for the final measurements of absorbance were selected based on the final step of the assay procedures common to diazepam injection, oral solution and rectal solution of the British Pharmacopoeia 2000, Volume II.

The technique of transcranial administration of diazepam 2 mg/3 ml oil solution:

One milliliter of the stock diazepam in oil solution 2 mg/ml was diluted with 2 ml of blank sesame oil to yield a dose of 2 mg in 3 ml of the oil at the time of the experiment. The preparation was done in an adjacent room by the investigator who applied the solution so that the volunteers, EEG technician and the investigator who did the sleep scoring were blinded as to whether a test dose or if a blank sesame oil placebo was applied.

Hair was parted manually within the electrode attachment area and about 2/3rd of the dose (2 ml) was poured on to the top of the scalp of the volunteer in sitting position. The 'rubbing in' of the oil was carried out immediately for 5 min with the convex side of a spoon-end of a stainless steel spatula, first within the electrode attachment points and then beyond to the periphery over the area of oil spread. The volunteers were rested for 10 min in sitting position followed by the application of the balance 1/3rd of the dose (1 ml) which was 'rubbed in' for another 5 min. The volunteers were then kept in the seated position for another 10 min prior to MSLT. This allows for a total of 30 min for the diffusion of diazepam into the brain tissue from the time of starting the application up to the commencement of MSLT at 09:00 h. Same MSLT protocol applied for 'control studies on normal untreated healthy volunteers' was followed. All the EEGs and MSLTs were done by the same technician in the same sleep lab. The EEG analysis and sleep scoring was done by the same investigator. The sleep scoring was carried out at the end of all the screenings in a particular phase to avoid bias.

Dimensions of the spatula are as follows. The overall length 120 mm; long axis of the elliptical spoon, 25 mm; short axis, 20 mm; radius of curvature long axis wise, 22 mm and short axis wise,17 mm. The equipment used for EEG and MSLTs was, Neurofax 9200K Digital Video EEG machine Nihon Kohden, Tokyo, Japan. Skin preparation for electrode placement was done using commercially available gel 'Nuprep', D. D. Weaver and Co, USA and electrodes were applied using electrode paste 'Elifex' Paste for EEG, Z-401CE F510, Japan. The electrode attachments were secured with hypoallergic 3M Plaster, micropore surgical tape. Diazepam BP raw material was obtained from MSJ Industries (Ceylon) Limited, Colombo 15, Sri Lanka.

The EEG background activities were visually examined using standard methods. Sleep scoring for MSLT was done manually as described under the section 'control studies on normal untreated healthy volunteers' and sleep latency of each nap was obtained. If sleep did not start, sleep latency was counted as 20 min. Mean sleep latency was calculated by averaging the sleep latencies of the 5 naps of each trial.

Phase-I test after a single transcranial application of diazepam in oil solution:

The same pre test protocol as described in the section 'control studies on normal untreated healthy volunteers' described above was observed by the subjects prior to the application of diazepam in oil. The subjects arrived at 07:30 h to the sleep lab and

a baseline 30 min EEG was performed at 08:00 h. At 08:30 h a single dose of diazepam in oil solution was applied on the scalp in the precise manner described in the previous section on 'the technique of transcranial administration of diazepam in oil solution'. All the subjects had diazepam in the oil preparation applied once at the same strength of 2 mg/3 ml. Flumezenil intramuscular injection as the antidote[19] and other emergency preparations were available bed side. An ICU bed was kept in reserve for any emergency. Volunteers were monitored for any side effects during the MSLT. Following the tests the volunteers were advised to avoid driving and operating machinery for 48 h.

Phase-II test after repeat transcranial application of diazepam in oil solution:

Phase II was designed to see if diazepam has a greater effect on sleep latency indicated by EEG criteria on repeat application of the drug. The Phase II was carried out after a rest period of three weeks from the Phase I study since diazepam is known to sustain EEG wave pattern changes on the brain for up to two weeks. Phase II study had to be restricted to two out of the five volunteers as three of the volunteers opted out of the test as per agreed ethics criteria.

One dose each consisting of 2 mg/3 ml of diazepam in oil was transcranially administered in three successive nights at 21:00 h prior to the test day in the manner described above under 'the technique of transcranial administration of diazepam in oil solution'. On the day of the test another dose was administered similar to the Phase I. Tests were carried out in a similar manner to the Phase I procedure above. Only two naps were completed as consented by the two volunteers.

Statistical analysis:

The results were statistically analyzed using standard Wilcoxon Test and the *P* values ≤0.05 were considered as significant.

RESULTS

After initial screening procedures 7 volunteers were recruited. Out of them two had to be rejected as they had low voltage beta range activity in the EEG at baseline recording. Remaining 5 subjects were recruited for the study. They were in the age group of 30 to 45 years. All of them were males.

All the subjects were those with alpha activity during awake EEG as specified in case selection criteria. All of them had alpha activity in posterior basic rhythm during screening subsequent to application of diazepam in oil. None had diffuse beta range activity which is usually seen in subjects who have had orally administered diazepam.

All the volunteers had individual base line sleep latencies of more than 20 min at the beginning of the test day and did not fall asleep prior to the application of the oil solution. Sleep latencies after a single application of diazepam preparation 2 mg in 3 ml are shown in (Table 1). All but one subject had sleep latencies shorter than 8 minutes during the first nap; mean being 7.45 min and median being 5 min. The subject with high value had a sleep latency of 19 min. The mean value is affected by this spuriously high value. These findings are significantly low in comparison to the expected value of 8.0 min.

The mean of the sleep latencies of all 5 naps (MSL) is less than 8 min in three of the subjects and more than 8 min in the other two (Table 1). Mean of these values is 8, median being 5.7. MSL is not significantly different from the expected value of 8 min. There is a tendency for the sleep latency to be high in 2nd to 4th naps while it again reduces in

TABLE 1: SLEEP LATENCY AT BASE LINE AND AFTER SINGLE TRANSCRANIAL APPLICATION OF DIAZEPAM IN SESAME OIL (PHASE - I)

Volunteer number	Baseline mean sleep latency (min)	Sleep latency after diazepam single application (min)					
		Nap 1	Nap 2	Nap 3	Nap 4	Nap 5	Mean sleep latency
1	20	19.0	9.0	5.0	10.0	14.0	11.4
2	20	5.0	6.0	8.5	8.5	8.0	7.2
3	20	3.7	5.0	8.0	12.0	10.0	7.7
4	20	4.5	20.0	20.0	20.0	5.3	14.0
5	20	5.0	3.5	2.0	10.5	3.5	4.9

Nap denotes a time period of 30 min after asking the volunteer to fall asleep.

the 5th nap. High sleep latencies in the 2nd to 4th naps seem to contribute to the lack of significant reduction of MSL values. However the MSLT values are significantly low ranging between 4.9 to 14 min when compared to the individual base line sleep latencies of 20 min.

The results of the Phase II are summarized in (Table 2). Although the Phase II study had to be restricted to two naps the reductions in sleep latencies due to repeated transcranial application of diazepam in oil are most convincing. The shortest sleep latency registered being 2.4 min against the expected value of 8 min for the normal adult population. No adverse effects were reported by any of the volunteers subsequent to the scalp application of diazepam preparation.

DISCUSSION

In oral administration of drugs the absorption of most of the drug molecules is nearly complete in the duodenum where the absorption surface area is enlarged six hundred times compared to an interior of a smooth tube of same length due to the presence of folds, villi and microvilli[20]. A similar enlargement of surface area is seen in the skin of the scalp due to the presence of a prolific hair growth and each hair follicle contributing to an additional surface area for the absorption. The epithelial layer of the hair follicles, sebaceous glands and the sweat glands are the main skin appendages that contribute to the enlargement of the surface area. The density of the sebaceous glands is between 400-900/cm^2 in the scalp compared to about 100/cm^2 or less in the skin of the rest of the body[21]. Therefore compared to the visible topographical area of the scalp, the overall three dimensional surface area of the scalp epithelium is at least ten times larger. The epidermal invaginations consisting of the hair follicles, sebaceous and sweat glands may be viewed as a form of 'ectodermal villi' that in effect increase the surface area in a manner similar to that of the mucous membrane of the duodenum facilitating drug absorption. The flat bones of the skull facilitate the process of drug absorption due to the presence of vascular haversian canals. The porous nature of the skull bones can be conveniently demonstrated by holding a small amount of gentian violet solution in alcohol in the concave surface of a skull bone when the blue color migrate within few minutes to the other side of the bone in progressively increasing patches.

TABLE 2: SLEEP LATENCY AT BASE LINE AND AFTER REPEATED TRANSCRANIAL APPLICATION OF DIAZEPAM IN SESAME OIL (PHASE - II)

Volunteer number	Base line mean sleep latency (min)	Sleep latency after diazepam repeat applications (min)			
		Nap 1	Nap 2	Average	% Reduction
1	20	3.0	4.4	3.7	81.5
4	20	3.0	2.4	2.7	86.5

Nap denotes a time period of 30 min after asking the volunteer to fall asleep.

Targeted drug delivery avoids first pass metabolism and the liver toxicity in the case of the oral drug delivery route and also minimizes the side effects on other organs due to the smaller doses employed. It must be noted that although 2 mg of diazepam in 3 ml of the oil was applied a portion of the oil remained adhering on to the hair. As a result it is possible that less than 2 mg was actually available for effective transcranial absorption.

During the Phase I study transcranially administered diazepam appears to act fast as detected by shortening of the sleep latency in the first nap which was allowed 30 min after starting and 10 min after completing the process of application of the dose. The reason for early onset of action may be due to rapid access of sufficient quantities of the drug into the target sites. The effect seems to wane off with time since the 2nd to 4th naps have sleep latencies longer than that of the first nap. However 5th nap again shows reduction of sleep latency. Rapid waning of the action may be due to small dose employed and the manner of redistribution of the drug within the brain and beyond. The Phase II study with repeat applications has resulted in much more pronounced shortening of sleep latencies down to 2.4 min (Table 2). This may be due to the response of a greater number of receptors as more of the drug has reached the brain with repeat applications.

Demonstration of diazepam in blood samples could have been the conclusive proof that the drug was absorbed by the transcranial route. However this was not possible due to the extremely low concentration of the drug that is expected to be present in blood since the study is brain targeted. The analyses of very low concentrations require sophisticated instruments and methods[6].

The present study demonstrates the reduction of sleep latency in human subjects following transcranial

administration of diazepam in oil. It also indicates the possibility of transporting diazepam molecules into the central nervous system by the transcranial route and exerting its sedative effect. A more elaborate study with a higher transcranial dose of diazepam including an administration of a parallel transdermal dose as a control has to be undertaken in the future. The present study forms the basis for a future study including measurements of centrally mediated muscle relaxant effect of diazepam. It may be concluded that transcranial route is effective and feasible for delivery of selected drugs to the brain tissue. The new route is also safe in the case of diazepam.

The greatest draw back in the electroencephalographic sleep latency study procedure is the difficulty in getting the volunteer participation in the day long screening with the attached electrodes. In the final determination of the effectiveness of transcranially administered diazepam, it may also be of interest to assess the mean sleep latency of each individual nap rather than the mean sleep latency of the naps of a given volunteer.

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