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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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Full Length Research Paper

Changes in plasma nitric oxide levels during migraine initial and attack periods in migraine patients

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The most important primary headaches are migraine. Migraine has a prevalence of 10% in the general population and its societal costs are high. The precise mechanisms underlying the pathophysiology of migraine are still elusive. Nitric oxide (NO) is believed to play a key role in migraine pathogenesis. In this study, we compared migraine patients with healthy controls by measuring plasma nitrite levels in order to investigate the effects of nitric oxide in migraine patients during headache free and attacks period. A total of 26 patients with migraine in headache free period and 24 patients with migraine in attack period participated in our study. Control group consisted of 24 healthy subjects. Plasma NO levels were measured spectrophotometrically. Plasma nitrite levels were found significantly higher than controls during headache free period ($p < 0.05$). By contrast, it was found significantly decreased in attack periods compared to headache free period. No significant change in plasma nitrite levels in migraine attacks period compared to controls was observed ($p > 0.05$). Plasma nitrite levels were not altered significantly after an attack in the patients with migraine. These results suggested that increased level of plasma NO during headache free period may play an important role in migraine pathogenesis.

Key words: Headache, migraine, nitric oxide, oxidative stress.

INTRODUCTION

Migraine is a common, chronic and disabling neurovascular disorder that is characterized by severe headache attacks with photophobia, nausea, vomiting, autonomic symptoms, and in some patients with aura involving neurological symptoms (Uzar et al., 2011). Migraine is the most prevalent neurological disorder, with an estimated 43 million sufferers in Europe and slightly smaller numbers in the USA (Andlin-Sobocki et al., 2005; Lipton et al., 2007). It is a very painful condition that often leads to absenteeism from work and more often to considerably decreased efficiency at work. The estimated cost of migraine in Europe is €27,000 million per year which, among the neurological disorders, ranks third after dementia and stroke (Andlin-Sobocki et al., 2005). Migraine is considered to be a disorder of neurovascular transmission without structural lesions (Olesen et al., 1993).

The molecular mechanisms of migraine have not been fully clarified yet. It has been hypothesized that increased oxidative and nitrosative stress may together take place in patients with migraine, especially during attacks (Yilmaz et al., 2007). There are many theories about the pathophysiology of migraine attacks. Most of these involve hereditary (Alexandrea, 1998; Lance and Goodsby,

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1998). Biological states that may cause increases in free fatty acids and blood lipids increased platelet aggregation, decreased serotonin levels and increased prostaglandin levels (Bic et al., 1998). Such changes can cause the vasodilatation that precedes migraine headache (Alexandrea, 1998; Bic, 1998).

Before the pain attack, cerebrovascular spasm induced by factors such as menstruation, lack of sleep, skipped meals, allergic reaction, and physical or mental stress, among others initially causes ischaemia which, in turn, produces prodromal symptoms such as nausea, photophobia or phonophobia (Alexandrea, 1998; Lance and Goodsby, 1998; Sadovsky, 1998).

Nitric oxide (NO) is a gaseous mediator synthesized mainly in the endothelium that exerts many important regulatory functions on the vessel wall and platelet (Moncada et al., 1991). NO can play a modulatory role on biological processes such as vasodilatation in migraine attacks (Bellantonio et al., 1998; Olesen, 1993). It has also been reported that NO can be released from endothelial cells, neurons, macrophages, and platelets. Because human platelets contain an L-arginine/NO pathway which may be associated with platelet aggregation (Radomski et al., 1990), NO may play a role in the pathogenesis of migraine and other vascular headaches. The final products of NO *in vivo* are nitrite (NO^{-2}) and nitrate (NO^{-3}), but the relative proportions of nitrite and nitrate are variable (Moncada et al., 1991). The best index of overall NO production, therefore, is the total concentration of both nitrate and nitrite (total nitrate/nitrite).

In our study, we compared migraine patients with healthy controls by measuring plasma nitrite levels in order to investigate the effects of nitric oxide in migraine patients during headache free and attacks period.

MATERIAL AND METHODS

Patients

In our hospital-based case-control study, 26 patients with migraine in headache free period and 24 patients with migraine in attack period participated. 24 controls without migraine diseases were recruited. The diagnosis of headache was made in accordance with the International Headache Society (IHS) criteria (Olesen, 2008). The migraine patients in headache free period group consisted of 26 subjects. The mean age was 33.3 ± 6.3 years [mean ± standard deviation (SD)]. The migraine patients in attack period group consisted of 24 subjects. The mean age was 31.4 ± 4.9 years (mean ± SD). The 24 normal controls without migraine diseases were recruited; the mean age was 30.1 ± 4.1 years (mean ± SD). Written informed consent was obtained from all subjects.

The study was carried out in the neurology clinic of Firat University Research and Application Hospital. Patient and healthy volunteers were recruited from the Department of Neurology, University of Firat. Furthermore, this study was approved by the Ethics Committee of the Medical Faculty of the same university. Medical, neurologic, and psychiatric evaluations were made on all of the patients. Patients having these characteristic were excluded; first-axis psychiatric disorder now or in the past, history of alcohol and cigarette consumption, history of any kind of medicine consumption within the last 2 weeks prior to the study (except simple analgesic), neurologic disorder and/or symptom in clinic and history, history of head trauma, history of cardiovascular, renal and endocrinologic disorder and existing medical disorder.

Measurement of plasma nitrite

Venous blood samples were taken from the antecubital vein with suitable vacutainers with ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The basal venous blood was obtained from all the participants in this study on the morning after 12 h of overnight fasting. In all cases, blood samples were taken according to the principles of the Helsinki declaration. The blood samples were drawn from the patients. These samples were centrifuged at +4°C at 3,000 rpm for 10 min, and after separating plasma they were kept at -20°C until analysis. Among all participants, information on demographic characteristics and risk factors was collected using a structured questionnaire. NO measurement is very difficult in biological specimens because it is easily oxidized to nitrite (NO_2) and subsequently to nitrate (NO_3) which serve as index parameters of NO production. Samples were initially deproteinized with NaOH and $ZnSO_4$. Total nitrite (NO_2 to NO_3) was measured by spectrophotometer at 545 nm after conversion of NO_2 to NO_3 by assay reactive. A standard curve was established by a set of serial dilutions of sodium nitrite. Results were expressed as µmol/L per plasma (Lyall et al., 1995).

Statistical analysis

The statistical package for the Social Sciences (SPSS) 15.0 (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. The plasma NO levels were evaluated statistically by Mann-Whitney U test. Results were given as mean ± SD, and $p < 0.05$ was accepted to indicate significant levels.

RESULTS

In control group, plasma nitrite levels were found to be 85.8 ± 3.2 µmol/L. In migraine patients, plasma nitrite level was detected to be 95.9 ± 21.2 µmol/L during headache free period and 85.2 ± 2.7 µmol/L during attacks. According to our results, slightly higher levels of nitrite in plasma of migraineurs during headache free period were found than in control subject. A statistically significant difference was observed in plasma nitrite level between migraine patients and control groups ($p = 0.004$). The plasma nitrite values of the patients and control groups are shown in Table 1.

When the patients were grouped as migraineurs with in headache free period and in attack period, plasma nitrite levels were significantly decreased in attacks periods compared to headache free period ($p < 0.05$). No significant change in plasma nitrite levels in migraine attacks period compared to controls was observed ($p > 0.05$). The plasma NO values in the attack period and initial period in migraine patients are shown in Table 2 and Figure 1.

Table 1. The values of plasma nitric oxide in migraine patients and control groups.

Variable	Control group (n=24)	Patient (n=26)	Total (n=40)	p	95% CI
Age (years)	30.1±4.1	33.3±6.3	31.6±5.2	0.287	0.488-0.283
Nitric oxide	85.8±3.2	95.9±21.2	89.29±13.3	0.004*	23.090-2.76

*p < 0.05 as significant level. CI = confidence interval.

Table 2. The values of plasma nitric oxide in the attack period and initial period in migraine patients.

Variable	Control group (n=24)	Patient (n=26)	Migraine during attack (n=24)
Age (years)	30.1±4.1	33.3±6.3	31.4±4.9
Nitric oxide	85.8±3.2[a]	95.9±21.2[b]	85.2±2.7[a]

[a,b]p < 0.05 as significant level.

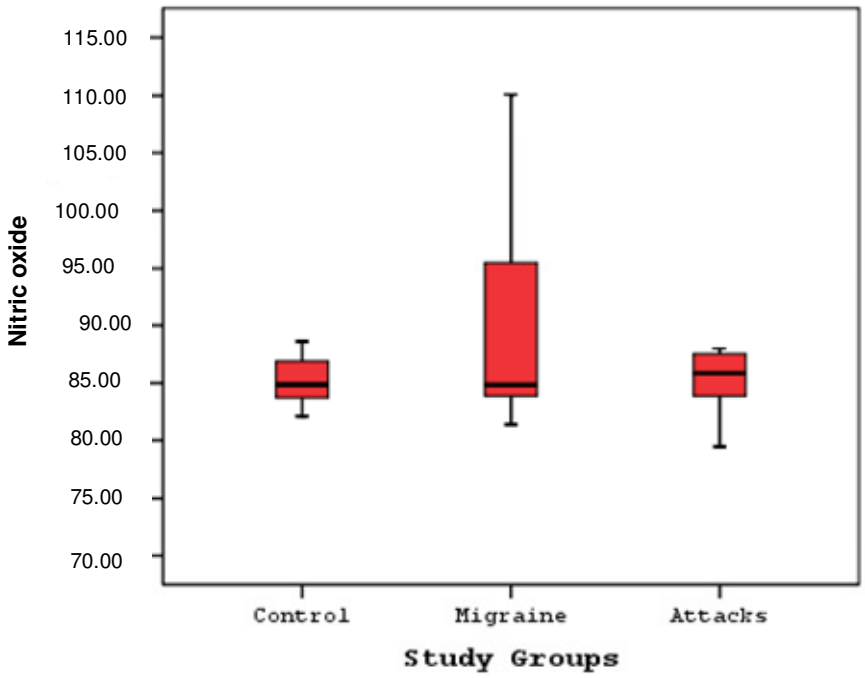

Figure 1. The distribution of values of plasma nitric oxide in the study groups. Group 1: healthy control (n = 24), Group 2: migraine patients in initial period (n = 26), Group 3: attack period (n = 24).

DISCUSSION

Migraine is a chronic disease with frequent attacks, high levels of pain and disability during attacks which causes reduced quality of life between attacks (Yilmaz et al., 2007). It is a widespread disorder, affecting about 10 to 15% general population. However, the mechanisms underlying the disease have not been clearly understood. Vascular disturbance in intracranial arteries plays a significant role in the migraine attacks. It was suggested that hemodynamic changes during the migraine attacks may be related to alterations in the level of NO. NO can precipitate the attacks by causing vasodilatation in cerebral vessels. It was shown that platelet hyperaggregation has an important role in migraine pathophysiology. Aggregated platelets produce NO levels which show a

counter effect to vasoconstrictors and finally cause vasodilatation and prevention of platelet aggregation (Yilmaz et al., 2007).

In our study, we measured NO activity during headache and headache-free period in migraine patients. Since NO is rapidly oxidized by tissue oxygen to the stable end products, nitrate (NO^{3-}) and nitrite (NO^{2-}), the best index of overall NO production is the total concentration of both nitrate and nitrite. Therefore, we measured total nitrite levels as an indicator of NO production. As similar to previous studies (D'Andrea and Cananzi, 1994; Sarchielli et al., 1996; Shimomura et al., 1999; Uzar et al., 2011), we found that plasma nitrite levels were significantly higher in migraine patients during headache-free period. Shimomura et al. (1999) showed that NO level decreased in headache-free period of migraine patients. In contrast to this study, we found slightly higher nitrite levels in headache-free period of migraine patients. In the light of our findings, we concluded that NO may be produced in plasma during headache-free period and increased NO may be related to the changes in the vascular vessels and pain during headache-free period. We would like to suggest that when the NO levels exceed a critical level, the headache-free period may begin.

On the other hand, simultaneous release of NO and of superoxide anions produces peroxynitrite anion which is a strong biological oxidant known to oxidize lipids, proteins and sulfhydryl (SH) groups particularly. In the most recent study on this issue, Taffi et al. (2005) evaluated platelet peroxynitrite levels a metabolite of nitric oxide as well as a potent oxidant in migraine patients to resolve uncertainty about NO activity in headache free period. They found increased peroxynitrite levels during headache-free period which are in concordance with our results. The main reason of increased peroxynitrite concentrations is concomitant overproduction of NO and superoxide anion, and these changes may result from platelet hyperaggregation. Therefore, increased nitrosative and oxidative stress may influence platelet function and cerebral microcirculation. It was suggested that NO may cause the headache through variations of cerebral blood flow by interacting with oxygen free radicals in migraine (Ciancarelli et al., 2003). It has also been proposed that NO may be involved in the pathophysiology of naturally occurring headaches such as migraine (Thomsen et al., 1993; Lassen et al., 1995). However, as of yet, there is no evidence that NO levels in the brain are elevated during the headache phase of migraine in humans (Lassen et al., 1997).

NO is one of the indicators of oxidative stress (Yilmaz et al., 2007). NO, a labile molecule with a half life of only a few seconds is synthesized generally in the endothelium. It is rapidly oxidized by tissue oxygen to the stable end products, nitrate and nitrite. Therefore, to the best index of overall NO production in the circulation is the total concentrations of both nitrate and nitrite (Yilmaz et al., 2007; Tain el al., 2010). NO is one of the substances that takes part in the pathophysiology of migraine (Yilmaz et al., 2007). NO is involved in the regulation of the cerebral vessels tone. NO may function as a signaling molecule in controlling neuronal activity and is important in controlling sensory inputs during migraine attack and interacting with reactive oxygen substances (ROS) which may induce headache through changes of cerebral blood flow (Ciancarelli et al., 2005).

The most important effect of NO is the activation of the soluble guanylate cyclase. This enzyme causes the synthesis of cyclic guanosine monophosphate (cGMP), and nitric oxide-cyclic guanosine monophosphate (NO-cGMP) pathway in smooth vascular muscles causes vascular dilation and relaxation (Olesen, 2008; Napoli et al., 2009). It is suggested that NO could be an important mediator in the initiation or the propagation of a neurogenic cranial vessel inflammatory response that might eventually result in a migraine attack (Napoli et al., 2009).

It has been found that the pathways consisting of NO synthesis are activated in the experimental headache models such as nitroglycerin-induced headache (Sarchielli et al., 2000). The increase of NO may be caused by interactions with free ROS. The primary product of the interaction between NO and superoxide anion is peroxynitrite. Peroxynitrite is an aggressive and potent cellular oxidant (Gruber et al., 2009). So, increased NO with migraine patients may be associated with oxidative stress. In addition, occurrence of peroxynitrite following NO increases the effects of endothelia function (Fo¨rstermann, 2008). Marked NO increase in migraine group in our study strengthened the correlation between oxidative stress and endothelia dysfunction in pathophysiology of migraine. The findings of increased NO in migraine may lead to new therapeutic strategies, including probable antioxidant use for management of migraine. In the future, development and enhancements of existing drugs may be accompanied by increased efforts to develop truly new migraine drugs based on knowledge of the pathophysiology (Stovner et al., 2009; Farinelli et al., 2009).

Conclusıon

Results obtained indicate that the plasma nitrite levels were not altered significantly after an attack in the patients with migraine. Nitrosative and oxidative stress are increased during headache-free period in plasma. Increased oxidative damage together with increased NO level in migraine attack may effect cerebral blood flow and cause headache. These results suggested that increased level of plasma NO during headache free period may play an important role in migraine pathogenesis, and NO may serve as useful markers to show the increased oxidative stress in migraine patients. Further clinical and

biochemical studies are needed to investigate the associations between migraine, stroke and to introduce new therapeutic modalities for migraine treatment.

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Full Length Research Paper

Effect of artesunate on the dissolution profile and antimicrobial activity of ciprofloxacin hydrochloride

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This study evaluated the *in vitro* physico-chemical implication of co-presentation of ciprofloxacin with artesunate. Thin layer chromatography (TLC) and ultraviolet (UV) analysis was used to monitor the co-presentation of the two drugs. The *in vitro* dissolution profile of ciprofloxacin hydrochloride tablet in the presence of artesunate tablet was evaluated in buffer with pH 1.2 and water at 37± 0.5°C as well as the effect artesunate on the antibacterial activity of ciprofloxacin. Physico-chemical interaction was not observed between ciprofloxacin and artesunate. However, a significant reduction in the dissolution rate of ciprofloxacin was observed in buffer pH 1.2 solution when compared with water as the medium ($p < 0.01$). Furthermore, the rate of dissolution of ciprofloxacin was significantly reduced in the presence of 200 mg artesunate in both media ($p = 0.0164$). Similarly, the extent of drug release at 30 min (C_{30}) was generally reduced in the presence of 200 mg artesunate in both water and buffer pH 1.2 solution ($p < 0.05$). The reduction in antimicrobial activity of ciprofloxacin on clinical isolates of *Staphylococcus aureus* and *Escherichia coli* was observed to be statistically insignificant in the presence of artesunate ($p > 0.05$). The results obtained in this study suggest that care should be taken during the co-administration of ciprofloxacin with the starting dose of artesunate.

Key words: Ciprofloxacin, artesunate, physico-chemical interaction, dissolution rate, *Staphylococcus aureus* and *Escherichia coli*.

INTRODUCTION

Ciprofloxacin [1 cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid], is one of the quinolone antibiotics which is a class of highly potent and orally active broad spectrum antibacterial compounds developed from the original 1,8-napthyridine urinary antibacterial agent, nalidixic acid (Smith et al., 1990). Ciprofloxacin is indicated for both Gram-negative and Gram- positive bacterial infections. It has been used in the treatment of a wide range of infections including biliary-tract infections, bone and joint infections, acute bacterial infections, gastro-enteritis, gonorrhea, immuno-compromised patients (neutropenia), skin disorders, typhoid and paratyphoid fever and urinary-tract infection (UTI) (Martindale, 2009). Ciprofloxacin have been reported to inhibit DNA gyrase activity, thereby causing significant antiparasitic effect against various strains of *Plasmodium falciparum* malaria (Mahmoud et al, 2003; Yeo and Rieckmann 1994). Malaria, a parasitic protozoal disease, is one of the most serious complex and refractory health problems currently facing humanity. The co-presentation of microbial infections and malaria is not uncommon in tropical developing countries, thus, the co-administration of antimicrobial drugs with antimalaria is a common practice. The co-administration of two or more drugs is usually accompanied by a variety of therapeutic implications ranging from opposition, alteration, synergism, potentiating as well as physical and chemical antagonism (Olaniyi, 2000). 3R,5aS,6R,8aS,9R,-10S,-12R,12aR)-decahydro-3,6,9-trimethyl-3,12-epoxy-12H pyrano(4,3-j)-1,2-benzodioxepin-10-ol hydrogen Artesunate, chemically known as (3R,5aS,6R,8aS,9R,10S,12R,12aR)-decahydro-3,6,9 trimethyl-3,12-epoxy-12H-pyrano[4,3-j]-

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1,2 enzodioxepin-10-ol hydrogen succinate, is a derivative of artemisinin which have been found useful in chloroquine-resistant malaria (O'Neill et al., 2010). Artesunate is about the most widely used of the artemisinin derivatives and is a key member of all the artemisinin combination therapies (ACT) compositions recommended by World Health Organization (WHO) for the management of malaria presently (WHO, 2006). The co-administration of ciprofloxacin with a variety of drugs has been reportedly accompanied with varied pharmacological effects. Probenecid was reported to increase the plasma concentration of ciprofloxacin as a result of a reduction of its excretion (Lim et al., 1995). Cyclosporine increases risks of nephrotoxicity when used concomitantly with ciprofloxacin (Van et al., 1990), while non-steroidal anti-inflammatory drugs (NSAIDs) and theophylline possibly increases risk of convulsion with ciprofloxacin (Polk, 1989). Antacids, calcium salts, sucralfate, zinc and oral iron reduce the absorption of ciprofloxacin; this may result in decreased plasma concentration (Taxeira et al., 1995; Joint Formulary Committee, 2003).

The possibility of decreased solubility resulting in decreased absorption as a result of physical interaction due to adsorption of drug particles has been reported (Galia et al., 1998). Similarly, chemical interactions in form of oxidation, hydrolysis, etc. of two or more drugs may result in significant effect on the therapeutic activity of the drug compounds (DiPalma, 1976). The co-administration of ciprofloxacin with antimalaria agents such as chloroquine and artesunate during the management of bacterial infection co-presented with malaria as a result of its broad spectrum of activity formed the basis for this study. In this study, the effects of artesunate on the dissolution profile and antimicrobial activity of ciprofloxacin against two strains of *Staphylococcus aureus* and *Escherichia coli* were determined.

MATERIALS AND METHODS

Materials and equipment

Ciprofloxacin hydrochloride powder was donated by Gemini Pharmaceuticals, Lagos, Nigeria, while artesunate powder was donated by Bond Chemical Industries Ltd, Awe, Oyo State, Nigeria. Artesunate tablet (50 mg) manufactured by Makophar Chemical Pharmaceutical Company Vietnam and ciprofloxacin HCl tablets (500 mg) manufactured by V.S. International PVT.LTD were obtained from retail pharmacy outlets in Ibadan. All the reagents used were of analytical grade.

UV Spectrophotometer (UNICAM) was use for drug content quantization, while Langford ultrasonics sonomatic® water bath and dissolution test apparatus USP standard were used for the interaction studies. The microbiological assay was carried out using Fisher Isotemp $^{(R)}$ 200 series incubator and Equitron autoclave carried out by determining the melting point, UV spectra between 200 to 400 nm. The chemical content were determined using the official method for artesunate (I.P. 2003), while an earlier reported method with very good interday and intraday precision was used for ciprofloxacin HCl samples (Adegbolagun et al., 2007).

Physico-chemical interaction evaluation

Ultraviolet spectroscopy

Stock solutions of ciprofloxacin HCl (5 mg/ml) (CIP) and artesunate (1 mg/ml) (ART) were prepared in buffer pH 1.2. Aliquot concentrations; 1000 µg/ml of CIP, 100 and 200 µg/ml of artesunate (ART) were prepared from the stock solutions. Solutions of the two drugs were combined at the following ratios to give a final concentration of; ART: CIP (50: 500 µg/ml and 200: 500 µg/ml), also individual solutions at ART (50 and 200 µg/ml) and CIP (500 µg/ml) were transferred into six labeled covered test-tubes per reaction mixture. The test tubes were placed in thermostated water bath at 37± 0.5°C. UV spectra (200 to 400 nm) of each set of the reaction mixture were run at 0, 15, 30, 45, 60 and 90 min using the buffer pH 1.2 as blank. The determination was carried out in duplicate. The procedure was repeated in distilled water.

In vitro dissolution profile evaluation

UV absorbance of solutions of CIP (0.1 - 5 × 10^{-4}%w/v) at 276 nm was used to generate a calibration curve of CIP in buffer pH 1.2 and distilled water. *In vitro* dissolution profile of CIP was determined according to B.P. 2010 method (British Pharmacopoeia, 2010). The determination was carried out in water at 37± 0.5°C using the one tablet (500 mg) and in the presence of artesunate tablets at 50 and 200mg. Aliquots of 10 ml were withdrawn at 10 min interval for 60 min and assayed. The dissolution fluid volume was maintained by adding 10 ml of the dissolution medium which had previously been maintained at 37± 0.5°C after each withdrawal. The withdrawn samples were assayed using UV spectrophotometer at 276 nm using the dissolution medium as blank. The procedure was repeated with four determinations, using buffer pH 1.2 at 37± 0.5°C as the dissolution medium.

Antimicrobial interaction evaluation

The effect of the *in vitro* co-presentation of artesunate and ciprofloxacin was evaluated on two strains each of *S. aureus* and *E. coli*. The bacterial strains include one clinical isolates of *E. coli* and *S. aureus* and their reference standards; American Type Culture Collection (*E. coli* ATCC 25922 and *S. aureus* ATCC 14563) obtained from the University College Hospital, Ibadan, Nigeria were used for the studies. All the bacterial cultures were grown in sterile nutrient broth at 37± 0.5°C for 24 h and maintained in nutrient agar at 4°C until use.

Sensitivity test

Overnight cultures (24 h) of *E. coli* and *S. aureus* (0.2 ml) were prepared by serial dilutions. Aliquots solutions (0.2 ml) of CIP (1.5625 × 10^{-3} – 4.0 µg/ml) was transferred into Mueller-Hinton broth (9 ml) followed by 0.2 ml of overnight culture of the bacterium and 0.6 ml of nutrient broth. The tubes were mixed and incubated at37°C for 24 h. The negative control contains the sterile broth, while the positive control contains the sterile nutrient broth, 0.8 ml sterile distilled water and 0.2 ml of the bacterium. The minimum inhibitory concentration (MIC) was defined as lowest concentration of the drug at which no turbidity was observed. The determination was done in triplicates. The procedure was also repeated with similar concentrations of artesunate.

Antimicrobial assays

Aliquots solutions of ciprofloxacin hydrochloride at 0.5, 1.0, 2.0 and

Table 1. Experimental designs for the antimicrobial drug - drug interaction studies between ciprofloxacin hydrochloride and artesunate.

CIP (µg/ml)	ART (µg/ml)			
	0.0	**0.1**	**0.2**	**0.3**
0.0	0.0/0.0	0.0/0.1	0.0/0.2	0.0/0.3
0.5	0.5/0.0	0.5/0.1	0.5/0.2	0.5/0.3
1.0	1.0/0.0	1.0/0.1	1.0/0.2	1.0/0.3
2.0	2.0/0.0	2.0/0.1	2.0/0.2	2.0/0.3
4.0	4.0/0.0	4.0/0.1	4.0/0.2	4.0/0.3

CIP, Ciprofloxacin hydrochloride; ART, artesunate.

4.0 µg/ml was prepared from the stock solution in buffer pH 1.2, while those of artesunate was similarly prepared at 0.1, 0.2, 0.3 µg/ml from its stock solution in buffer. Diluted overnight cultures of the different micro organism (0.2ml) was individually introduced into 20 ml of warm molten agar, which was carefully mixed and poured into sterile Petri plates and allowed to set for about 45 to 60 min. Wells of 8 mm were aseptically cut in the set agar in Petri plates inoculated with the microbial suspension.

The pure drugs (CIP and ART) and their mixtures at the different combination ratios (Table 1) were prepared in test tubes which were covered and placed in thermostated water bath set at 37 ±0.5°C. Samples (0.2 ml) were carefully withdrawn from the reaction mixtures and transferred into the labeled wells of the seeded agar plates at 0, 15, 30, 45 and 60 min. The agar plates were kept for 1 to 2 h to allow diffusion before incubation at 37 ±0.5°C for 24 h. The diameters of zones of inhibition were measured in mm. The procedure was done in triplicates and also repeated for the drug solutions in distilled water.

Statistical analysis

The results obtained in the research work were expressed as mean ± standard deviation. The results were analysed using one way ANOVA and Student t-test (Instat 3, Statistical Package) with $p< 0.05$ for level of significance.

RESULTS AND DISCUSSION

The identification and chemical content determination of the drug compounds authenticated the quality of the two drug compounds. CIP melted at 240 – 241°C with a UV λ_{max} at 276 nm, while the melting point of the pure ART was 131 - 132°C and did not show any UV absorbance between 210 to 400 nm. Their chemical contents obtained were also within the official specification for the two drugs [ciprofloxacin HCl (100.8 and 98.9%w/w); artesunate (100.5 and 96.2%w/w) for pure drug and tablet dosage form, respectively] (I.P. 2003, B.P. 2010). The physico-chemical interaction evaluation monitored by using the UV spectrophotometer did not reveal any effect on the UV spectrum of CIP as there was no change in the λ_{max} and absorbance reading at 276 nm in the presence of ART. This may indicate an absence of observable physicochemical interaction between CIP and ART.

Dissolution of drugs is a very important step in the absorption of drugs from dosage forms. It is one of the fundamental parameters controlling the rate and extent of drug absorption (Gordon et al., 1995). Dissolution profiles of some drugs have been reportedly affected by the dissolution medium (Galia et al., 1998; Lobenberg et al., 2000). Buffer pH 1.2 was used in this study to provide a similar medium to the normal pH of the stomach which is the site of drug dissolution prior to absorption. On the other hand, water was selected as the second medium because it is the specified medium for the evaluation of the dissolution profile of CIP (B.P. 2010). The experimental design for the dissolution profile evaluation used CIP tablet (500 mg), while ART at 200 mg is the starting dose for the management of malaria while 50 mg was used to observe the effect at the lower concentration of ART.

From the results obtained, the official monographs specified an *in vitro* release of 80%w/v for CIP at 30 min; that is, the time to release/dissolve 80%w/v of the drug (T_{80}) was 30 min. The dissolution profile of CIP in buffer pH 1.2 and water showed a delayed rate of release from the tablet dosage form in the presence of ART (Figure 1; Table 2). The rate of release of drugs from dosage forms was obtained from the time to reach 80%w/v concentration (T_{80}). A significant increase in T_{80} of CIP alone in buffer pH 1.2 compared with water was observed in this study with a T_{80} of 12.0 ± 4.5 and 35.3 ± 2.2 min in water and buffer, res-pectively ($p = 0.0013$). This indicates a significant reduction in the rate of release of CIP in buffer solution. However, no significant difference was observed in the % CIP released at 30 min (C_{30}) in the two media in the absence of ART ($p > 0.05$).

Furthermore, a significant increase in T_{80} of CIP was observed in the presence of 200 mg ART in both water and buffer solution ($p < 0.05$). This indicates a significant reduction in the rate of release/ dissolution from the tablet dosage form in both media. The specified *in vitro* dissolution profile of CIP tablet dosage form should be 80%w/v at 30 min (T_{80} of 30 min and C_{30} of 80%w/v) (B.P. 2010). A highly significant increase in T_{80} of CIP was observed in the presence of 200 mg ART when compared with 50 mg in both media ($p < 0.05$). Moreover, the CIP release in the presence of 200 mg ART at 30 min was lower than specified 80%w/v in both media (Table 2), while the time to achieve 80%w/v dissolution was also

Table 2. Dissolution profiles (T_{80} and C_{30}) of ciprofloxacin hydrochloride tablet in the presence of artesunate tablets at 50 and 200 mg (mean ± S.D.).

Sample	T_{80} (% ± S.D.)		C_{30} (min ± S.D.)	
	Buffer pH 1.2	Water	Buffer pH 1.2	Water
CIP alone	35.3 ± 2.2	12.0 ± 4.5	73.3 ± 1.3	89.8 ± 14.8
CIP + 50 mg ART	25.6 ± 11.9	13.7 ± 8.4	83.9 ± 10.9	97.9 ± 7.5
CIP + 200 mg ART	53.5 ± 7.6	46.5 ± 0.9	21.7 ± 6.9	65.9 ± 6.1
p value (ANOVA)	0.0164	0.0029	0.0001	0.0565

CIP, Ciprofloxacin hydrochloride; ART, artesunate.

Table 3. Zones of inhibition (mm) of ciprofloxacin hydrochloride (CIP) (0 – 4ug/ml) in the presence of artesunate (ART) (0.1 – 0.3ug/ml) at 0 and 60minutes against two strains of *S. aureus* and *E. coli*.

Organism	ART concentration (ug/ml)	Zone of inhibition at different concentrations of CIP (ug/ml) at 0 and 60 min (mm)							
		0.5		1.0		2.0		4.0	
		0	60	0	60	0	60	0	60
SAT	0.1	15.7 ± 3.21	17.7 ± 1.15	22.3 ± 3.51	22.0 ± 0.1	23.3 ± 2.1	23.5 ± 2.0	24.7 ± 2.1	23.5 ± 0.7
	0.2	20.0 ± 2.82	16.3 ± 7.23	18.7 ± 3.2	20.3 ± 3.5	22 ± 1.0	22.5 ± 3.5	26.3 ± 1.5	24.0 ± 2.7
	0.3	18.3 ± 2.08	22.3 ± 2.31	18.3 ± 7.0	26.7 ± 0.6	21.7 ± 1.2	21.0 ± 1.0	24.7 ± 0.6	24.7 ± 2.9
SAC	0.1	17.3 ± 3.0	16.3 ± 3.1	22.7 ± 4.7	20.3 ± 1.5	22.7 ± 4.0	17.7 ± 5.7	28.0 ± 2.0	23.7 ± 3.8
	0.2	20.7 ± 0.6	11.3 ± 1.5	21.3 ± 4.2	12.0 ± 5.0	20.3 ± 0.6	21.0 ± 1.0	28.0 ± 3.6	22.7 ± 0.6
	0.3	19.7 ± 4.5	16.7 ± 1.5	24.0 ± 1.7	18.3 ± 2.5	22.7 ±2.5	21.0 ± 1.0	27.7 ± 2.1	23.3 ± 1.5
ECT	0.1	24.3 ± 1.5	25.7 ± 5.5	28.3 ± 1.5	22.3 ± 1.2	30.7 ± 0.6	29.7 ± 1.5	31.0 ± 3.5	32.0 ± 1.0
	0.2	24.7 ±1.5	20.7 ± 2.1	30.7 ± 4.0	28.7 ± 4.9	30.7 ± 1.2	26.3 ± 7.7	26.0 ± 4.4	30.7 ± 1.5
	0.3	24.3 ± 3.2	22.0 ± 5.1	32.3 ± 4.1	27.3 ± 4.6	31.7 ± 1.5	26.7 ± 1.5	31.0 ± 2.7	33.0 ± 1.0
ECC	0.1	26.0 ± 4.0	27.3 ± 2.3	33.7 ± 2.9	30.0 ± 2.5	32.0 ± 1.0	27.7 ± 0.6	34.7 ± 0.6	33.3 ± 0.6
	0.2	33.3 ± 3.2	25.0 ± 3.0	30.7 ± 1.2	28.7 ± 2.5	33.7 ± 2.9	28.7 ± 1.2	36.7 ± 0.6	28.0 ± 1.7
	0.3	32.7 ± 2.5	22.0 ± 1.0	31.7 ± 2.5	31.0 ± 1.7	30.7 ± 1.2	26.7 ± 2.1	37.0 ± 1.0	31.3 ± 3.1

higher than the 30 min in the presence 200 mg ART. The increased T_{80} and decreased C_{30} with ART at 200mg imply an increase in the time to achieve appreciable biological concentration as a result of delayed rate of *in vivo* drug absorption. This may cause a decrease in the rate of achieving desired therapeutic effect, which may result in a compromise of the therapeutic outcome. On the other hand, the significant difference in the dissolution profile of CIP alone in buffer pH 1.2 and water is an indication that the use of water to define the dissolution profile may not be a good indicator of the dissolution profile of CIP in biological medium.

In addition, the microbial sensitivity test showed that the micro organisms used in this study were sensitive to the CIP at 0.25 to 4.0 µg/ml, while ART expectedly did not show any antimicrobial activity even at the highest concentration of 0.4 µg/ml. The effect of ART on *in vitro* antimicrobial effect of CIP on *E. coli* and *S. aureus* revealed a decrease in the antimicrobial activity on the clinical isolates which were however found to be statistically insignificant ($p > 0.05$) (Table 3).

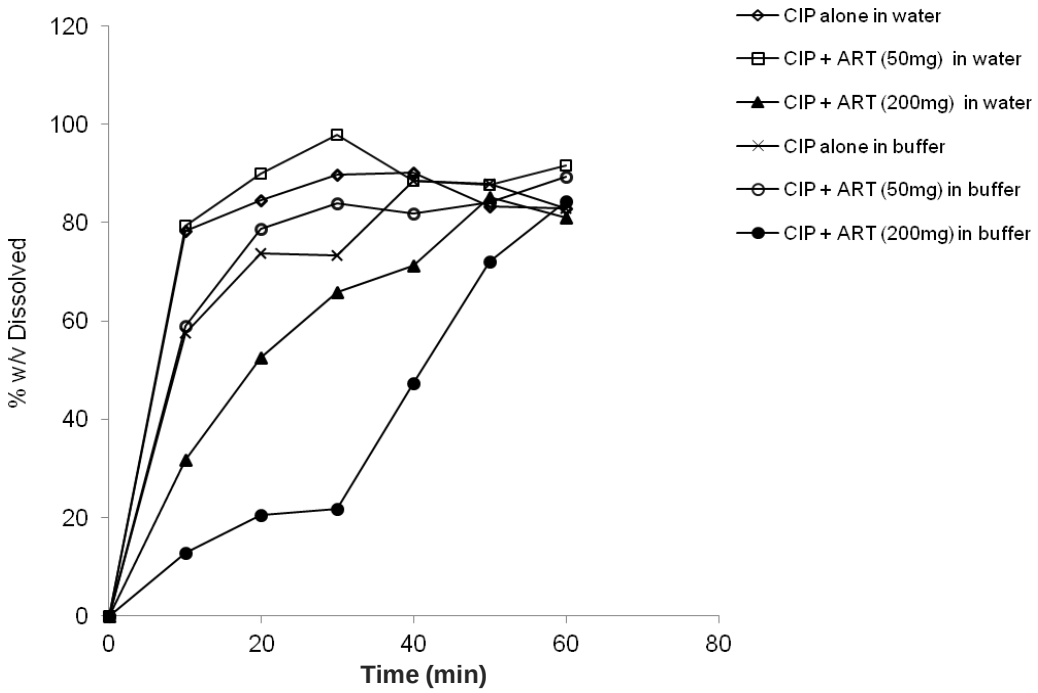

Figure 1. Dissolution profile of ciprofloxacin hydrochloride tablet (500 mg) in the presence of artesunate tablet at 50 and 200 mg in water and buffer pH 1.2 at 37± 0.5°C (CIP: ciprofloxacin hydrochloride; ART: artesunate).

Nevertheless, no difference in antimicrobial activity was observed with the reference micro organisms (SAT and ECT) across the different concentrations used in this study.

Conclusion

Although no significant difference was obtained with the physico-chemical interaction study, the alteration in the dissolution profile of CIP in the presence of 200 mg ART calls for caution during the administration of CIP with starting dose of ART. This is further important in view of the obtained result of non-statistically significant reduction in antimicrobial activity obtained with the clinical isolates. It is hereby suggested that care should be taken during the co-administration of CIP with the starting dose of ART. An adequate time interval should be observed in order to avoid compromising the therapeutic outcome.

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Full Length Research Paper

Comparative excretion of vitexin-2"-*O*-rhamnoside in mice after oral and intravenous administration

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The aim of the present study was to characterize comparative excretion of pure vitexin-2"-*O*-rhamnoside (VR) in mice following oral and intravenous administration at dose of 30 mg/kg, therefore, a sensitive and specific high-performance liquid chromatography (HPLC) method using vitexin-4"-*O*-glucoside as internal standard developed and validated for quantitative analysis of VR. The results of elimination of VR in urinary and fecal excretion following oral and intravenous dosing indicated that VR was mainly excreted as prototype for both routes of administration, and biliary and renal excretions are two major ways of elimination of VR.

Key words: Excretion, high-performance liquid chromatography (HPLC), Vitexin-2"-*O*-rhamnoside.

INTRODUCTION

Vitexin-2"-*O*-rhamnoside (VR) abundantly exists in fruits and leaves of *Crataegus pinnatifida* Beg. var *major* (hawthorn and hawthorn leaves), both of which are very popular herbal materials in traditional Chinese medicine (TCM) and are well used in treating cardiovascular diseases (PRC, 2010). As VR is one of the main components of flavonoid of hawthorn leaves (Ding et al., 1990) and also a bioactive constituent on cardiovascular system in hawthorn (Liang et al., 2007), many pharmacological studies of VR have been reported until now, such as protective effect on the injured cardiac myocytes and endothelial cells (Zhu et al., 2003, 2006) and strongly inhibiting deoxyribonucleic acid (DNA) synthesis in MCF-7 human breast cancer cells (Ninfali et al., 2007). In recent years, many articles focus on *in vitro* study and pharmacokinetic studies (Cheng et al., 2007; Ying et al., 2007, Du et al., 2011). However, there is little research on the comparative excretion of pure VR isolated from hawthorn leaves following oral and intravenous routes of administration. In our study, a sensitive and specific high-performance liquid chromatography (HPLC) method using vitexin-4"-*O*-glucoside (VG) as internal standard thereby was first established to fully evaluate the urinary and fecal excretion content of VR following oral and intravenous route of administration. In addition, the differences of excretion after two forms of administration can be identified.

MATERIALS AND METHODS

Reagents and chemicals

The water used in all experiments was purified by a Milli-Q® Biocel Ultrapure Water System (Millipore, Bedford, MA, USA). Methanol, acetonitrile and tetrahydrofuran were all of HPLC grade and purchased from Xinxing Chemical Reagent Plant (Shanghai, China). All other chemicals of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

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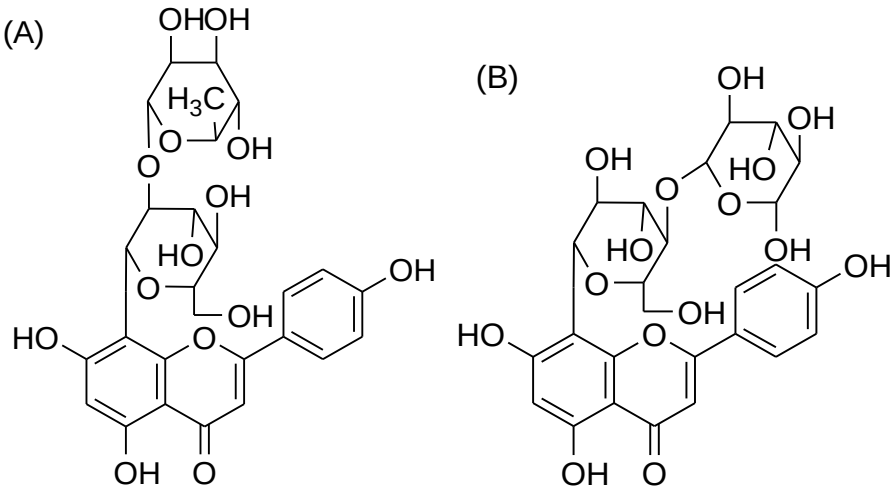

Figure 1. (A) Chemical structure of vitexin-2"-O-rhamnoside. (B) Chemical structure of vitexin-4"-O-glucoside (I.S.).

Plant

Leaves of *C. pinnatifida* Bge.var *major* were collected in Shenyang, Liaoning Province, China and identified by Prof. Ting-Guo Kang. A voucher specimen (20110921) was deposited in Liaoning University of Traditional Chinese Medicine.

Extraction and isolation

A sample of the leaves (2 kg) of *C. pinnatifida* Bge. var. *major* was cut in small pieces and refluxed with 60% aqueous ethanol for two times, each for 2 h. The crude extract after concentrated under reduced pressure was then adsorbed on a porous-polymer resin (AB-8, Tianjin, China) column, removed impurity with water and eluted with a gradient of 30, 50 and 70% ethanol. The fraction eluted with 30% ethanol was evaporated under reduced pressure to obtain extract, which was then chromatographed on silica gel column and eluted with ethyl acetate/butanone/formic acid/water 3:3:1:1. Fractions of similar composition were pooled on the basis of TLC analysis (UV monitoring at 365 nm) were repeatedly subjected to silica gel column chromatography and eluted with ethyl acetate/butanone/formic acid/water 5:3:1:1 to directly obtain crystal of VR (1 g) and VG (0.2 g) internal standard (I.S.), and purities of them were both over 99% by HPLC analysis. The chemical structures of VR and VG, confirmed by ^{1}HNMR, ^{13}CNMR and MS data, are shown in Figure 1.

Chromatographic system

The analysis was carried out on an Agilent 1100 series HPLC system (Agilent technology, Palo Alto, CA, USA) which consisted of a quaternary Pump (G1310A), a vacuum degasser (G1322A), a UV-VIS spectrophotometric detector (G1314A) and Chemstation software (Agilent). The analytical column was a Diamonsil C18 column (150 mm × 4.6 mm i.d., 5 µm, Diamonsil, USA) protected by a KR C18 guard column (35 mm × 8.0 mm, i.d., 5 µm, Dalian Create Science and Technology Co., Ltd., China). The optimal mobile phase used for separation is a mixture consisting of methanol-acetonitrile-tetrahydrofuran-1% glacial acetic acid (6:2:18:74, v/v/v/v). All the chromatographic measurements were performed at room temperature and a flow rate of 1 ml/min with the detection wavelength of 330 nm.

Preparation of standards and quality control samples

Standard stock solutions of VR and I.S. were both prepared in methanol to yield the concentrations of 4000 µg/ml and 276 µg/ml, respectively. Stock solution of VR was serially diluted with methanol to desired concentrations over the range of 0.16 to 3200 µg/ml. All the solutions were stored at 4°C before use. The calibration samples were prepared by spiking 200 µl blank mouse urine or 500 µl blank feces homogenates successively with appropriate amount of working solutions of VR (50 µl), acetic acid (20 µl) and I.S.. The volume of I.S. is 40 µl for urine samples and 80 µl for feces samples. The quality control samples of VR in the method validation were similarly prepared to the standard calibration samples.

Animals and dosing

SPF male Kunming strain mice (20 ± 2 g) were obtained from the Experimental Animal Center of Liaoning University of Traditional Chinese Medicine (Shenyang, China) and housed in an air-controlled breeding room for a week. Before starting the experiment, all the mice formerly free access to standard laboratory food as well as water ad libitum were fasted for more than 12 h. All experiments involving animals were approved by the animal ethics committee of Liaoning University of traditional Chinese medicine and performed according to the Guidelines for Animal Experimentation of this institution. For excretion studies, ten mice were randomly divided into two groups to be administrated VR at dose of 30 mg/kg. Among the two groups, one was orally administrated and the other intravenously administrated. All the mice were respectively housed in stainless-steel metabolism cages to collect urine and feces at different time. Two hours after administration, water and standard laboratory food were offered. Urine and feces samples were collected at 0 to 2, 2 to 4, 4 to 6, 6 to 8, 8 to 12 and 12 to 24 h post-dosing. The volume of each collected urine sample and the weight of each collected feces sample were separately recorded. All the sample s were stored at -20°C until analysis.

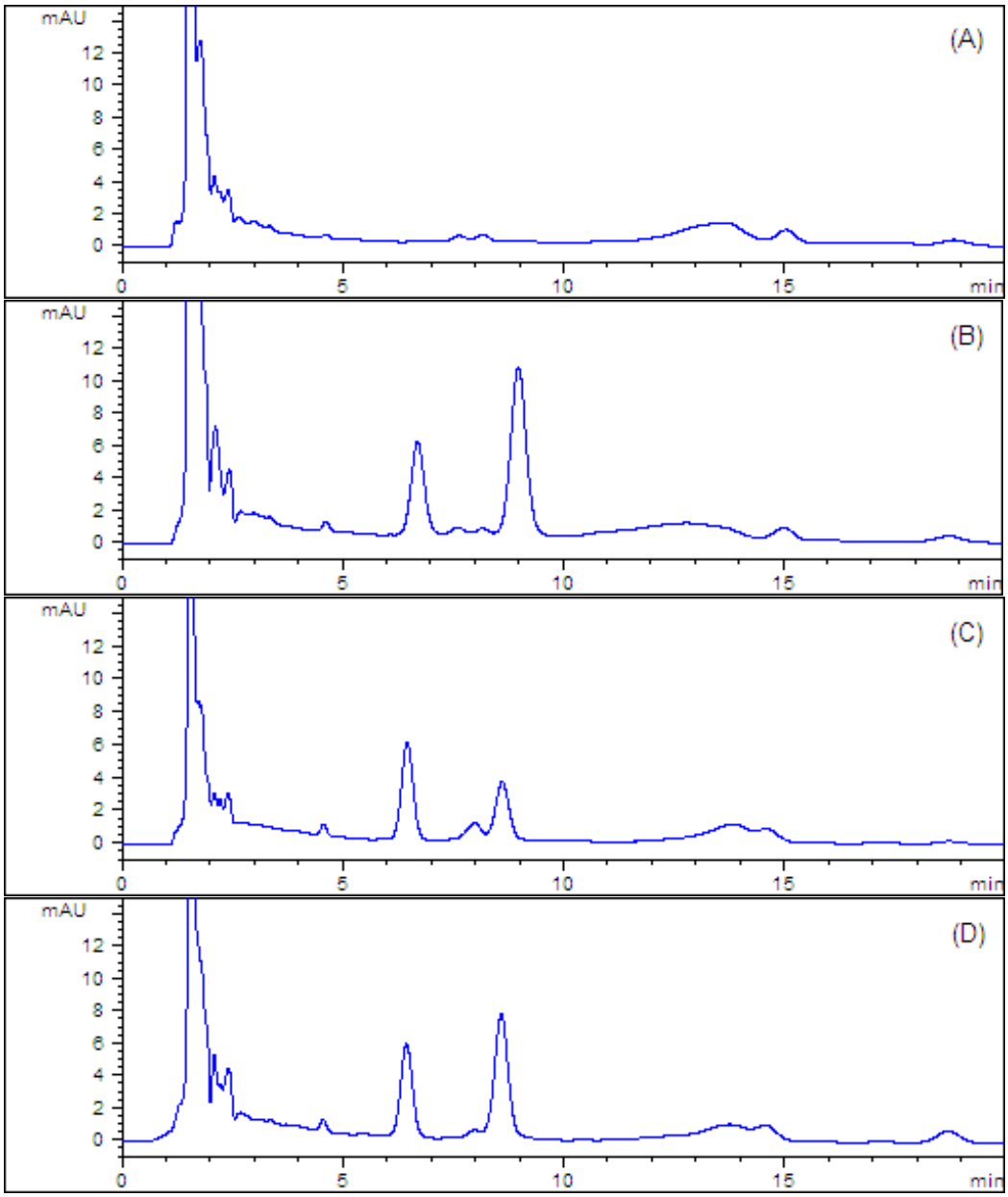

Figure 2. Typical chromatograms of urine excretion study (A-D) respectively obtained from blank urine sample, blank urine sample spiked with standard analyte and I.S., and urine samples collected from 4 to 6 h following oral and intravenous routes of administration of VR at dose of 30 mg/kg.

Sample processing

To 200 µl urine samples, 20 µl of acetic acid, 40 µl of I.S. and 1 ml of methanol were successively pipetted, followed by vortex mixing for 1 min. After samples being centrifuged at 890 g for 15 min, the supernatant was separated and evaporated to dryness under a gentle stream of nitrogen at 50ºC. The residue was diluted in 200 µl of mobile phase and again centrifuged at 15,092 g for 10 min. Then an aliquot (20 µl) of clean supernatant was injected into HPLC column for analysis. Feces (0.2 g) were homogenized in 0.5 ml of saline solution. The homogenate was successively added 20 µl of acetic acid, 80 µl of I.S. and 2 ml of methanol and then treated as urine samples.

RESULTS AND DISCUSSION

HPLC chromatograms

Typical chromatograms of urinary and fecal excretion studies are shown in Figures 2 and 3, respectively. The total run time was no more than 20 min and the retention times of I.S. and VR were approximately 6.6 and 8.7 min, with no interfering peaks detected at the retention times of VR or I.S.

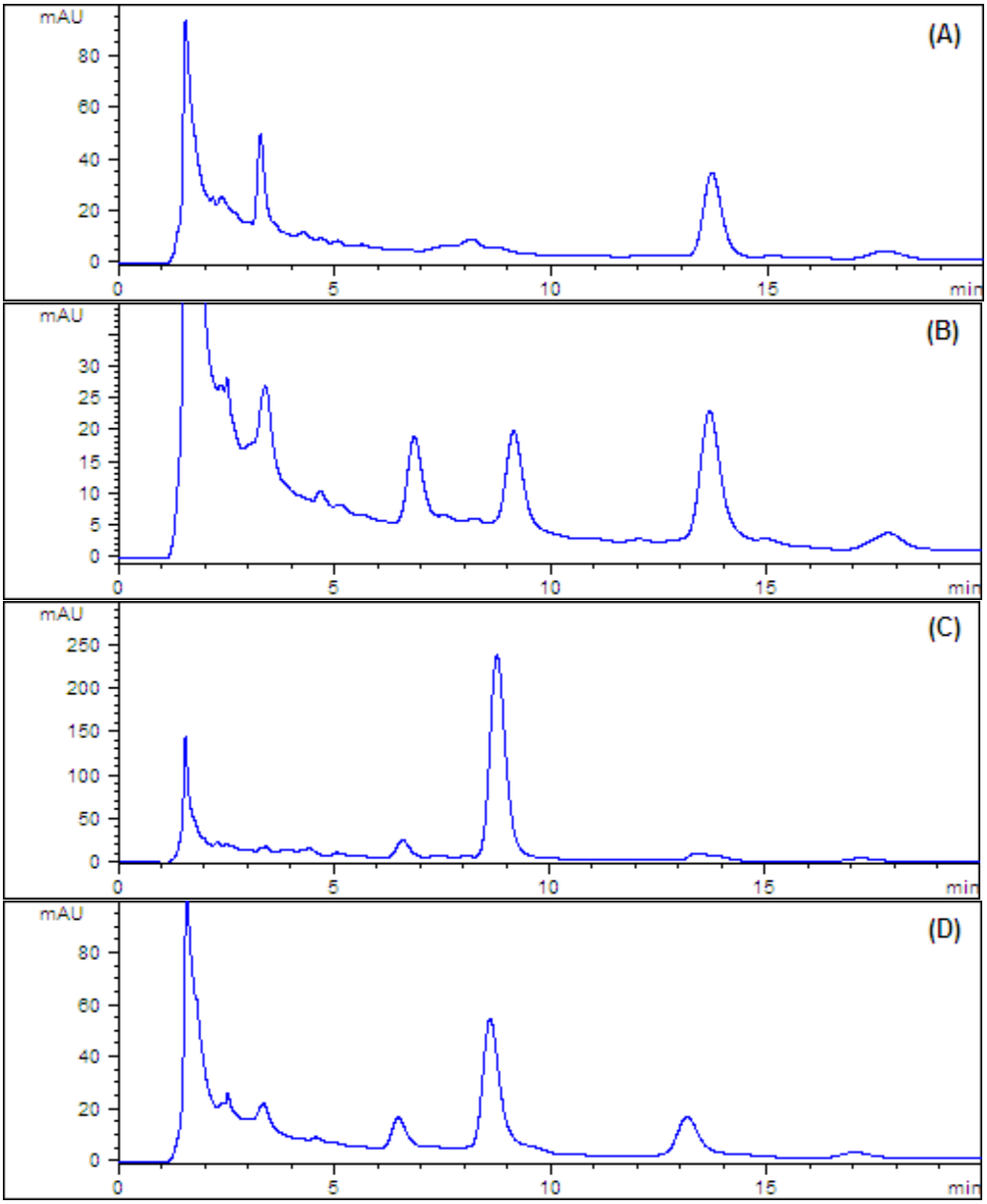

Figure 3. Typical chromatograms of fecal excretion study (A-D) respectively obtained from blank feces sample, blank feces sample spiked with standard analyte and I.S., and feces samples collected from 6 to 8 h following oral and intravenous routes of administration of VR at dose of 30 mg/kg.

Method validation

Method validation involves linearity, precision, limit of detection (LOD), limit of quantification (LOQ), recovery and stability. The linear range for urine and feces were within 0.4 to 400 µg/ml with R^2 > 0.99. The LOD (*S/N* > 3) and the LOQ (*S/N* > 10) were respectively 0.121 and 0.363 µg/ml in urine and 0.121 and 0.363 µg/g in feces. Both of the precision (RSD%) and accuracy (RE%) were below 15%, conforming to the criteria for the analysis of biological sample according to guidance of USFDA. The extraction recoveries of VR in urine and feces ranged from 82.36 ± 5.82% to 108.9 ± 8.47%. The results of short-term stability, long-term stability and freeze-thaw stability indicated that no remarkable degradation occurred during chromatography, extraction and sample storage processes for excreta samples.

Excretion studies

Figure 4 shows the urinary and fecal excretion time profiles

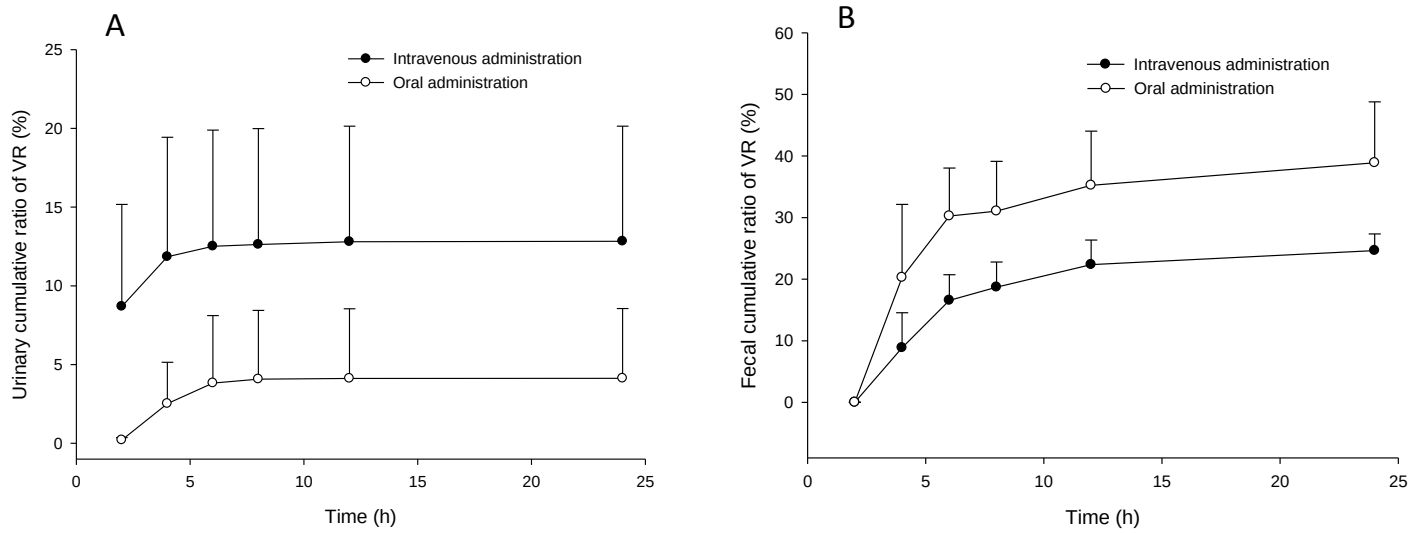

Figure 4. (A) Urinary cumulative ratio of vitexin-2"-*O*-rhamnoside in mice (mean ± S.D., *n* = 5) following oral and intravenous routes of administration at dose of 30 mg/kg. (B) Fecal cumulative ratio of vitexin-2"-*O*-rhamnoside in mice (mean ± S.D., *n* = 5) following oral and intravenous routes of administration at dose of 30 mg/kg.

of VR following oral and intravenous administration at dose 30 mg/kg. The urinary and fecal cumulative ratios of unchanged VR amounted to 4.13 ± 0.01% and 38.89 ± 3.04% after oral administration, and amounted to 12.83 ± 0.03% and 24.65 ± 1.75% after intravenous administration. VR as prototype was detected in excreta by comparing HPLC chromatogram of blank biological samples with the tested one. The total VR recoveries of excreta were 37.48 ± 1.78% (12.83 ± 0.03% in urine; 24.65 ± 1.75% in feces) following intravenous dosing and 43.02 ± 3.05% (4.13 ± 0.01% in urine; 38.89 ± 3.04% in feces) following oral dosing, demonstrating that biliary excretion and renal excretion are two major ways of elimination of VR and VR underwent extensive first-pass effect after oral administration and that not being absorbed was mainly excreted as feces. Recently, Ma et al. (2010) studied the excretions of VR after orally administrated hawthorn leaves extract and found that the total recovery of the dose in 24 h was 89.01% (0.72% in urine; 88.29% in feces) for VR (Ma et al., 2010), which is different from the results of monomer administration of VR were 43.02 ± 3.05% (4.13 ± 0.01% in urinary excretion; 38.89 ± 3.04% in fecal excretion) following oral dosing, suggesting that various kinds of components in hawthorn leaves extract affect the concentration and duration of VR *in vivo*.

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Full Length Research Paper

Preparation and evaluation of sustained release pellets of Tramadol

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Preparation of sustained release dosage forms is one of the main objectives in drug formulation. Tramadol is a centrally acting synthetic opioid analgesic used in treating severe pain. In this study, Tramadol sustained release pellets were prepared using two methods and the effect of type, ratio of polymers and plasticizers on drug loading and drug release profile was studied. From the results obtained, it seems that Eudragit RL is not a suitable choice and when Tween was used as plasticizer. Moreover, when mixture of Eudragit S and RS were applied as polymers, the optimum drug release profile was obtained and after 10 h, 95% of loaded drug was released.

Key words: Eudragit, Tramadol, sustained release, plasticizer, pellet.

INTRODUCTION

Multi-particulate drug delivery systems are mainly oral dosage forms consisting of a multiplicity of small discrete units, each exhibiting some desired characteristics. In these systems, the dosage of the drug substances is divided on a plurality of subunit typically consisting of thousands of spherical particles with diameter of 0.05 to 2.00 mm. Thus multi-particulate dosage forms are pharmaceutical formulations in which the active substance is present as a number of small independent subunits (Dey et al., 2008). Multiparticulate systems show better reproducible pharmacokinetic behavior than conventional (monolithic) formulations. Some other studies were done to investigate pharmacokinetics of sustained release dosage forms prepared using ion exchange resins (Liu et al., 2012). Drug safety may also be increased by using multiparticulate dosage forms, particularly for modified release systems (Laila and Chandran, 2006; Hu et al., 2006).

Multiparticulates may be prepared by several methods. Some of these methods may be broadly classified as pelletization, granulation, spray drying, and spray congealing (Bechegaard and Nielson, 1978). Pelletized delivery system (PDS) is a sustained release system using pellets or beads manufactured using marumerization/ spheronization/ pelletization techniques, or by layering powders or solutions on nonpareil seeds

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(Dey et al., 2008; Hu et al., 2006) and controlled drug release systems can be assembled from either polymers or pumps. Because of their small size and lower cost, polymers are most widely employed (Garala et al., 2009). Pelletization is a technique that enables the formation of spherical beads or pellets. These pellets can eventually be coated and very often used in controlled-release dosage forms (Hirjau et al., 2011).

Pelletization methods used in the pharmaceutical industry can be grouped by various criteria, e.g. by the type of equipment used, the intensity of the mechanical forces involved or the techniques employed for the production of pellets. The success of these methods depends on the complex relations between the equipment, the formulation and process variables (Hirjau et al., 2011; Bechegaard and Nielson, 1978). Different pelletization methods are as follows:

(a) Extrusion / spheronization (Liew et al., 2000; Ghai et al., 2009; Zhang et al., 2012).
(b) Fluid-bed Granulation.
(c) Rotogranulation is one of the most recent methods for the production of spheroids.
(d) Layering a suspension or a solution of a drug on a seed material.
(e) Dry powder layering is some method in which process is similar to the solution or suspension layering.
(f) Spray-drying represents another process.
(g) Spray-congealing (spray-chilling) is a technique similar to spray-drying (Schaefer and Kristensen, 1993; Bechegaard and Nielson, 1978; Haritha, 2012).

Tramadol hydrochloride is a centrally acting synthetic opioid analgesic used in treating severe pain. The drug has a wide range of applications, including treatment for rheumatoid arthritis, restless legs syndrome and fibromyalgia. Tramadol comes in many forms, including capsules, tablets, suppositories, effervescent tablets, powders, and ampoules for subcutaneous injection (SC), intra-muscular injection (IM), and intravenous (IV) injection and liquids (Trease, 1964). In this study, sustained release pellets of Tramadol were prepared using two different methods and in addition to comparing the suitability of designed methods, the effect of type of polymers, ratio of polymer to drug as an important factor which was studied in other sustained release formulations (Jan et al., 2012), type of emulsifiers, amount of used emulsifiers and core particle size on drug loading and drug release profile were studied.

MATERIALS AND METHODS

Tramadol hydrochloride (Figure 1) was purchased from Palace, Italy. Eudragit RS100, S, L and RL were purchased from Rohm Pharma, Germany. Sucrose, polyvinylpyrrolidone (PVP), isopropanol, acetone, ethanol, polyethylene glycol 400 (PEG 400), Tween 20 and 40, potassium hydrogen phosphate, sodium hydroxide, talc, titanium dioxide and acid chloride were purchased from Merck, Germany.

Preparation of pellets

For preparation of pellets in this study, two different methods were applied. In the first method, primary cores were designed using sieved sucrose and the addition of PVP suspension in isopropyl alcohol, and then the suspension of other components including: calcium carbonate, talc, titanium dioxide and corn starch in water. The prepared cores were separated by sieving method using a sieving machine (Teb Azma, Iran), after which loading of drug was done on the cores and finally polymers were used to coat designed drug loaded pellets. This method was designed to prepare spherical core with high ratio of surface to volume and to increase probability of more drug loading efficiency on them. For this approach, sucrose was sieved using sieves with 16 - 50 mesh size and the fraction of sucrose that remained on sieve with 40 mesh size was chosen to be as core. Table 1 shows the US standard for relation of mesh size and particle size that was applied in this study. From the table, sucrose was 420 micron in size. Three different formulations for first layer were designed and added to sucrose to reach more spherical cores in shape and to protect sucrose as a core. Tables 2 to 5 show different suspension of the first layer on sucrose. For loading of drug on the designed cores, mixture of drug and excipients was sprayed on cores using hand spray gun and coating pan (Erweka, Germany) in different steps to load enough drug. Although some other studies investigated the effect of different experimental parameters on characterization of microcapsules (Esposito et al., 2000), in this study experimental parameters fixed and the effect of presented parameters like ratio and type of polymers and solvents were studied. Tables 6 to 9 show the drug and different polymers in different ratio mixture that were sprayed on the cores to prepare controlled release pellets.

In second method for the preparation of cores, mixture of drug and excipients were used instead of sucrose, using pelletiser (Pelletiser GTE, Erweka, Germany) and then polymers were added to the surface of desired pellets. For preparation of pellets based on the second method, first mixture of drug and some excipients were poured into the pelletiser to make primary cores as it is shown in Table 10. As for the first layer, solution of PVP (10%) in ethanol was sprayed on the desired cores to help powders to adhere to each other and make cores. The second layer was formulated as described in Table 11 and sprayed to the surface of the cores. Desired cores were sieved with sieve sizes of 16 - 40. The cores remaining on the sieve size 20 and 25 were selected and mixed to continue the study. Also, cores remaining on sieve size 18 were separately collected for further studies. Then formulations as described in Tables 12 and 13 were prepared to spray on selected cores. Finally, analyses of desired pellets were done.

Determination of λ max of Tramadol

Absorbance of serial dilution of Tramadol in water and phosphate buffer solution (PBS) pH: 6.8 were studied to determine λ max of Tramadol.

Drug loading efficiency

Briefly, 100 mg of cores and 100 mg pellets containing drug were weighed separately, extruded and dissolved in 100 ml purified water. Pure cores solution was used as blank and Tramadol assay was done in detected λ max.

Drug release study

Dissolution test was done at a desired condition as follows: medium of dissolution was 500 ml water, apparatus number I, 100 RPM at

Table 1. US standard for relation between mesh size of sieves and particle size of materials.

US standard	
Mesh size	**Particle size (µ)**
10	2000
12	1680
14	1410
16	1190
18	1000
20	840
25	710
30	590
35	500
40	420
45	350
50	97

Table 2. Component of formulation 1 that applied as first layer on sucrose core in the first method.

Material	Percentage (%)
Sucrose	50
Talk	10
Calcium carbonate	10
PVP	1
Titanium dioxide	0.5
Water	Up to 100

Table 3. Component of formulation 2 that applied as first layer on sucrose core in the first method.

Material	Amount (g)
Sucrose	35
Calcium carbonate	30
Talk	5
PVP	1
Titanium dioxide	0.5
Corn starch	10
water	30

37.5±0.5°C. The sampling interval was 1 h, in each interval 5 ml samples was gathered and fresh medium was replace. The medium of dissolution for pellets containing Eudragit S and L was changed to HCL 0.1 N for the first 2 h and then PBS, pH:6.8 because these Eudragits are pH sensitive.

RESULTS AND DISCUSSION

Calibration curve of tramadol in water and PBS

The λ max of 272 nm was selected for Tramadol and

Table 4. Component of formulation 3 that applied as first layer on sucrose core in the first method.

Material	Amount (g)
Sucrose	35
Calcium carbonate	30
Talk	8
PVP	1
Titanium dioxide	0.5
Corn starch	10
Water	35
Ethanol	5-10%

Table 5. Components of tramadol containing solution which was sprayed on the cores in firs pellet preparation method.

Material	Amount
Tramadol HCL	5 (g)
Eudragit RS100	5(g)
TEC	15%
Ane	50 (ml)
Ethanol	50 (ml)

calibration curve was designed as shown in Figure 2. The R2 of the both curves was 0.999.

Drug loading on pellets

The amount of drug loaded on the desired pellets is shown in Table 14. According to the results, formulations F1, 5, 7, 8, 10 and 11, F13 to 16 and also F19-21 and 24 had drug loading more than 11%.

Drug release

Figure 3 shows drug release profile of pellets that was studied in water medium. As shown, formulation number 5, 7 and 9 had a significant burst effect and more than 50% of loaded drug was released through them in the first hour. In formulation F8, at the first sampling time, 81% of loaded drug was released and no significant change was observed in 8 h (data not shown). Study of drug release profile through desired pellets showed that usage of polymeric mixture with concentration of 90% Eudragit RS and 10% Eudragit S could be suitable formulation to design sustained release pellets. Usage of total electron content (TEC) and Tween 40 in ratio of 20 to 5%, respectively in coating solution could cause the preparation of pellets with spherical shape, reasonable drug loading and sustained drug release profile. Figures 3 to 5 show the drug release profile; comparison of F1 –

Table 6. Formulations of pellets which containing Eudragit RS.

Formulation number	Ratio of polymer to drug	Acetone (ml)	Ethanol (ml)	Tween 20 (g)	PEG 400	TEC (g)	Eudragit RS (g)
F1	0.25	50	50	-	-	0.75	5
F2	0.27	50	50	-	-	0.75	5
F3	0.39	50	50	-	-	0.75	5
F4	0.88	50	50	-	-	0.75	5
F5	0.25	50	50	-	0.75	-	5
F6	0.30	50	50	-	0.75	-	5
F7	0.20	50	50	0.75	-	-	5
F8	0.26	50	50	0.75	-	-	5
F9	0.31	50	50	0.75	-	-	5

Table 7. Formulations of pellets which containing Eudragit RS and S.

Formulation number	Ratio of polymer to drug	Acetone (ml)	Ethanol (ml)	Tween 20 (g)	TEC (g)	Eudragit S (g)	Eudragit RS (g)
F10	0.3	50	50	-	0.75	0.5	4.5
F11	0.35	50	50	-	0.75	0.5	4.5
F12	0.45	50	50	-	0.75	0.5	4.5
F13	0.20	50	50	0.25	1	0.5	4.5
F14	0.31	50	50	0.25	1	0.5	4.5
F15	0.36	50	50	0.25	1	0.5	4.5
F16	0.44	50	50	0.25	1	0.5	4.5
F17	0.70	50	50	0.25	1	0.5	4.5
F18	0.76	50	50	0.25	1	0.5	4.5

Table 8. Formulations of pellets which containing Eudragit RS and L.

Formulation number	Ratio of polymer to drug	Acetone (ml)	Ethanol (ml)	TEC (g)	Eudragit L(g)	Eudragit RS (g)
F19	0.36	50	50	0.75	0.5	4.5
F20	0.40	50	50	0.75	0.5	4.5
F21	0.45	50	50	0.75	0.5	4.5
F22	0.47	50	50	0.75	0.5	4.5
F23	0.55	50	50	0.75	0.5	4.5

Table 9. Formulations of pellets which containing Eudragit RL.

Formulation number	Ratio of polymer to drug	Acetone (ml)	Ethanol (ml)	TEC (g)	Eudragit RL (g)
F24	0.28	50	50	0.75	10
F25	0.31	50	50	0.75	10
F26	0.42	50	50	0.75	10
F27	0.51	50	50	0.75	10

Table 10. Materials which used to prepare primary cores based on second method.

Material	Amount (g)
Tramadol HCL	10
Lactose	20
Corn starch	20
PVP	2.5

Table 11. Materials which used as second layer to prepare pellets based on second method.

Material	Amount (g)
Sucrose	25
Calcium carbonate	30
Talk	8
PVP	2
Titanium dioxide	0.5
Corn starch	15
Water	30
Ethanol	30

Table 12. Materials used to spray on cores of second method with size of 710-840 µ.

Formulation no	Eudragit RS (g)	Eudragit S (g)	TEC (g)	Tween 40 (g)	Ethanol (ml)	Acetone (ml)	Ratio of polymer to drug
F'13	4.5	0.5	1	0.25	50	50	0.20
F'14	4.5	0.5	1	0.25	50	50	0.30
F'15	4.5	0.5	1	0.25	50	50	0.36
F'16	4.5	0.5	1	0.25	50	50	0.45
F'17	4.5	0.5	1	0.25	50	50	0.70

Table 13. Materials used to spray on cores of second method with size of 1000 µ.

Formulation no	Eudragit RS (g)	Eudragit S (g)	TEC (g)	Tween 40 (g)	Ethanol (ml)	Acetone (ml)	Ratio of polymer to drug
F"14	4.5	0.5	1	0.25	50	50	0.30
F"15	4.5	0.5	1	0.25	50	50	0.35
F"16	4.5	0.5	1	0.25	50	50	0.44
F"17	4.5	0.5	1	0.25	50	50	0.70
F"18	4.5	0.5	1	0.25	50	50	0.75

Table 14. Results of drug loading on pellets.

Formulation no.	Amount of drug (mg %)
F1	11.06 ± 0.079
F2	10.91 ± 0.089
F3	9.47 ± 0.369
F4	8.21 ± 0.373
F5	11.06 ± 0.195
F6	10.74 ± 0.130
F7	11.39 ± 0.284
F8	11.01 ± 0.079
F9	10.47 ± 0.065
F10	12.13 ± 0.073
F11	11.56 ± 0.186
F12	10.97 ± 0.146
F13	12.62 ± 0.075

Table 14. Contd.

F14	12.06 ± 0.107
F15	11.48 ± 0.132
F16	11.1 ± 0.064
F17	8.89 ± 0.2407
F18	7.89 ± 0.140
F19	12.32 ± 0.167
F20	11.95 ± 0.188
F21	11.63 ± 0.193
F22	10.98 ± 0.293
F23	10.64 ± 0.603
F24	11.25 ± 0.117
F25	10.82 ± 0.172
F26	10.52 ± 0.186
F27	10.14 ± 0.235

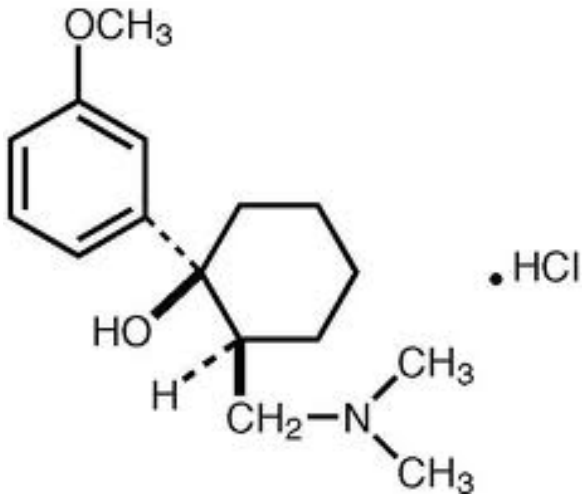

Figure 1. Chemical structure of Tramadol HCL.

F4 shows that in these formulations, increasing the ratio of polymer reduces the amount of drug release, especially at first release times. Comparison of F1, F5 and F8 shows that different plasticizers may cause different drug release profile; all parameters were equal in these formulations and just the type of plasticizer was changed. The desired changes showed that TEC with hydrophobic character could be responsible for less drug release in formulation F1 versus Tween with hydrophilic character, which used in F8 and led to more drug release.

Furthermore, in F5 with PEG as a plasticizer, release profile shows intermediate condition in comparison with F1 and F8. Comparison of F2, F6 and F9 shows the same results. TEC was selected as a better plasticizer to achieve pellets with steady release condition and no significant burst effect. Regarding the drug release results, it seems that Eudragit RS and Tween in legal ratio of polymer usage could not help to design controlled release formulation and most of the drug was released at the first sampling times.

Study on drug release profile of F10 - 12 in which just ratio of polymer was increased, shows that increasing polymer could sustain the release profile and when mixture of Eudragit RS and S was used; results were more reasonable. A study of F19 - 23 shows that formulations which were prepared using mixture of Eudragit RS and L were not suitable to prepare pellets with predicted drug release profile. Based on F24 – 27 we estimated that usage of Eudragit RL could cause higher range of drug release at initial sampling point. Comparison of Eudragit RS with RL shows that ammonium groups in Eudragit RL are more than RS and this reason causes more solubility of Eudragit RL in equal ratio. The formulation containing Eudragit RS could retain the release of drug more than Eudragit RL. These two types of Eudragit are not pH dependent. In comparison of Eudragit S and L, Eudragit S could release drug in pH ranges more than 7 and this range for Eudragit L is more than 6. Hence, Eudragit S could be use as polymer for colon drug delivery and the drug release through it is more sustained. To compare the effect polymer type on drug release profile, formulations F3, 11, 20 and 26 were studied in which the amount of polymers was 0.4 and formulations contained Eudragit RS, mixture of RS and S, RS and RL, respectively. Results showed that more water permeability and more solubility of Eudragit RL does not mean that it will be a suitable polymer for sustained drug release. The sustainability of these Eudragit was in the condition as shown: Mixture of S and RS > RS > mixture of RS and RL > RL. Therefore, the study was continued using mixture of Eudragits S and RS.

In addition, based on the results of plasticizers which were used (TEC, PEG 400 and Tween), TEC as an insoluble plasticizer could cause more sustained formulations. More studies showed that the best condition to achieve formulation with desired drug release profile will be when the mixture of Eudragit S and RS in of ratio of 90, 10 and the mixture TEC and Tween in ratio of 20, 5 were applied. Formulations F13 - 16 had such a condition. In comparison, F16 showed more reasonable drug release profile and drug release profile was studied for 10 h on it; Figures 6 and 7 shows the result. Studies showed that the kinetic of drug release through formulation F16 matched with zero order. The R2 of zero, first and Higushi kinetic was 0.963, 0.910 and 0.955, respectively.

Previous studies have been done to evaluate the ratio of different kinds of Eudragits on the *in vitro* release profile of drug (Hu et al., 2006; Bidah and Vergnaud, 1991; Golman and Jalilpak, 2008; Yadav and Jain, 2011; Adibkia et al., 2012). Some studies showed that the combination of two different acrylic polymers showed better effect on the release kinetics of drug than any individual polymer coated pellets to sustain the release of

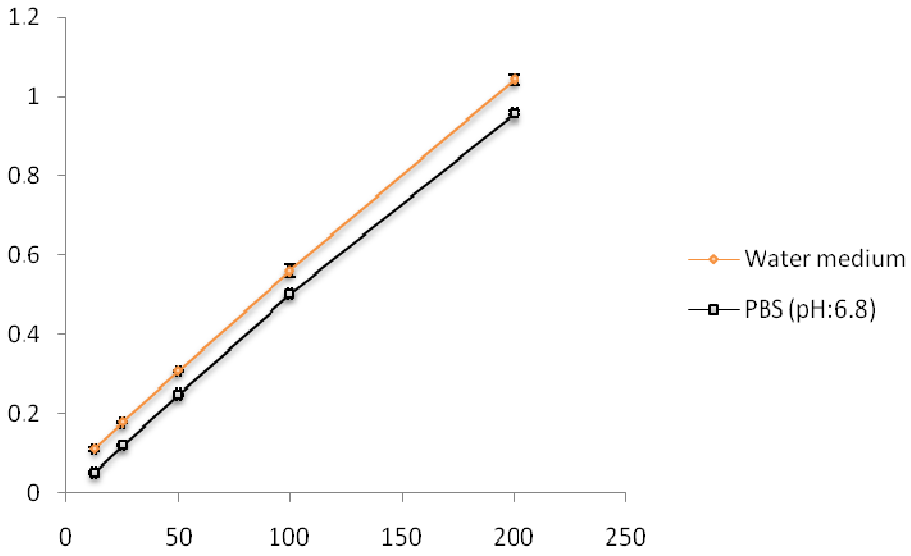

Figure 2. Calibration curve of Tramadol in water in λ_{max}:272 nm.

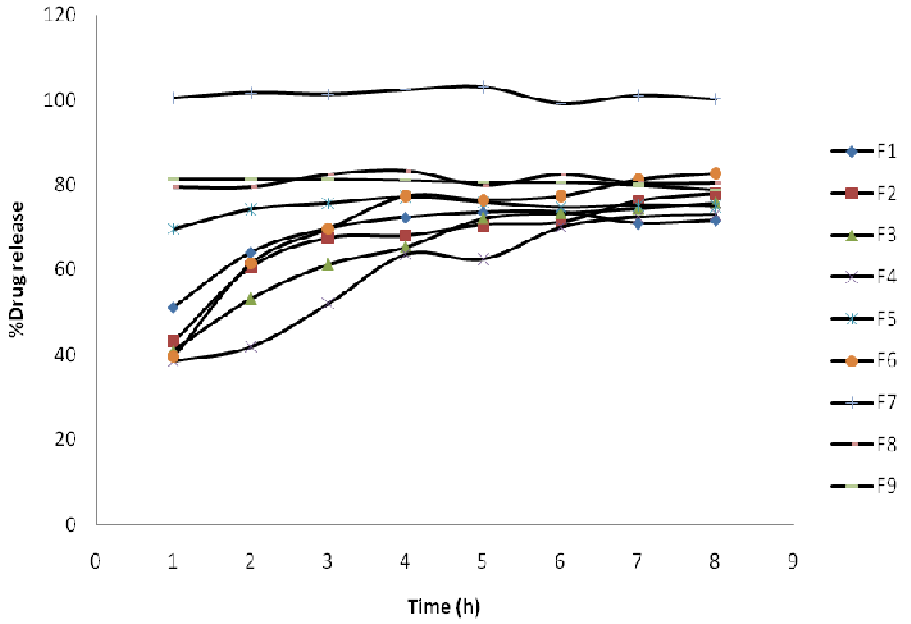

Figure 3. Drug release profile through formulation F1 - F9 in water medium.

drug over a period of time, with better dissolution profile as well as better linearity in drug (ketoprofen) release kinetics (Golman and Jalilpak, 2008). Some other studies showed that when Eudragites were used in formulation, results were affected by the type and ratio of used Eudragit S and also the solvents in which Eudragit RL and RS were suggested to prepare sustained release microcapsules (Kibria and Jalil, 2008; Ghaffari et al., 2011). Compatibility of Eudragits with tramadol was established before by Shinde et al. (2008).

Conclusion

The polymers used in this study are used widely in pharmaceuticals to control the release of drug. The approach of the present study was to evaluate the effect of type and ratio of polymer and plasticizers on the characters of desired sustained release pellets. It seems that Eudragit could be a suitable choice to prepare sustained release pellets containing Tramadol and with decreasing of drug taking intervals, the desired system

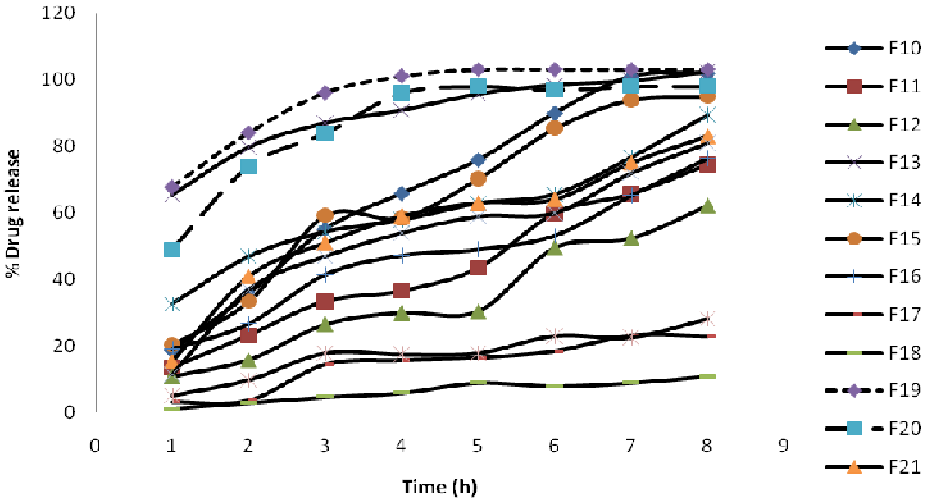

Figure 4. Drug release profile through formulation F10 - F21 in medium of acid and then PBS (pH: 6.8).

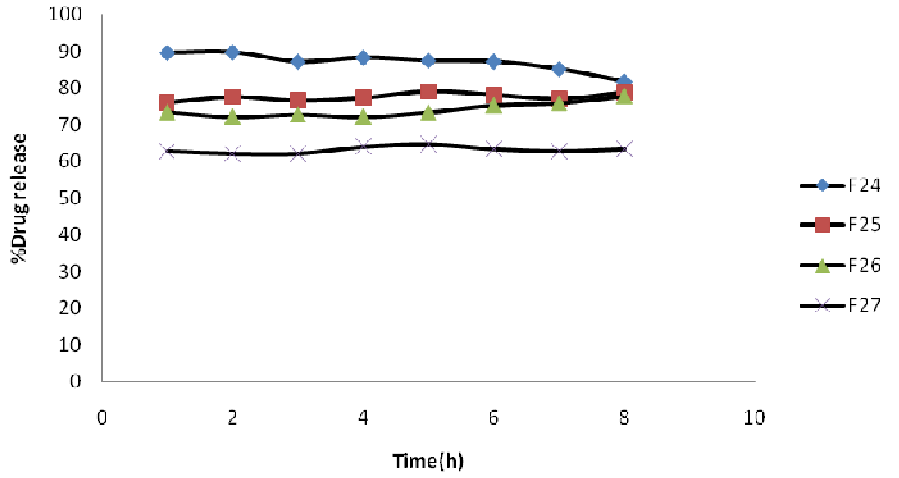

Figure 5. Drug release profile through formulation F24 - F27 in water medium.

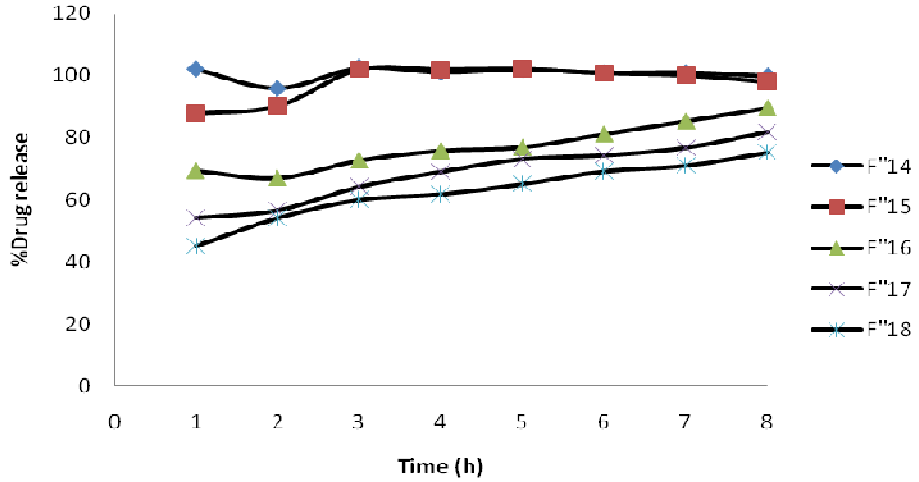

Figure 6. Drug release profile through formulation F"14 - F"18 in medium of acid and then PBS (pH: 6.8).

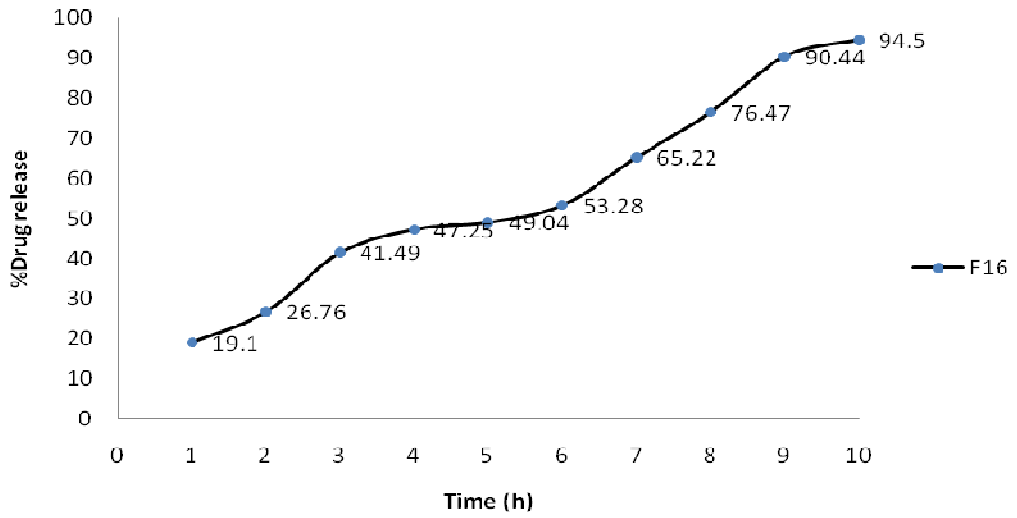

Figure 7. Drug release profile through formulation F16 in medium of acid and then PBS (pH: 6.8).

could increase compliance of patients and reduce doses and drug toxicity. Desired pellets could be filled in hard gelatin capsules to take by patients in the future after some modifications on drug loading efficiency and scale up procedure.

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Full Length Research Paper

Phytochemical constituents and antidiarrhoeal effects of the aqueous extract of *Terminalia superba* leaves on Wistar rats

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The aqueous extract of *Terminalia superba* leaves was subjected to phytochemical screening. Antidiarrhoeal property of the extract was determined at 50, 100 and 150 mg/kg body weight in castor oil-induced diarrhoeal Wistar rats. Phytochemical screening revealed the presence of saponins, cardenolides, triterpenes, flavonoids, steroids, phenolics and tannins, whereas alkaloids, anthraquinones and phlobatanins were not detected. The time of induction of diarrhoea was significantly ($P < 0.05$) prolonged at all the doses of the extract. The frequency of stooling and feacal parameters (total number of feaces and number of wet feaces), intestinal fluid accumulation (enteropooling) and the weight of intestinal content were significantly reduced. The data in the present study indicate that the aqueous extract of *T. superba* leaves possessed antidiarrhoeal properties.

Key words: Phytochemicals, *Terminalia superba*, aqueous extract, diarrhoea, rats.

INTRODUCTION

Diarrhoea is an increase in the frequency of bowel movements, a common cause of death in developing countries (including Nigeria) and a second common cause of infant deaths worldwide (Schiller and Sellin, 2010). In the year 2001, diarrhoea claimed 1,793,000 lives in European countries (Venkat, 2010). In the developing world, infectious diarrheoa is a serious life-threatening illness that results in 4 to 6 million deaths each year, mostly in children (Groulez and Wood, 1985). Diarrhoea is caused by viruses like rotavirus, norovirus, cytomegalovirus and viral hepatitis (Dennehy, 2000; Rheingans et al., 2006; Sompson et al., 2007). Other causes are bacteria like E*scherichia coli, Shigella flexneri,* and *Salmonella* spp. (Ryan and Ray, 2004; Potter, 2006), parasites (*Giardia lamblia, Entamoeba histolytica* and *Cryptosporidium*) and adverse reaction to medicines or food (Santosham et al., 1997). Diarrhoea is managed in a variety of ways. These include the use of drugs such as antibiotics like loperamide, diphenoxylate, atropine, and host of others. Nutrition and supplements have also been used (Ehrlich, 2010). Certain astringent herbs such as *Rubus fruticosus, Rubus idaeus, Matricaria recuitta,* and *Althea officialis* have been used for the treatment of diarrhoea (Ehrlich, 2010).

Terminalia superba is a large tree in the family Combretaceae claimed locally to possess antidiarrhoeal activity. It grows up to 60 m tall, with a domed or flat crown and a trunk typically clear of branches for much of its height, buttressed at the base. In Nigeria, it is widespread, where it is used for making chewing sticks and furniture (Groulez and Wood, 1985). In this research, aqueous extract of *T. superba* leaves was investigated

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for the treatment of diarrhoea in Wistar rats.

MATERIALS AND METHODS

Plant material

T. superba was collected from Ilesa town in Oriade Local Government Area, Osun State, Nigeria. The dried powdered (250 g) of *T. superba* leaves was soaked in 1000 ml of distilled water for 24 h. It was filtered using Muslin cloth and then suction pumped with Watchman No. 2 filter paper and air-dried in Petri dishes. The residual powder from each Petri dish was then scooped and then reconstituted in distilled water to prepare different doses (50, 100 and 150 mg/kg body weight) that were administered to different groups of animals.

Experimental animals

Wistar rats (*Rattus norvegicus*) of both sexes weighing 157.40 ± 14.68 g used in this experiment were purchased from Ayo Ola farms, Ilorin, Kwara State, Nigeria.

Qualitative phytochemical screening

The qualitative phytochemical screening of *T. superba* leaves was carried out as described by Odebiyi and Sofowora (1978) and Sofowora (1993) for the detection of tannins, saponins, phenolics, alkaloids, steroids, cardiac glycosides, flavonoids, anthraquinones, terpenoids and cardenolides.

Castor oil-induced diarrhoea

Diarrhoea was induced in the rats by the process as described by Gerald et al. (2007). The rats were fasted for 18 h prior to the experiment but were allowed free access to water. Twenty-five rats were grouped into five containing five rats each. Group I (negative control) was administered orally with 1 ml of distilled water while group II (positive control) was orally administered with 2.50 mg/kg body weight of loperamide chloride. Groups III, IV and V were administered with 50, 100 and 150 mg/kg body weight of aqueous extract of *T. superba* leaves. At the 30 min post-treatment, each animal from all the groups was orally administered 1 ml of castor oil. The time between oil administration and appearance of the first diarrhoeal feaces was noted. Observations for determining the severity of diarrhoea were accessed for 6 h by monitoring the diarrhoeal feaces on a pre-weighed white paper placed beneath the individual rat cages. The total number of feaces, diarrhoeal feaces and total weight of feaces excreted were expressed as average and compared with the controls. The percentage inhibition of diarrhoeal feaces in each group was calculated using the following equation:

$$\% \text{ Inhibition} = \frac{\text{No. of diarrhoeal feaces in control} - \text{No. of diarrhoeal feaces in treated rat}}{\text{No. of feaces in control}} \times 100$$

Castor oil-induced enteropooling

Intestinal fluid accumulation was determined by the method described by Havagiray et al. (2004). Twenty-five rats were divided into five groups of five animals each. Group I (negative control) received 1 ml of distilled water while group II (positive control) received loperamide at a dose of 2.50 mg/kg body weight. The test Groups III, IV and V were administered the aqueous extract of *T. superba* leaves at oral doses of 50, 100 and 150 mg/kg body weight. Castor oil (1 ml) was immediately orally administered to each of the rats in all groups. After 30 min, the rats were sacrificed according to the method of Yakubu et al. (2005) and the small intestine from the pylorus to the caecum was isolated. The intestinal contents were collected by milking into a graduated measuring cylinder, weighed and their volume measured.

Statistical analysis

The results were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Duncan multiple range tests. $P < 0.05$ was considered significant.

RESULTS

The phytochemical qualitative analysis of the aqueous extract of *T. superba* leaves revealed the presence of saponins, cardenolides, triterpenes, flavonoids, steroids, phenolics and tannins whereas alkaloids, anthraquinones and phlobatanins were not detected (Table 1).

In the castor oil-induced diarrhoeal experiment, the aqueous extract of *T. superba* leaves significantly prolonged ($P < 0.05$) the time of diarrhoeal induction. The highest dose of the extract (150 mg/kg body weight) has the most significant time of diarrhoeal induction. The frequency of stooling and faecal parameters (total number of feaces and number of wet feaces) decreased significantly compared with distilled water-treated animals. Similarly, the fresh weight and water content of the feaces decreased significantly with a greater reduction in these parameters at 150 mg/kg body weight when compared with water-treated animals. Inhibition of defecation was significantly increased in extract-treated animals compared with the water-treated ones (Table 2).

There was a significant decrease ($P < 0.05$) in the masses and volumes of the intestinal content in the extract-treated animals compared with distilled water-treated animals. The decrease was dose dependent. Similarly, the percentage inhibition of the intestinal content of animals treated with the extract increased dose dependently with 150 mg/kg body weight of the extract producing the highest percentage of inhibition of intestinal content. The percentage inhibition in animals treated with 150 mg/kg body weight is compared favourably with loperamide-treated animals (Table 3).

Table 1. Qualitative phytochemical results of the aqueous extract of *T. superba* leaves.

Phytochemical	Status
Saponins	+
Alkaloids	_
Cardenolides	+
Anthraquinones	_
Phlobatanins	--
Triterpenes	+
Flavonoids	+
Steroids	+
Phenolics	+
Tannins	+

(+), Present; (-), absent. Results are means ± SEM of three determinations.

Table 2. Time of induction and frequency of stooling due to the effect of aqueous extract of *T. superba* leaves against castor oil-induced diarrhoeal rats.

Parameter / Dose	Water (1 ml)	Loperamide (mg/kg body weight)	Plant extract (mg/kg body weight)		
	0	2.5	50	100	150
Onset time (min)	35.46 ± 0.43^{a}	-	50.13 ± 0.05^{b}	55.44 ± 0.01^{c}	65.41 ± 0.07^{c}
Total no. of feaces	18.13 ± 0.04^{a}	2.00 ± 0.02^{e}	14.54 ± 0.08^{b}	5.76 ± 0.04^{c}	4.33 ± 0.09^{d}
Number of wet feaces	5.45 ± 0.06^{a}	0.86 ± 0.01^{e}	5.21 ± 0.01^{b}	3.01 ± 0.06^{c}	1.00 ± 0.03^{d}
Fresh weight of wet feaces (g)	2.32 ± 0.02^{a}	0.42 ± 0.02^{e}	2.23 ± 0.07^{b}	2.09 ± 0.02^{c}	1.32 ± 0.02^{d}
Water content of feaces (ml)	1.14 ± 0.03^{a}	0.19 ± 0.04^{e}	1.12 ± 0.03^{b}	1.02 ± 0.04^{c}	0.64 ± 0.08^{d}
Inhibition of defeacation (%)	0.00^{a}	84.22^{e}	4.40^{b}	44.77^{c}	81.65^{d}

Values are means ± SD of five determinations. Values carrying different superscripts from the control are significantly different ($P < 0.05$).

Table 3. Anti-enteropooling activity of aqueous extract of *T. superba* leaves on castor oil-induced diarrhoeal rats.

Parameter / Dose	Water (1 ml)	Loperamide (mg/kg body weight)	Plant extract (mg/kg body weight)		
	0	2.5	50	100	150
Mass of intestinal fluid (g)	1.41 ± 0.02^{a}	1.00 ± 0.07^{e}	1.11 ± 0.02^{b}	1.07 ± 0.04^{c}	1.02 ± 0.03^{d}
Volume of intestinal fluid (ml)	1.60 ± 0.05^{a}	1.05 ± 0.06^{e}	1.37 ± 0.01^{b}	1.08 ± 0.03^{c}	1.06 ± 0.01^{d}
Inhibition of mass intestinal content (%)	-	29.08^{b}	21.28^{e}	24.11^{d}	27.66^{c}
Inhibition of volume intestinal content (%)	-	34.38^{b}	14.38^{d}	32.50^{c}	33.75^{b}

Values are mean of five determinations ± SD. Values carrying different superscripts from the control are significantly different ($P < 0.05$).

DISCUSSION

T. superba is used in the traditional system in managing diarrhoea by traditional medicine practitioners mostly in Western Nigeria. The present study sought to assess the antidiarrhoeal activity of aqueous extract of *T. superba* leaves. Our findings showed that the extract inhibited significantly ($P < 0.05$) castor oil-induced diarrhoea in Wistar rats.

It has been observed that the most active component of castor oil, ricinoleic acid produces an irritating action that stimulates the peristaltic activity of the small intestine (Rouf et al., 2003). This causes changes in the electrolytic permeability of the intestinal mucosa (Rouf et al., 2003). Its effect also caused the liberation of several endogenous mediators such as prostaglandins, which stimulates motility and secretion, thereby decreasing the absorption of sodium and potassium ions (Zavala et al.,

1998; Rouf et al., 2003); and nitric oxide, which has been claimed to contribute to the diarrhoeal effect of castor oil (Mascolo, et al., 1996).

The prolonged time of induction of diarrhoea, decreased frequency of stooling and faecal parameters (total number, fresh weight, water content and number of diarrhoeal faeces) observed with the extract in this study are indications of antidiarrhoeal potential. The inhibition of castor oil-induced intestinal fluid accumulation (enteropooling) and the weight of the intestinal content may be due to the ability of he extract to increase the re-absorption of electrolytes and water as also observed with the standard drug, loperamide. It can also be assumed that the antidiarrhoeal action of the extract was mediated by an antisecretory mechanism. The findings in this research agree with those of Tangu and Yadav (2004) and Appidi et al. (2010).

Antidiarrhoeal properties of medicinal plants may be due to bioactive agents such as tannins, flavonoids, steroids and terpenoids (Havagiray et al., 2004; Venkatesan et al., 2005). Others include alkaloids, saponins and reducing sugar (Longanga et al., 2000). For instance, the antidiarrhoeal activity of tannins has been ascribed to their ability to denature proteins in the intestinal mucosa by forming tannates that make intestinal mucosa more resistant to chemical alteration, thereby reducing secretion (Havagiray et al., 2004). In addition, flavonoids are known to inhibit intestinal motility and hydro-electrolytic secretion (Perez et al., 2005).

Conclusion

T. superba leaves exhibit antidiarrhoeal activity in the pharmacological models used, and thus, its use in traditional medicine is justified. The dose related antidiarrhoeal activity shown by the aqueous extract of *T. superba* leaves probably suggests the presence of some active ingredients (phytochemicals), which act through one or more antidiarrhoeal protecting mechanism.

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Full Length Research Paper

The comparison of multilevel models, method of moment and restricted maximum likelihood in assessing population bioequivalence

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The determination of bioequivalence is very important in the pharmaceutical industries because the regulatory agencies like the Food and Drug Administration (FDA) allow a generic drug to be marketed only if its manufacturer can demonstrate that the generic product is bioequivalent to the brand-name product. Up to date, there is a lack of widely accepted statistical procedure for assessing population bioequivalence. We propose multilevel models (MLMs) for evaluating and estimating parameters to assess population bioequivalence (PBE), and compare statistical properties of PBE estimators between MLMs and current approaches recommended by the FDA-the method of moment (MOM) and REML. The approach developed is illustrated using a real data set from the FDA. Statistical properties of MLM estimators are further explored using simulation studies as compared with MOM and restricted maximum likelihood (REML) estimators. The performance of MLM appeared to be much comparable to the existing REML procedure. The results suggest that MLM estimators that are fully comparable with REML estimators can be an adequate approach for assessing PBE. The MLMs approach proposed in the study provides an alternative and yet more flexible and powerful method than existing methods in assessing bioequivalence (BE) for complex study designs and data structures.

Key words: Population bioequivalence, multilevel models, simulation, estimation procedure, restricted iterative generalized least square (RIGLS), restricted maximum likelihood (REML), method of moments (MOM), food and drug administration (FDA).

INTRODUCTION

The goal of a bioequivalence study is to show that two formulations of a drug have similar bioavailability (Ashby, 2006). The determination of bioequivalence is very important in the pharmaceutical industries because the regulatory agencies like the United States Food and Drug Administration (FDA) allow a generic drug to be marketed only if its manufacturer can demonstrate that the generic product is bioequivalent to the brand-name product. Thus, the design, performance and evaluation of bioequivalence studies have received major attention among the health authorities and pharmaceutical industry, as well as, statisticians (Ashby, 2006; Blume and Midha, 1993).

During the last few decades, the assessment of average

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bioequivalence (ABE), which emphasizes comparing the means of two drug formulations has been well established (Ashby, 2006; Chen et al., 2000, 2001). The major concern was then of course how population bioequivalence (PBE) and individual bioequivalence (IBE) should be statistically assessed (Chow and Liu, 2008; Davit et al., 2008). In contrast to ABE, PBE requires equivalence on both the averages as well as variances, whereas IBE not only requires equivalence on similarity of averages and variances, but also on the homogeneity of subject-by-formulation interaction. In 2001, The FDA guidance recommended the use of the method of moments (MOM) for variance component estimation, as well as, the restricted maximum likelihood (REML) estimation of data including random missing records based on a two-sequence, four-period (2 × 4) cross-over design (Carrasco and Jover, 2003).

During the last decade, although, ABE is now a relatively matured field and is theoretically well understood, there is still a lack of widely accepted statistical procedure for assessing PBE. Many researchers have argued that the current FDA's statistical procedures are unsatisfactory in terms of their statistical properties (Endrenyi et al., 2003; FDA, 2001, 2003; Ghosh and Gönen, 2008; Ghosh and Ntzoufras, 2005). In addition, complete data sets are not common in bioequivalence studies, and MOM estimation is known to be limited in such situations. Furthermore, little information regarding the statistical test procedure is provided with the exception of 2 × 4 cross-over design, while for PBE and IBE, McNally et al. (2003) examined the generalized *P*-value approach for making inferences concerning the FDA-recommended PBE and IBE criteria. Carrasco et al. (2003) introduced the structural equation model (SEM) approach for parameter estimation and constructing criterion to assess IBE. Dragalin et al. (2003) proposed the Kullback–Leibler divergence (KLD) approach for parameter estimation and defining criterion in evaluating PBE and IBE. In 2005, Bayesian methods of assessing IBE and PBE using the FDA criteria were proposed (Ghosh and Ntzoufras, 2005). However, none of these methods are robust and sensitive enough to be accepted as the standard approach by current FDA guidance. Moreover, since bioequivalence evaluations are often based on the logarithmic transformation of AUC and C_{max}, the FDA consider two drugs to be bioequivalent only if they are similar in both AUC and C_{max}. It would be better to consider a test which included several endpoints together. However, none of the proposed methods including MOM, REML, SEM or KLD gave examples in assessing ABE, PBE and IBE with multiple endpoints. Research in this area is almost blank.

The theory by Harvey (2010) suggested that bioequivalence is a natural field to be assessed using MLMs. In principle MLMs can easily disentangle multiple variance components as well as covariance required by PBE assessment. To our knowledge, there was no thoroughly study discussing its applications in PBE studies.

This paper serves two purposes: (1) to introduce multilevel models using restricted iterative generalized least square (RIGLS) algorithm in estimating parameters to assess PBE, and (2) to compare the statistical properties of PBE estimators between multilevel models and two current approaches recommended by FDA. Our concern is not on the inference of the PBE criterion but merely estimating the components in the current FDA-proposed PBE criterion.

MATERIALS AND METHODS

Current methods for PBE

The 2001 FDA guidance proposed the following aggregated, scaled moment-based one-sided null hypotheses as:

$$H_0 : \frac{(\mu_T - \mu_R)^2 + (\sigma_T^2 - \sigma_R^2)}{\max(\sigma_{T0}^2, \sigma_R^2)} \geq \theta_p$$

Note that μ_T and μ_R, σ_T^2 and σ_R^2 are population means and variances in the logarithm scale. For convenience, we define $\Delta = \mu_T - \mu_R$ as mean difference, $\sigma_T^2 = \sigma_{BT}^2 + \sigma_{WT}^2$ as total variance of T, $\sigma_R^2 = \sigma_{BR}^2 + \sigma_{WR}^2$ as total variance of the R. σ_{BR}^2 and σ_{BT}^2 are between-subject variances, σ_{WR}^2 and σ_{WT}^2 are within-subject variances respectively for the T and R. The expression is linearised as:

$$H_0 : \nu_{PBE} = \Delta^2 + \sigma_T^2 - (1 + \theta_p)\sigma_R^2 \geq 0$$

When $\hat{\sigma}_R^2 > 0.04$ or $H_0 : \nu_{C.PBE} = \Delta^2 + \sigma_T^2 - \sigma_R^2 - \theta_p \cdot \sigma_{T0}^2 \geq 0$, $\hat{\sigma}_R^2 \leq 0.04$. θ_p is the bioequivalent limit with a recommended value of 1.7448. If the upper bound of the 95% one-sided confidence interval of criterion for both ln (AUC) and ln (C_{max}) are below zero, PBE can be claimed. The measure of PBE is a mixture of the mean and variance of the ln (AUC) and the ln (C_{max}). In the guidance, FDA recommended MOM to estimate variance component for complete data, as well as, REML estimation for data including missing records. After the estimation of the mean difference and the variances has been completed, 95% one-sided upper confidence bound for a linearised form of the PBE criterion can be obtained. FDA indicated that the method for the upper confidence bound should be consistent with the method used for estimating the variances.

Multilevel models (MLMs)

Models and notations

When measurements are repeated on the same subjects, as the data from 2 × 4 replicated cross-over design, a 2-level hierarchical structure is established. The measurement replicates are regarded as level 1 units, and subjects as level 2 units. Therefore, multilevel models (MLMs), proposed by Harvey Goldstein could be a powerful tool to analyze BE data. For the j^{th} subject at i^{th} replicate, we can fit

Table 1. Variance components for PBE criteria in multilevel model.

Parameter	Drug R	Drug T
Between-subject (Level 2)	$\sigma^2_{u0}\ (\sigma^2_{BR})$	$\sigma^2_{u0}+2\sigma_{u01}+\sigma^2_{u1}\ (\sigma^2_{BT})$
Within-subject (Level 1)	$\sigma^2_{e4}\ (\sigma^2_{WR})$	$\sigma^2_{e5}\ (\sigma^2_{WT})$
Total	σ^2_R	σ^2_T

a two-level random slope model in which factors for sequence and period are included, and the variances for T and R groups are defined at level 1 as:

$$y_{ij}=\beta_{0j}+\beta_{1j}x_1+\beta_2\text{sequence}_j+\beta_3\text{period}_{ij}+e_{4ij}z_{2ij}+e_{5ij}z_{3ij}$$

$$\beta_{0j}=\beta_0+u_{0j},\quad \beta_{1j}=\beta_1+u_{1j}$$

$$\begin{bmatrix}u_{0j}\\u_{1j}\end{bmatrix} \sim N(0,\ \Omega_u):\quad \Omega_u=\begin{bmatrix}\sigma^2_{u0} & \\ \sigma_{u01} & \sigma^2_{u1}\end{bmatrix}$$

$$\begin{bmatrix}e_{4ij}\\e_{5ij}\end{bmatrix} \sim N(0,\ \Omega_e):\quad \Omega_e=\begin{bmatrix}\sigma^2_{e4} & \\ \sigma_{e45} & \sigma^2_{e5}\end{bmatrix}$$

X_1 = 1 for drug T and 0 for drug R.
$Z4ij$ = 1 for drug R and 0 for drug T.
$Z5ij$ = 1 for drug T and 0 for drug R.

The intercept term is denoted by β_0 which estimates the mean of drug R, and the slope β_1 estimates the difference of means between T and R, (that is, $\beta_1=\mu_T-\mu_R=\Delta$). The '$\beta$s' are always referred to as "fixed" parameters of the model, such as period and sequence effects. What makes the two-level model different from standard linear regression model are the additional random effect, '*u*s' and '*e*s'. The '*u*s' parameters denote the residual in level 2, and the '*e*s' denote the residual in level 1.

Variance components for PBE criteria

To obtain variance components of PBE as shown in Equation 2, we can specify the level 2 variance as function of x_1 as represented:

$$\text{var}(u_{0j}+u_{1j}x_1)=\sigma^2_{u0}+2\sigma_{u01}x_1+\sigma^2_{u1}x_1^2.$$

Thus, the level 2 variance of between subjects for drug R (x_1 = 0) is σ^2_{u0}, and $\sigma^2_{u0}+2\sigma_{u01}+\sigma^2_{u1}$ for drug T (x_1 = 1). Since a subject cannot take both test and reference drug in each measurement, so $\text{cov}(e_{4ij},e_{5ij})=0$. Hence, the level 1 variance (or within-subject) for drug R (Z_{4ij} = 0, Z_{5ij} = 1) is:

$$\sigma^2_{WR}=\sigma^2_{e4}$$

for drug T (Z_{4ij} = 0, Z_{5ij} = 1) is:

$$\sigma^2_{WT}=\sigma^2_{e5},$$

The total level 1 variance is:

$$\text{var}(e_{4ij}z_{4ij}+e_{5ij}z_{5ij})=\sigma^2_{e4}z^2_{4ij}+\sigma^2_{e5}z^2_{5ij}.$$

The total variance of R is:

$$\sigma^2_R=\sigma^2_{BR}+\sigma^2_{WR}=\sigma^2_{u0}+\sigma^2_{e4}.$$

The total variance of T is:

$$\sigma^2_T=\sigma^2_{BT}+\sigma^2_{WT}=\sigma^2_{u0}+2\sigma_{u01}+\sigma^2_{u1}+\sigma^2_{e5}.$$

The variance components in the model above are summarized in Table 1.

Parameter estimation methods in MLMs

Goldstein and his colleagues developed the MLwiN software package (Version 2.23, Centre for Multilevel Modeling, University of Bristol, UK) for multilevel modeling (Rashbash et al., 2008). MLwiN uses the iterated generalized least squares (IGLS) and restricted iterated generalized least squares (RIGLS) algorithms to estimate parameters (Goldstein, 1986, 1989). In considering the small size of samples in both the illustrative example and our simulation data, we used RIGLS algorithm for all multilevel models presented in the paper. It is noted that we also use the term MLMs and RIGLS exchangeable for convenience throughout the paper.

Table 2. Parameter estimations (SE) from fitting multilevel models.

Parameter	ln (AUC)	ln (C_{max})
Fixed effect		
β_0 intercept	7.823(0.325)	5.894(0.370)
β_1 treatment	-0.043(0.060)	-0.109(0.074)
β_2 sequence	-0.186(0.204)	-0.302(0.231)
β_3 period	0.047(0.020)	0.056(0.027)
Random effect		
Level 2 (subject)		
σ^2_{u0} (σ^2_{BR})	0.366(0.093)	0.478(0.126)
σ^2_{u1}	0.053(0.034)	0.060 (0.053)
σ_{u01}	-0.009(0.039)	-0.035(0.058)
Level 1 (replicate)		
σ^2_{WR}	0.065(0.015)	0.119(0.028)
σ^2_{WT}	0.094(0.022)	0.165(0.038)

Table 3. Comparison of three methods in variance components estimates.

Variance component	ln (AUC)			ln (C_{max})		
	MOM	REML	MLM	MOM	REML	MLM
σ^2_{BT}	0.400(0.096)	0.400 (0.107)	0.401(0.105)	0.472 (0.113)	0.468(0.133)	0.468(0.129)
σ^2_{BR}	0.378(0.090)	0.366(0.095)	0.366(0.093)	0.515 (0.123)	0.478 (0.129)	0.478(0.126)
σ^2_{WT}	0.098 (0.024)	0.094(0.022)	0.094(0.022)	0.171 (0.041)	0.165(0.039)	0.165(0.038)
σ^2_{WR}	0.067 (0.016)	0.065 (0.015)	0.065(0.015)	0.126 (0.030)	0.119 (0.028)	0.119(0.028)
ν_{PBE}	-0.723	-0.686	-0.691	-1.106	-0.996	-1.019
95 percent upper bound	-0.360	-0.355	-0.431	-0.603	-0.524	-0.650

* The MLM's 95 percent one-sided upper bound was estimated using asymptotic normal procedure.

An illustrative example

To demonstrate the methods described in this paper, we acquired data set 17a for drug#17: Antihypertensive on the FDA website, http://www.fda.gov/drugs/scienceresearch/researchareas/biostatistics/ucm081434.htm. It is a 2 × 4 cross-overdesign (RTTR and TRRT) with 19 and 18 subjects per sequence. Thus, there are two replications for each drug. The two response variables are the natural logarithm of the reported value for AUC and C_{max}. We fitted two MLMs in MLwiN by using Equation 4 for ln(AUC) and ln(Cmax) respectively. The results obtained are presented in Table 2. Since total variance of the R group for both ln(AUC) and ln(Cmax) in this case were greater than 0.04, that is, $\hat{\sigma}^2_R = \hat{\sigma}^2_{BR} + \hat{\sigma}^2_{WR} > 0.04$, the reference–scaled (FDA, 2001) was used to test for the PBE criterion. We compared the scale calculated based on parameter estimates from the MOM, REML and RIGLS in MLM. As shown in Table 3, the estimators of variance components in REML and MLM are similar, while MOM provided slightly larger estimations .Using all the aforementioned methods, the upper bounds of the 95% one-sided confidence intervals for both ln(AUC) and ln(C_{max}) are less than 0, so PBE can be claimed.

Simulation study

Study design

The simulation study was designed to investigate the performance of RIGLS estimation in multilevel models in comparison to that of MOM and REML. We simulated log-transformed data for the two sequence (TRTR/RTRT), replicated design assuming a sample size

Table 4. Parameter settings for simulations (n=36, $\rho = 0.9$, 500 runs per scenario).

Scenario	Δ	$\sigma_{WT} = \sigma_{WR}$	$\sigma_{BT} = \sigma_{BR}$
1	0.05	0.3	0.3
2		0.3	0.6
3		0.5	0.5
4		0.5	1.0
5	0.223	0.3	0.3
6		0.3	0.6
7		0.5	0.5
8		0.5	1.0

of 36 subjects, 18 per sequence. This sample size corresponded to the illustrative example earlier mentioned, which also represented a moderate study size suggested in the FDA guidance.

Scenarios to be investigated

Scenarios were assumed based on drugs with typical characteristics and statistical distributions in practice and in previous studies. Six different parameters are to be defined to generate sample datasets for the simulation: Δ, σ_{WR}, σ_{WT}, σ_{BR}, σ_{BT} and ρ. Following other examples, we assumed same standard deviation for the two formulations, namely $\sigma_{WT} = \sigma_{WR}$ and $\sigma_{BT} = \sigma_{BR}$ (FDA, 2001; Endrenyi et al., 2000). We then considered the values for $\Delta = \mu_T - \mu_R$ =0.05 and ln1.25 (≈0.223), which corresponds to two prototypical situations in ABE studies, and were also used in the simulation research of PBE in the GlaxoSmithKline technical report by Patterson and Jones (2002).

The log-transformed parameters σ_{WR} and σ_{WT} can be calculated from the within-subject coefficients of variation (CV) on the original scale using the equation:

$$\sigma_{WR} = \sigma_{WT} = \sqrt{\ln(1 + CV^2)}$$

A CV of 30% is generally considered the threshold for a highly variable drug or drug product (HVD). In 2003 to 2005, the FDA's Office of Generic Drugs (OGD) reviewed 1010 acceptable bioequivalence studies of 524 different drug products. Among them, the highest CV was 55% (Davit et al., 2005). Since the topic of BE for highly variable drugs is one that has been intensely debated in many recent articles, conferences and meetings nationally and internationally (Haidar et al., 2008), our simulations were conducted for moderate high and very high variability products with CV of 30 and 55% respectively, which derives the values for σ_{WR} and σ_{WT} approximately 0.3 and 0.5.

In many studies, σ_{BR} and σ_{BT} are generally larger than σ_{WR} and σ_{WT} (Riviere and Papich, 2009; Willavize et al., 2006), we considered scenarios including cases where the σ_{BR} and σ_{BT} ranged from 1 to 2 times greater than the σ_{WR} and σ_{WT}.This range was also used by the sample size determination tables for PBE given in the Appendix of the FDA guidance document, and it also appeared to correspond to some retrospective analyses of AUC in replicate cross-over bioequivalence studies.

Finally, all scenarios considered a strong correlation ρ between R and T at 0.9. The reason for examining cases with such seemingly high correlation is that responses within a subject to different formulations of the same drug are still likely to have a strong degree of correlation even if the formulations are only borderline bioequivalent (Shao et al., 2000).

In summary, given the consideration of six parameters, eight scenarios were generated (Table 4). The chosen parameter values were considered to be representative of typical bioequivalence data and these are quite natural and common in practice.

Data generation and data analysis

For each scenario, 500 samples were simulated using SAS (Version 9.1.3, Cary, NC). We used SAS to perform MOM and REML estimation procedures and fitted MLMs using RIGLS procedure in MLwiN. Performances of different estimation procedures were evaluated using the following quantities that are frequently regarded as benchmark accuracy and precision measures; bias, mean square error (MSE) and coverage of 95% confidence interval.

SIMULATION RESULTS

The summarized results of parameter estimates, bias, MSE and CI coverage from 500 run samples were shown in Table 5 and Figures 1 to 3.

Parameter estimates

From Table 5, we observed that all three estimation procedures yielded same values of Δ for all scenarios. The estimates of variance components are close to their true values from all three methods. In nearly every

Table 5. Parameter estimates of MOM, REML and MLM (RIGLS) from 500 runs.

Scenario	Parameter settings	MOM		REML		MLM	
		Mean estimation	Standard error	Mean estimation	Standard error	Mean estimation	Standard error
1	$\Delta = 0.05$	0.052	0.046	0.052	0.046	0.052	0.046
	$\sigma^2_{BR} = 0.09$	0.095	0.034	0.092	0.033	0.093	0.032
	$\sigma^2_{BT} = 0.09$	0.092	0.036	0.090	0.035	0.092	0.033
	$\sigma^2_{WR} = 0.09$	0.091	0.021	0.088	0.020	0.087	0.019
	$\sigma^2_{WT} = 0.09$	0.093	0.022	0.090	0.021	0.089	0.019
2	$\Delta = 0.05$	0.052	0.055	0.052	0.055	0.052	0.055
	$\sigma^2_{BR} = 0.36$	0.375	0.101	0.364	0.098	0.364	0.098
	$\sigma^2_{BT} = 0.36$	0.369	0.103	0.359	0.100	0.359	0.100
	$\sigma^2_{WR} = 0.09$	0.091	0.021	0.088	0.020	0.088	0.020
	$\sigma^2_{WT} = 0.09$	0.093	0.022	0.090	0.021	0.090	0.020
3	$\Delta = 0.05$	0.054	0.076	0.054	0.076	0.054	0.076
	$\sigma^2_{BR} = 0.25$	0.264	0.095	0.257	0.093	0.259	0.088
	$\sigma^2_{BT} = 0.25$	0.256	0.099	0.249	0.096	0.255	0.091
	$\sigma^2_{WR} = 0.25$	0.252	0.058	0.244	0.056	0.241	0.053
	$\sigma^2_{WT} = 0.25$	0.259	0.060	0.251	0.057	0.246	0.054
4	$\Delta = 0.05$	0.053	0.092	0.053	0.092	0.053	0.092
	$\sigma^2_{BR} = 1$	1.041	0.279	1.012	0.272	1.011	0.271
	$\sigma^2_{BT} = 1$	1.026	0.286	0.997	0.278	0.998	0.278
	$\sigma^2_{WR} = 0.25$	0.252	0.058	0.244	0.056	0.244	0.056
	$\sigma^2_{WT} = 0.25$	0.259	0.060	0.251	0.057	0.250	0.057
5	$\Delta = 0.223$	0.225	0.046	0.225	0.046	0.225	0.046
	$\sigma^2_{BR} = 0.09$	0.095	0.034	0.092	0.033	0.093	0.032
	$\sigma^2_{BT} = 0.09$	0.092	0.036	0.090	0.035	0.092	0.033
	$\sigma^2_{WR} = 0.09$	0.091	0.021	0.088	0.020	0.087	0.019
	$\sigma^2_{WT} = 0.09$	0.093	0.022	0.090	0.021	0.089	0.019
6	$\Delta = 0.223$	0.225	0.055	0.225	0.055	0.225	0.055
	$\sigma^2_{BR} = 0.36$	0.375	0.101	0.364	0.098	0.364	0.098
	$\sigma^2_{BT} = 0.36$	0.369	0.103	0.359	0.100	0.359	0.100
	$\sigma^2_{WR} = 0.09$	0.091	0.021	0.088	0.020	0.088	0.020
	$\sigma^2_{WT} = 0.09$	0.093	0.022	0.090	0.021	0.090	0.020
7	$\Delta = 0.223$	0.227	0.076	0.227	0.076	0.227	0.076
	$\sigma^2_{BR} = 0.25$	0.264	0.095	0.257	0.093	0.259	0.088
	$\sigma^2_{BT} = 0.25$	0.256	0.099	0.249	0.096	0.255	0.091
	$\sigma^2_{WR} = 0.25$	0.252	0.058	0.244	0.056	0.241	0.053
	$\sigma^2_{WT} = 0.25$	0.259	0.060	0.251	0.057	0.246	0.054

Table 5. Contd.

	$\Delta = 0.223$	0.226	0.092	0.226	0.092	0.226	0.092
	$\sigma^2_{BR} = 1$	1.041	0.279	1.012	0.272	1.011	0.271
8	$\sigma^2_{BT} = 1$	1.026	0.286	0.997	0.278	0.998	0.278
	$\sigma^2_{WR} = 0.25$	0.252	0.058	0.244	0.056	0.244	0.056
	$\sigma^2_{WT} = 0.25$	0.259	0.060	0.251	0.057	**0.250**	**0.057**

corresponding estimates from REML and RIGLS (we use the term exchangeable with MLMs), while the latter two showed similar results as expected because RIGLS provides REML estimators. In cases of scenarios 1, 3, 5 and 7 where the between-subject variances are the same as within-subject variances, the between-subject variances estimated from MLMs are slightly larger than that of REML, while the within-subject variances from MLM are slightly smaller. This trend is not evident in scenarios 2, 4, 6 and 8.

Assessment of bias

Figure 1 graphically summarizes the bias of variance components versus their true population values. It can be scenario, estimates from the MOM were greater than seen that the biases for all three methods are rather small. However, MOM has the largest biases for both between-subject variances and within-subject variances, that is consistent with the previous simulation results (Patterson and Jones, 2002), whilst REML and RIGLS showed similar bias. In Figure 1, we can see that the bias for MOM is consistently larger (in magnitude), and increases steeply when the variance increases. This suggests that compared to RIGLS and REML, the MOM seems more prone to overestimate as the drug effects become more highly variable. RIGLS and REML estimates demonstrated less bias than MOM with similar patterns in bias as drug effects change in the simulation.

MSE

The MSE from three methods are quite small, suggesting good performance of all methods. Overall, the differences observed in MSE could be by chance in the simulation sample and ignorable in practice. MLM estimates are very close to REML with slightly better accuracy or less bias in some parameter estimates in certain scenarios based on the simulation samples.

Coverage of 95% CI

Three methods demonstrated differences in the CI coverage and those differences seemed grouped by scenarios 1, 3, 5 and 7, and scenarios 2, 4, 6 and 8. So we drew Figures 2 and 3 for these situations separately. Regarding the four variance components, although, the values are different among Figures 2 and 3, they showed the same patterns. The coverage of all the four variance components are constant, completely unaffected by the increasing drug variability. MOM gains the largest coverage, while MLM and REML showed similar results which are close to the nominated 95% for σ^2_{BR}, σ^2_{BT} and σ^2_{WR}, while for σ^2_{WT}, MLM has the same coverage with MOM, which is slightly lower than REML.

In summary, for the assessment of PBE, we can still conclude that the MLM and REML showed similar performance characteristics, and the latter two performed better than MOM.

DISCUSSION

This paper served two purposes; to propose multilevel models (MLMs) using the RIGLS algorithm for evaluating and estimating parameters to assess PBE, and to compare statistical properties of PBE estimators between MLMs and current approaches recommended by the FDA. To our knowledge, this paper is the first to present findings from simulation using the RIGLS algorithm in the software package MLwiN to analyze bioequivalence data. The results indicate that the performance of MLM (RIGLS) appeared to be much comparable to the existing REML procedure, because RIGLS is equal to REML in large sample (Goldstein, 1989). These two procedures are frequently indistinguishable and often provide better performance than MOM.

However, MLM exhibits three advantages over the existing methods. First of all, it has the ability to decompose the total variance into components for complex data structure, that is, more than three levels of hierarchy, more than two treatment and more than two periods.

The second advantage is its easy extension for assessing not only PBE, but also IBE and ABE. IBE is a criterion for deciding if a patient who is currently being treated with R can be switched to T. When IBE is considered, it assesses an aggregate measure involving the means and variance of T and R, as well as the subject-by-formulation interaction. The key difference from PBE is

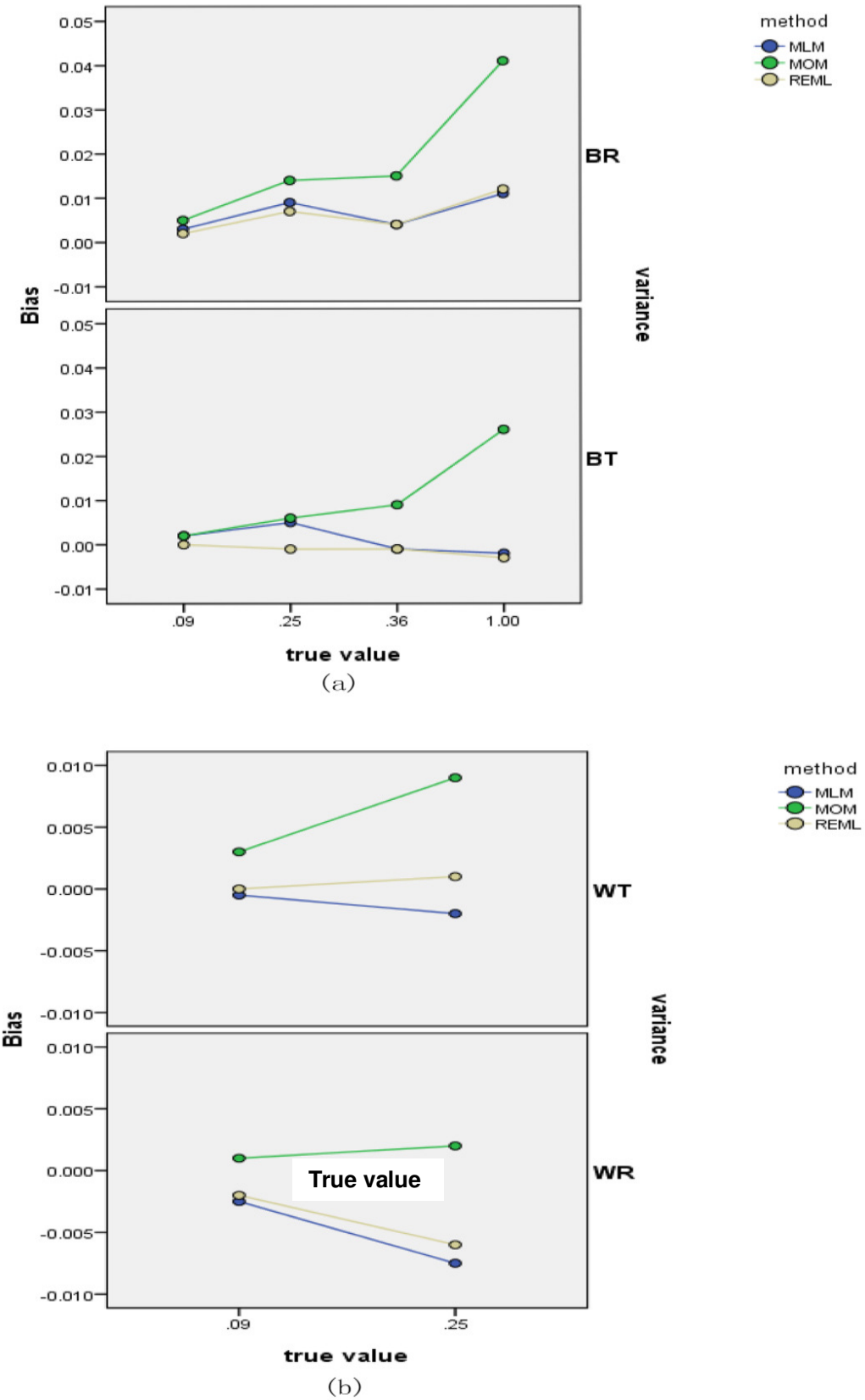
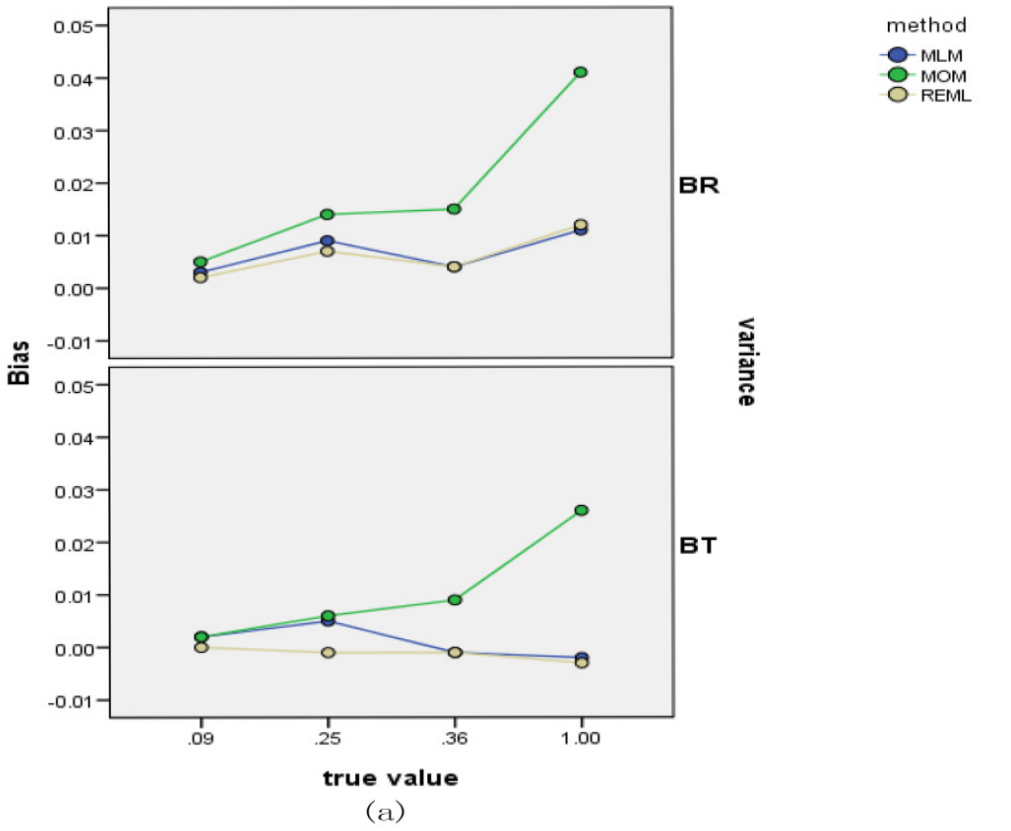


Figure 1. The mean bias in the estimates of four variance components by MLM, MOM and REML. BT stands for σ^2_{BT} , BR for σ^2_{BR} , WT for σ^2_{WT} ,and WR for σ^2_{WR} .



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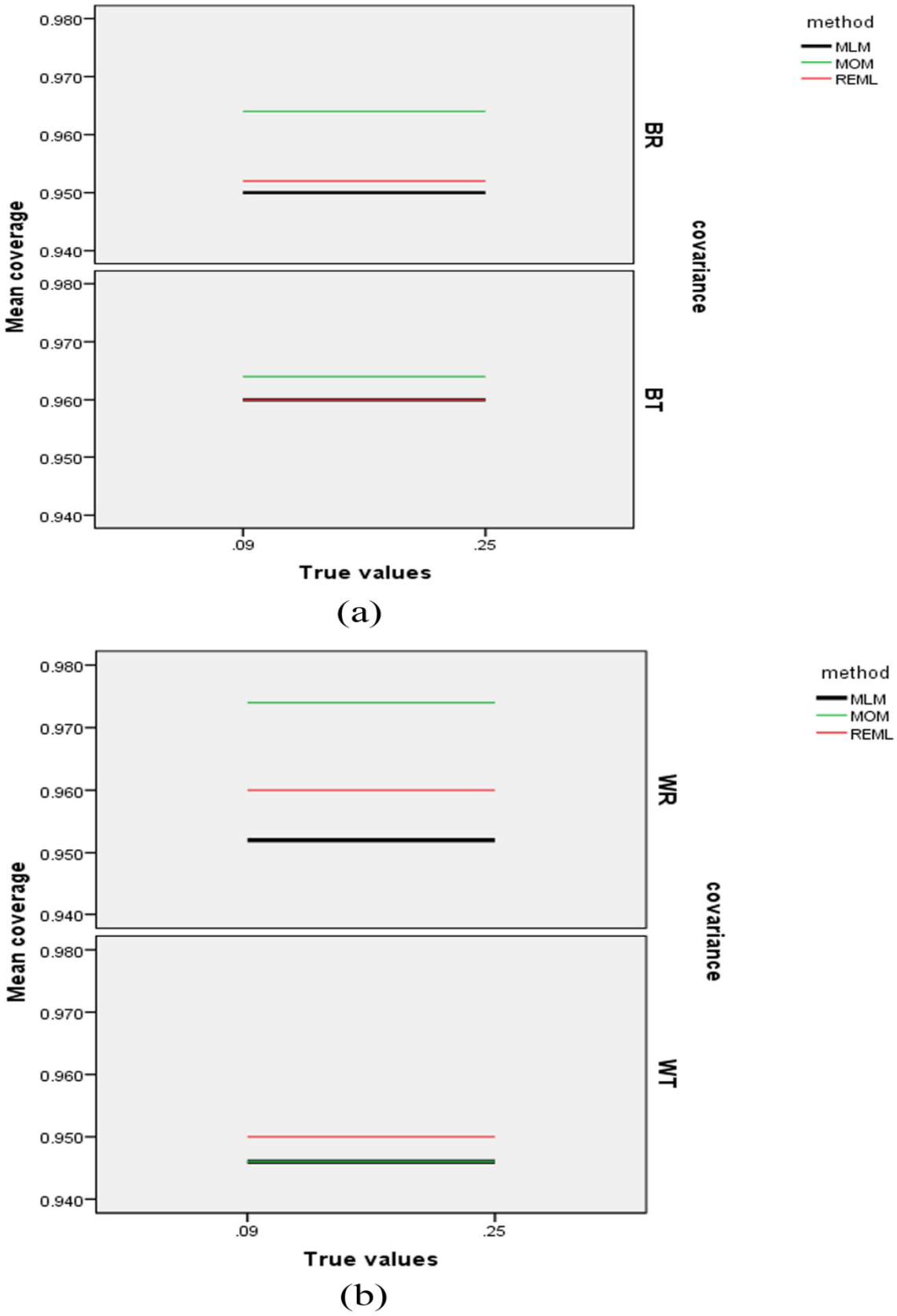
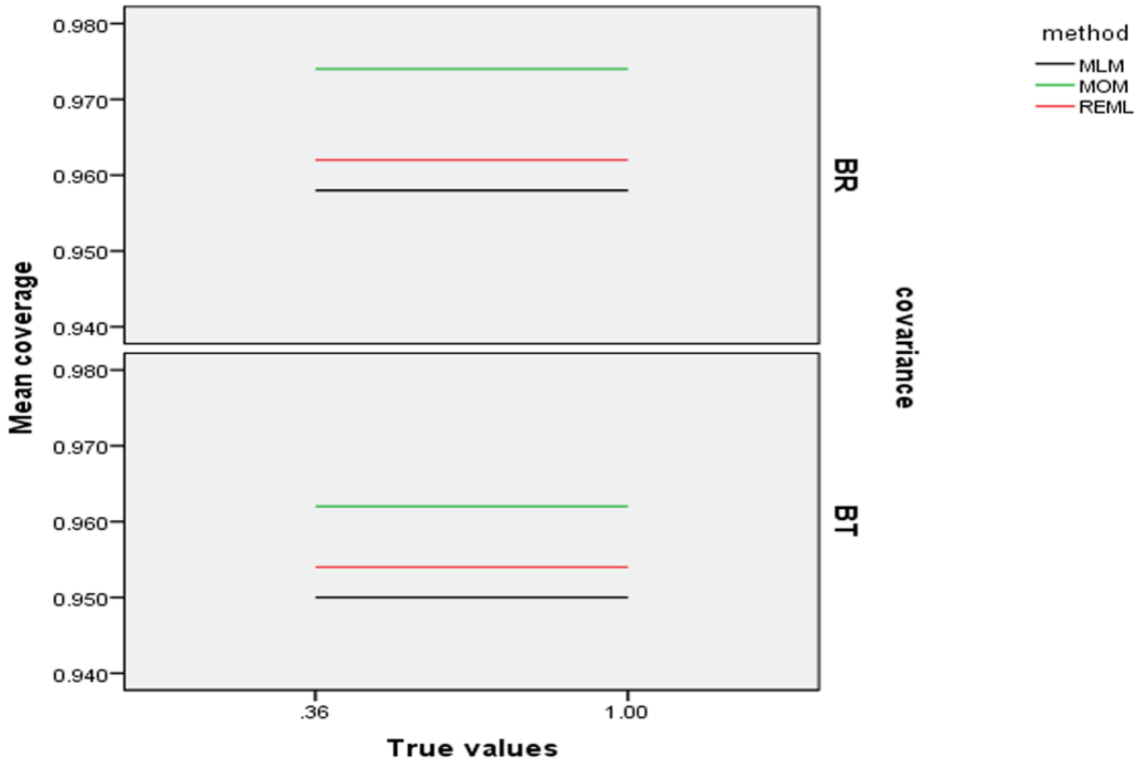
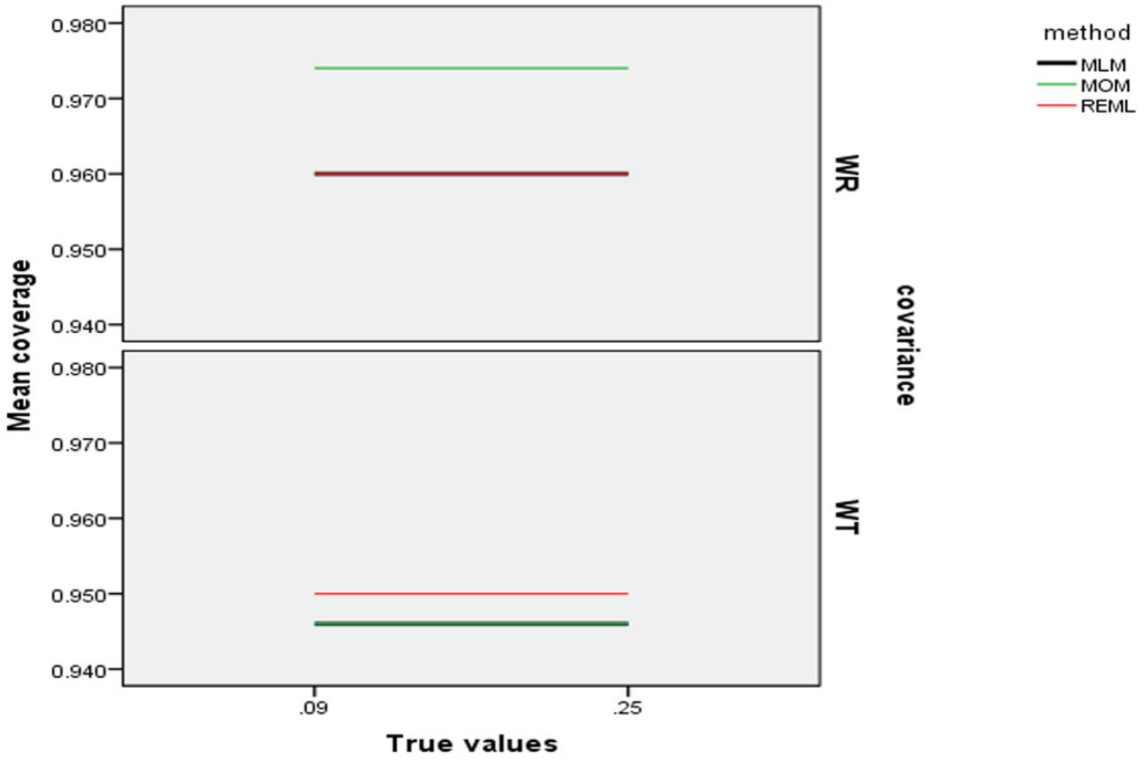


Figure 2. Coverages of 95% CI for four variances components according to MLM, MOM and REML on Scenario 1, 3, 5, 7.



(a)



(b)

Figure 3. Coverages of 95% CI for four variances components according to MLM, MOM and REML on Scenario 2, 4, 6, 8.

that IBE assumed individual difference in responding to formulation. Such difference can be captured and measured by the subject-by-formulation interaction σ_D^2 :

$$\sigma_D^2 = \mathrm{var}(\eta_{jT} - \eta_{jR}) = \sigma_{BT}^2 + \sigma_{BR}^2 - 2\rho\sigma_{BT}\sigma_{BR},$$

Where η_{iT} and η_{iR} are the random subject effect for subject j on T and R especially.

Multilevel model is readily expanded for assessing IBE. For instance, consider a two-level random slope model as an example, similar to the one we mentioned in simulation result. In Equation 4, the random effect u_{1j} is the slope residual in subject *j*, which measured the subject-specific increment to the treatment effect. It is easy to prove that $u_{1j} = \eta_{jT} - \eta_{jR}$, $\mathrm{var}(u_{1j}) = \mathrm{var}(\eta_{jT} - \eta_{jR}) = \sigma_D^2$. Therefore the key ingredient in the IBE criteria σ_D^2 can be expressed as σ_{u1}^2, which is a variance component in multilevel model. The extension of MLMs for assessing IBE is straightforward. A preliminary examination of MLMs in BE, including assessing IBE by Shen et al. (2009), has been published most recently. As the study only illustrated the MLMs approach by applying it on real example, further simulation studies to demonstrate the performance of such model in assessing IBE will be helpful.

The third advantage is the flexibility to cover multiple endpoints for simultaneous bioequivalence assessment. It should be noted that, in the example of FDA dataset illustrated in this paper, both AUC and C_{max} met the criterion for establishment of PBE. However, in practice, it is not uncommon to pass AUC but fail C_{max}. In this case, the regulatory authorities and researchers proposed several alternative measures (Wang et al., 1999; Ghosh and Gönen, 2008; Chen et al., 2001). One proposal was that for highly correlated AUC and C_{max}, one should obtain a combined estimate of the drug effects by considering the two outcomes simultaneously. In a multilevel model framework, data with multiple outcomes at certain time period within-subject can be viewed as a 3-level structure: the outcomes measured on each occasion are considered nested at the lowest level, within replicated measurement (now at level 2) within subject (now at level 3). MLMs approach can simply link the marginal models of AUC and C_{max} through a variance-covariance structure of the two at level 1 to form a simultaneous model to assess either ABE or PBE. Further simulation study should be conducted to examine performance of multivariate MLMs and the statistical features of the parameter estimates.

This research offers many exciting new directions for future research. A number of issues in the proposed MLMs approach are the remaining debatable. First of all, the 95% upper bound of the linearised PBE criterion in this study was calculated based on asymptotically normal assumption. The standard error for each variance component and function of a particular variance provided directly by the software MLwiN of RIGLS algorithm is based on asymptotic properties and may be unreliable when sample size is small. The advantage of MLM's capacity to assess IBE or multivariate outcomes would be penalized by more complex variance-covariance structure, more complex criterion, hence more uncertainty in the upper bound of the criterion. Recently FDA becomes more accepting of Bayesian (Li and Xu, 2011; Ashby, 2006). The use of the Bayesian approach allows us to obtain credible intervals and density plots for both random effect variances and standard deviations. The MLwiN was built in tools for MCMC and bootstrapping modeling. Hence, further research should explore those models within the MLMs framework in estimating upper bound of the PBE and its extendibility for IBE or multivariate MLMs criterion. Other methodological study in order to establish the MLMs approach in the BE field could be the rate of type 1 error over the effect size, sample size determination and the power of test. Further investigations in those areas are also suggested.

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Full Length Research Paper

Pharmacokinetics of omeprazole and its metabolites in premenopausal and postmenopausal females

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The pharmacokinetic (PK) of omeprazole (OMP), 5-hydroxy-omeprazole (5-OH-OMP) and omeprazole sulphone (OMP-SUL), was investigated in healthy premenopausal and postmenopausal females. A single oral dose, open-label, non-controlled, pharmacokinetic study was conducted in healthy premenopausal (n = 16) and postmenopausal (n = 8) females. The samples were analyzed using reversed phase high performance liquid chromatography (HPLC), different pharmacokinetics (PK) parameters were determined and compared statistically to evaluate the difference between two groups. The activities of CYP2C19 and CYP3A4 were determined as $AUC_{OMP}/AUC_{OH\text{-}OMP}$ and $AUC_{OMP}/AUC_{OMP\text{-}SUL}$, respectively. The significant differences ($P < 0.05$) between C_{max}, t_{max}, $[AUC]_0^t$, $[AUC]_0^\infty$, $t_{1/2}$ and metabolic ratios (MR) of OMP, 5-OH-OMP and OMP-SUL were observed between premenopausal and postmenopausal females. The present studies showed the higher AUC and longer $t_{1/2}$ in postmenopausal subjects. The increase in MR for 5–OH-OMP and also OMP-SUL determined as $AUC_{OH\text{-}OMP}/AUC_{OMP}$ and $AUC_{OMP\text{-}SUL}/AUC_{OMP}$, respectively, was also observed in postmenopausal females compared with the premenopausal females.

Key words: Omeprazole, pharmacokinetics, menopause.

INTRODUCTION

The perimenopause and menopause are associated with manifestation of hyperacidity, along with hot flashes (Pachman et al., 2012; Machowska et al., 2004), depression and insomnia (Cuadros et al., 2012; Freeman et al., 2007). Postmenopausal females have higher propensity for gastric hyperacidity (Machowska et al., 2004), which is associated with increased use of gastric antisecretary drugs including proton pump inhibitors (PPIs). The increased risk of hip bone fracture has been reported in postmenopausal females, accompanied with the use of OMP (Roux et al., 2009). Menopause is associated with hormonal transitions (Davison et al., 2005), which affects metabolism of drugs (Simpson and Davis, 2001) and this may lead to altered pharmacokinetics (PK) in postmenopausal females.

The OMP is metabolized in the liver by CYP2C19 to 5-OH-OMP (70%) (Andersson et al., 1994; Karam et al., 1996; Cederberg et al., 1989) and by CYP3A4 into OMP-SUL (30%) and a major secondary metabolite is hydroxyl sulphone (OH-SUL) obtained from 5-OH-OMP and OMP-SUL (Andersson et al., 2012).

PK of OMP has been accomplished considering genetic polymorphism (Andersson, 1990; Ramsjö, 2010) racial or interethnic differences (Kim et al., 2004; Ieiri et al., 1996), and population PK (Gonzalez et al., 2003; Chang et al., 1995), but in these studies PK were evaluated without

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Table 1. Demographic characters of pre and postmenopausal subjects.

Group	Pre (n=16)	Post (n=8)
Age (years)	24.0±0.30	55.90±0.74*
Weight (kg)	52.60±2.13	66.50±2.04
Height (cm)	148±3.2	150.40±5.1
BMI	24.5±0.43	29.6±0.52

*Significant at P<0.05; NS, Non significant; Pre, premenopausal females; Post, postmenopausal females; BMI, body mass index. Demographic characters have been shown

considering genders differences or menopausal status of females. Though, PK profile of OMP has been investigated in males; however, in females it is obscure, including the postmenopausal females and this needs further exploration (Sakai et al., 2001). Different variables, which may affect PK and clinical outcome of drugs, must be investigated in order to optimize the dose regimen (Weston and Hood, 2004).

The relationship between the CYP2C19 genotype and PK of OMP in elderly people was established; however, menopause or gender difference was not considered as a potential factor (Ishizawa et al., 2005). The extensive literature survey reveals that available data on the subject of PK of OMP is not sufficient to explain or predict PK of OMP in postmenopausal females.

This study was conducted to investigate the PK differences for OMP and its metabolites 5-OH-OMP and OMP-SUL, as well as to determine the differences of activities of CYP2C19 and CYP3A4 between premenopausal and postmenopausal female.

MATERIALS AND METHODS

An optimized and validated high performance liquid chromatography-ultraviolet (HPLC-UV) method was used for quantitative analysis of OMP, 5-OH-OMP and OMP-SUL (Ahmad et al., 2011). The applied method was validated by determining accuracy, precision, sensitivity and specificity (validation parameters are shown in Table 3 and Table 6). A HPLC system of Perkin Elmer Series 200 (Norwalk, USA), operational with online vacuum degasser, Perkin Elmer Series 200, column oven, and UV-VIS detector were used. Total-chrome workstation software (version 6.3.1) was linked with the LC system by network chromatography interface (NCI) 900.

OMP, 5-OH-OMP, OMP-SUL and pantoprazole, used as internal standard (IS), were provided by Astra Zeneca (Sweden) and Medicraft (Pvt.) Ltd. (Peshawar, Pakistan), respectively. HPLC grade methanol, potassium dehydrogenate phosphate and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich (oslo, Norway), Fischer Scientific (Leicestershire, UK) and Merck (Darmstadt, Germany), respectively. Distilled water prepared by Millipore (Milford, USA) distillation apparatus was used throughout this study.

Study design

A single dose, open-label, non-controlled, premenopausal and postmenopausal cohort PK study was designed to investigate the PK of OMP and the activities of CYP2C19 and CYP3A4 in premenopausal (n = 16) and postmenopausal (n = 8) healthy female volunteers. This study was conducted in agreement with the principles of international conference on harmonization for conducting good clinical practice and declaration of Helsinki. This study was approved by ethical committee of Department of Pharmacy, University of Peshawar. Application number 13/EC/Pharm.ref number 12/Pharm.

Selection of volunteers

Healthy premenopausal (n = 16) and postmenopausal (n = 8) females were selected. The design and purpose of the study was explained to volunteers and a written informed consent was obtained. The mean ± standard deviation (SD) age of the premenopausal volunteers was 24.0 ± 0.23 years and for postmenopausal females, it was 55.90 ± 0.74 years. The body weights of premenopausal and postmenopausal female volunteers were 52.60 ± 2.13 and 66.50 ± 2.04 kg, respectively. All premenopausal female subjects had a regular menstrual cycle of 28 days. The postmenopausal females had entered the menopause completely for minimum one year passed after the cessation of their last menstrual cycle. The overall health status of postmenopausal females was satisfactory, free of any gross level pathological evidence, but fluctuation in their blood pressures was compromised. The demographic data of volunteers is shown in Table 1. Prior to inclusion of volunteers in the experiment, complete medical histories were obtained and full body screening by various laboratory tests was performed like blood hemoglobin, serum cholesterol, serum bilirubin, serum creatinine, low density lipoprotein (LDL)-cholesterol, triglyceride, high density lipoprotein (HDL)-cholesterol, very low density lipoprotein (VLDL)-cholesterol to determine their health status. The clinical data is shown in Table 2.

Drug administration and blood sampling

The subjects were instructed to avoid intake of any medication including herbal medicines a week before the study. The volunteers were kept on overnight fasting before trial, no juice, caffeine or food intake was allowed except water before studies. At about 8.00 AM, each volunteer received OMP capsules (40 mg) with a full glass of water (ca ≈ 250 ml). The blood samples (≈ 5 ml) were collected from cubital vein and occasionally collected from cephalic vein, at 0.0, 0.5, 1.0, 2, 3, 4, 6 and 8 h. The blood samples were centrifuged and the plasma was separately stored at -80°C till analysis. The standard breakfast and lunch was served after 3 and 5 h, respectively, following the drug administration.

Standard solutions

Stock solutions (1 mg/ml) of OMP, 5-OH-OMP and OMP-SUL were prepared by dissolving the drug and metabolites in methanol. Working solutions were prepared by diluting the stock solution in Eppendorf tubes (2 ml) in the concentration ranges of 5 to 500 ng/ml for each OMP and OMP-SUL, while 10 to 1000 ng/ml for 5-OH-OMP, respectively. The concentration of internal standard (IS) (1 µg/ml) was kept constant in all dilutions. While preparing plasma sample, plasma (150 µl) and 50 µl of each of analyte and internal standard were taken (Ahmad et al., 2011).

Sample preparation

Plasma sample thawed at room temperature and sample (150 µl) were transferred to Eppendorf tube. Then pantoprazole, used as an

Table 2. Clinical data of pre and postmenopausal female participants of pharmacokinetic study of OMP and its metabolites.

Clinical value	Unit	Pre (n=16)	Post (n=16)	P value
Hb	gm/dl	14.44±0.23	12.84±0.32	0.0014**
Serum bilirubin	mg/dl	0.56±0.3	0.77±0.5	0.0021**
Serum creatinine	mg/dl	0.66±0.05	0.8±0.05	0.1142
Serum cholestrol	mg/dl	133.8±8.5	144.2±4.2	0.3
Serum triglyceride	mg/dl	84.63±10.32	114.0±7.8	0.04*
HDL cholesterol	mg/dl	31.25±4.4	81.13±4.4	0.2
LDL cholesterol	mg/dl	71.88±5.3	78.5±4.3	0.84

*Significant at P<0.05, **Significant at P<0.005, ***Significant at P<0.0005, Hb, hemoglobin value; HDL cholesterol, high density lipid cholesterol; LDL cholesterol, Low density lipid cholesterol.

Table 3. Pharmacokinetic parameters of OMP (40 mg) following oral administration in pre and postmenopausal female volunteers.

PK	Unit	OMP		P-value	95% confidence interval
		Pre (n=16)	post (n=8)		
C_{max}	µg/ml	2.9±0.60	3.7±0.50	0.003**	-1.4 to -0.34
t_{max}	h	2.3±0.2	2.583±0.4	0.14	-0.60 to 0.10
$[AUC]_0^t$	µg.h/ml	8.7±2.24	15.3±2.24*	0.0001***	-12.81to -7.62
$[AUC]_0^\infty$	µg.h/ml	9.7±3.31	19.38±2.9*	0.0001***	-18.8 to -0.6
Vd	ml/kg	315.4±97.9	469.3±125.1	0.011*	-265.5 to -42.3
CL	ml/h/kg	111.6±20.69	58.40±8.62*	0.0001***	40.5 to 65.5
$t_{1/2}$	h	2.3±0.8	2.9±0.24	0.01*	-1.06 to -0.2

*significant at $p < 0.05$, ** significant at $p < 0.005$, ***significant at $p < 0.0005$; PKs , pharmacokinetic parameters, compared in pre and postmenopausal females for OMP ;Cmax, maximum plasma concentration:tmax, time to achieve the maximum plasma concentration;t1/2 ,elimination half life;AUC0-t ,Area under the plasma concentration time curve from 0 time to last sampling time;AUC∞ , Area under the plasma concentration time curve from 0 hr to infinity time,Vd,volume of distribution; CL, clearence of analyte from body

internal standard, was added and vortexed for 30 s. Three parts of methanol (450 µl) was added, vortexed for further 3 min and then centrifuged at 5000 rpm at 0˚C for 5 min. The separated clear layer of plasma was transferred to Eppendorf tube (Ahmad et al., 2011).

Chromatography

Chromatographic separation was achieved using Supelco/discovery C_{18} (150 × 4.6 mm, 5 µm) analytical columns protected by a Perkin Elmer C_{18} (30 × 4.6 mm, 10 µm; Norwalk, USA), pre-column guard cartridge, the temperature of the column was 45°C and the detector wavelength was adjusted at 302 nm. The mobile phase, ratio of methanol and buffered (pH 7.2) aqueous phase of potassium dehydrogenate phosphate (42:58 v/v) were pumped, at the flow rate of 0.8 ml/min. Different optimized parameters are shown in Table 3.

PK analysis

Plasma concentration of OMP, 5-OH-OMP, and OMP-SUL as a function of time was plotted on semi-log scale to evaluate various PK parameters. The data was also analyzed using PK-Solution® 2.0 software for non-compartmental data analysis.

Statistical analysis

The software programme used for statistical analysis was graph pad prism 5 and Statistical Package for Social Sciences (SPSS) 16. The data was presented as mean ± standard deviation (SD) and the difference between various groups was studied using student *t*-test at 95% confidence interval.

RESULTS

PK of OMP and its metabolites

Volunteers characteristics

The demographic and clinical data of the volunteers is shown in Tables 1 and 2, respectively. The age of postmenopausal females was significantly higher when compared with the premenopausal females ($P < 0.05$), while the rest of the demographic characters did not display any significant difference. Hemoglobin value was significantly higher in premenopausal females ($P < 0.005$)

Table 4. Pharmacokinetic parameters of metabolite, 5-OH-OMP following oral administration of OMP (40 mg) in pre and postmenopausal female volunteers.

PK	Unit	5-OH-OMP		P-value	95% CI
		Pre (M)	Post (F)		
C_{max}	µg/ml	1.11±0.2	1.252±0.5	0.02*	-0.60 to 0.4
t_{max}	h	2.62±0.20	3.06±0.34	0.020*	-0. 8to -0.099
$[AUC]_0^t$	µg.h/ml	3.13±0.53	3.90±0.7	0.0001***	-1.41 to -0.15
$[AUC]_0^\infty$	µg.h/ml	3.6±0.73	9.486±2.51*	0.0001***	-7.05 to -4.6
Vd	ml/kg	664.3±110.1	640.1±48.5	0.0001***	43.09 to 91.7
CL	ml/h/kg	271.3±52.30	93.88±19.8 *	0.0001***	146.7 to 208.3
$t_{1/2}$	h	2.3±0.79	7.64±2.7*	0.0001***	-6.8 to -3.8
MR	AUC_{OMP}	2.8± 0.63	3.72± 1.3	0.06	-1.9 to 0.04

*Significant at p < 0.05, ** significant at p < 0.005, ***significant at p < 0.0005, MR: metabolic ratio, pharmacokinetic parameters compared in pre and postmenopausal females for 5-OH-OMP.

Table 5. Pharmacokinetic parameters of metabolite, OMP-SUL following oral administration of OMP (40 mg) in pre and postmenopausal female volunteers.

PKs	Unit	OMP-SUL		P-value	95% CI
		Pre	Post		
C_{max}	µg/ml	0.25±0.032	0.18±0.082	0.012*	0.030 to 0.2
t_{max}	Hr	3.5±0.42	4.040±0.8	0.1069	-1.24 to 0.14
$[AUC]_0^t$	µg.hr/ml	0.75±0.2	0.8±0.2	0.3821	-0.3 to 0.11
$[AUC]_0^\infty$	µg.hr/ml	1.4±0.42	3.653±1.6	0.0062**	-3.63 to -0.9
Vd	ml/kg	5155±1715	5996±2051	0.3383	-2676 to 995.6
CL	ml/hr/kg	1527±610.2	779.7±467.3	0.004**	273.4 to 1223
$t_{1/2}$	Hr	4.06±1.07	26.58±11.9*	0.0001***	-28.89 to -16.17
MR	AUC_{OMP} /$AUC_{OMP-SUL}$	13.08± 6.4	18.16± 6.91	0.08	-10.8 to 0.61

*significant at p < 0.05, ** significant at p < 0.005, ***significant at p < 0.0005, MR: metabolic ratio , pharmacokinetic parameters compared in pre and postmenopausal females for OMP-SUL.

while serum bilirubin (P < 0.005) and serum triglyceride levels (P < 0.05) were significantly higher in postmenopausal females. Rest of the clinical data also did not show any significant difference. Plasma proteins and plasma albumins were insignificantly higher in postmenopausal females.

Gender based PK analysis of omeprazole and its metabolites 5-OH-OMP and OMP-SUL

The plasma OMP, 5-OH-OMP and OMP-SUL concentrations as a function of time are shown in Figures 1, 2 and 3. The PK data of OMP, 5-OH-OMP and OMP-SUL between premenopausal and postmenopausal female were then compared (results are shown in Tables 3, 4 and 5).

In postmenopausal females, 27% increase in C_{max} for OMP (P < 0.005) and 13% increase in t_{max}, (P > *0.05*) compared with the premenopausal females was observed. Also, results in the 75% increase in the $[AUC]_0^t$ and 99% increase in the $[AUC]_0^\infty$ in postmenopausal females (P < 0.0005). The elimination $t_{1/2}$ was also significantly (P < *0.05*) higher (26% higher) in postmenopausal females. The significant increase in volume of distribution (Vd) and decrease in clearance of analyte from body (CL) of OMP was observed in postmenopausal women compared with the premenopausal female volunteers.

The C_{max} and t_{max} of 5-OH-OMP were significantly (P < 0.05) higher in postmenopausal female compared with premenopausal females. The metabolic ratios (MR) exhibited a 32% raise in postmenopausal females. The elimination $t_{1/2}$ value was significantly higher (232% raise) in postmenopausal females, (P < 0.05). Vd and CL increased significantly in premenopausal females.

C_{max} of OMP-SUL decreased upto 28% in postmenopausal

Table 6. HPLC Method validation parameters (Ahmad et al., 2011)

Parameter	Calibration range (mg/mL)	Regression equation	Correlation coefficient	Spiked plasma samples	Regression equation	Correlation coefficient sensitivity	Limit of detection, LOD (ng/mL)	Limit of quantification, LOQ (ng/mL)
OH	0.01–1	Y =1.71 x +0.00	0.999	24.5±0.43	Y = 1.720 x +0.011	0.998	3	10
5-OMP -OMP	0.05—.5	Y = 3.675 x -0.002	0.999	29.6±0.52	Y = 3.581 x -0.05	0.999	1.5	5
OMP-SUL	0.05–.5	Y = 4.430 x -0.00	0.999	0.53	Y = 4.234 x +0.027	0.998	1.3	5

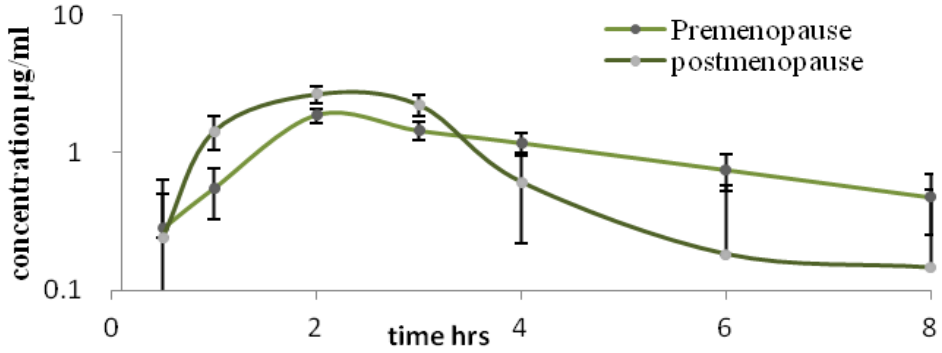

Figure 1. The plasma concentrations of OMP as a function of time in pre and postmenopausal females.

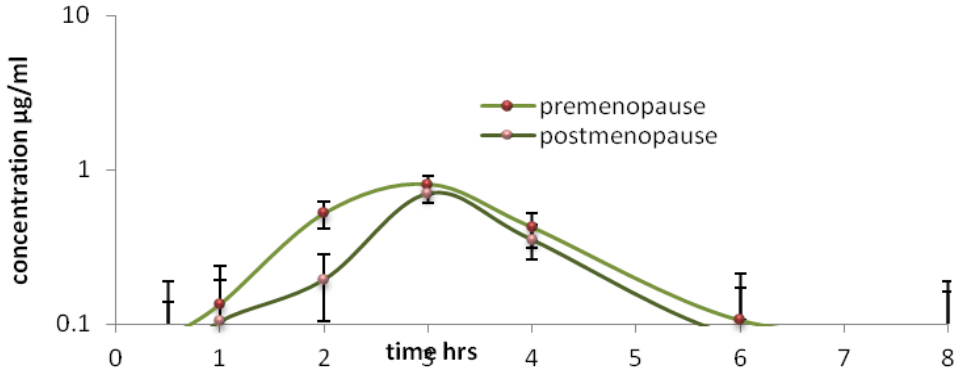

Figure 2. The plasma concentrations of 5-OH-OMP as a function of time in pre and postmenopausal females.

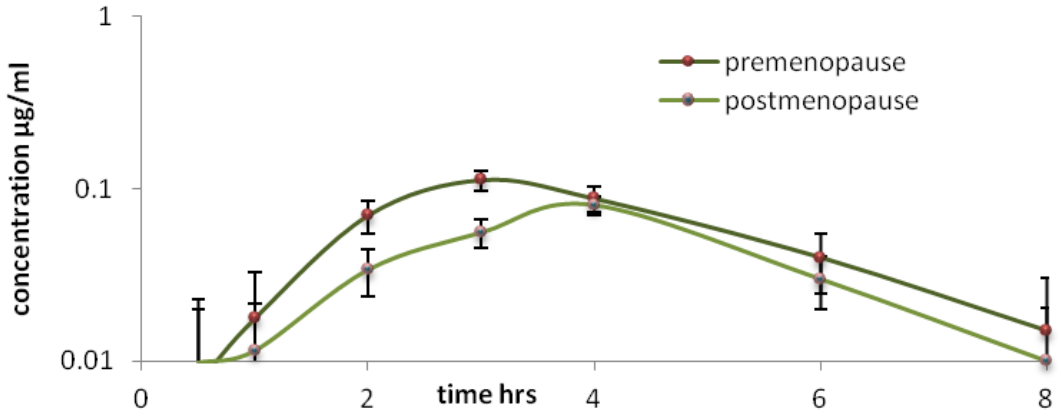

Figure 3. The plasma concentrations of OMP-SUL as a function of time in pre and postmenopausal female.

females compared with premenopausal females ($P < 0.05$) while the t_{max} was 15% higher in postmenopausal females with no significant difference ($P > 0.05$). $[AUC]_0^t$ increased insignificantly ($P > 0.05$) in postmenopausal females compared with premenopausal females. $[AUC]_0^\infty$ showed a significant 163% rise in postmenopausal females. MR was 38% higher in postmenopausal females. $t_{1/2}$ was significantly higher in postmenopausal females. The insignificant increase in Vd in postmenopausal females was observed. CL of OMP-SUL was also significantly higher in premenopausal females compared with the postmenopausal female volunteers.

DISCUSSION

The PK differences of OMP, 5-OH-OMP and OMP-SUL and the activities of CYP2C19 and CYP3A4 in premenopausal and postmenopausal females were determined. Menopause is associated with age therefore the geriatric dose may not be sufficient for the treatment of various ailments in these patients (Schwartz, 2007) and there is need for the adjustment of dose or dosage regimen (Gurwitz et al., 2005). OMP is a highly interactive drug and inhibit various cytochrome P450 enzymes. It is a potent competitive inhibitor of CYP2C19, moderate competitive inhibitor of CYP2C9, very weak competitive inhibitor of CYP2D6 (Ko et al., 1997), and weak non-competitive inhibitor of CYP3A (Tateishi et al., 1995). It has no effect on CYP1A2 or 2E1, but it induces CYP1A1 (Quattrochi and Tukey, 1993). It is possible that OMP may interact with other drugs in multiple drug therapy and PK of the drug may vary in old age, particularly in postmenopausal female. The physiological and biochemical changes that appear in old age are also associated with menopause; like slower gastrointestinal motility (Graff et al., 2008), higher body fat ratio (Carr, 2003) and decreased renal clearance (Christiansen et al., 1971) that may also affect absorption, distribution and clearance of xenobiotics in postmenopausal females.

The C_{max} of OMP in postmenopausal was significantly higher ($P < 0.002$) compared with premenopausal females and the t_{max} slightly increased ($P > 0.14$) in postmenopausal volunteers. Similarly, significant increase ($P<0.0001$) in $[AUC]_0^t$ of the OMP was observed in postmenopausal female compared with the premenopausal volunteers. The AUC in postmenopausal female was increased by 199.78%, that is, revealed by the significantly ($P<0.0001$) higher $[AUC]_0^\infty$ in postmenopausal females. That may be due to the either increase in the absorption of the drug or slower metabolism of the OMP by the postmenopausal female. AUC is determined by gastrointestinal absorption, hepatic metabolism, first-pass effect and volume of distribution. It is known that gastrointestinal motility decreases in postmenopausal females (Graff et al., 2008), that may be the possible reason for increased systemic absorption of OMP. It is also possible that the decrease in the metabolism or decrease in the activities of efflux transporters may increase the systemic exposure of the OMP. The drugs that are substrate of CYP3A4 or P-gp showed higher bioavailability in older females (Krecic-Shepard et al., 2000). OMP is a substrate of P-gp which decreased in old age and may increase the absorption of the OMP. Similarly, the activity of the CYP3A4 also decreased in old age (Krecic-Shepard et al., 2000) and may reduce the metabolism of the drug and results in higher C_{max}. The elimination half-life $t_{1/2}$ was also significantly higher in postmenopausal females, suggesting elimination of OMP is slower in post-menopausal females. The reduced elimination of OMP does not depend on renal clearance/function, as orally administered omeprazole is metabolized completely and 80% is excreted as metabolites in the urine and the remainder 20% is found in the feces, primarily originating from bile secretion.

Therefore, determination of MR plays very important role in evaluating the enzymes activities and metabolites formation which reflect the conversion of OMP into its respective metabolites. MR for both metabolites ($[AUC]_0^\infty$ OMP/$[AUC]_0^\infty$ 5-OH-OMP and $[AUC]_0^\infty$ OMP/$[AUC]_0^\infty$ OMP-SUL, was higher in postmenopausal females when compared with the premenopausal subjects; however, the difference was not significant. Apparently, it suggests that CYP2C19 activity is reduced in postmenopausal females. While the ratio of the metabolites ($[AUC]_0^\infty$ 5-OH-OMP/$[AUC]_0^\infty$ OMP-SUL), increased significantly from 0.389 to 2.6 and may be due to significant decrease in the CYP3A4 activity in the postmenopausal female as it has been observed that the bioavailability of CYP3A4 substrates is higher in such females (Krecic-Shepard et al., 2000).

The Vd was higher while CL of OMP was lower in postmenopausal female when compared with the premenopausal and may be due to the plasma protein binding of the drug as the OMP is the highly protein bound drug (96%). Moreover, the body mass index (BMI) and weight were higher in postmenopausal females and may be a quite plausible reason for higher Vd in postmenopausal females. The change in the protein level may also be responsible for higher concentration of unchanged drug in the postmenopausal volunteers.

The 5-OH-OMP displayed higher values for C_{max}, $[AUC]_0^t$ and $[AUC]_0^\infty$ and t_{max} in postmenopausal females. Previously, no PK study on OMP was conducted, particularly in postmenopausal females but studies in older females suggest that CYP2C19 activity is lower in females (Bachmann and Belloto, 1999). The results of this study support the previous findings. A significantly higher value of $t_{1/2}$ in postmenopausal females was observed. This suggests that CL and Vd of 5-OH-OMP is reduced, as is evident from the higher serum creatinine value of postmenopausal females, indicating reduced glomerular filtration rate (GFR). Aging is associated with decrease in all modes of clearance like glomerular filtration, tubular reabsorption or secretion (Cockcroft and Gault, 1976) and in older females, menopause combined with aging, precipitates decrease in renal clearance (Christiansen et al., 1971).

The OMP-SUL C_{max} and $[AUC]_0^\infty$ displayed a significantly higher value in premenopausal females, though $[AUC]_0^t$ values were not significantly higher. The MR for OMP and OMP-SUL was higher in postmenopausal females suggesting an overall decreased metabolic activity of CYP3A4 in postmenopausal females. The significantly higher value of $t_{1/2}$ in postmenopausal females suggests the same fact that renal CL and Vd were reduced which may be due to increase in the serum creatinine level leading to reduced GFR.

Though, in this study, genetic polymorphism of volunteers was not determined but apparently it is not affecting the status of the results evaluation. The PK studies previously conducted for OMP, neither gender based analysis was performed nor menopause was given any important consideration and the differences between premenopausal and postmenopausal females were not ruled out. Though few studies have been conducted taking OMP as probe drug to determine CYP2C19 and CYP3A4 activities in males and females, but these did not provide most of the information regarding female's PK profile (Laine et al., 2000; Zhang et al., 2006).

Conclusion

The PK differences of OMP and metabolic activities of CYP2C19 and CYP3A4 in premenopausal and postmenopausal female volunteers were evaluated. These findings suggest that postmenopausal females need to be considered as special group of subjects and dose should be adjustment if necessary, particularly in multiple drug therapy where omeprazole exhibits drug interactions.

ACKNOWLEDGEMENTS

The authors are grateful to University of Peshawar for financial support for this study. They also acknowledge Astra Zeneca and Medicraft for providing the standards of OMP, metabolites and internal standard pantoprazole, respectively.

ABBREVIATIONS

PK, Pharmacokinetics; **OMP,** omeprazole; **5-OH-OMP,** 5-hydroxy omeprazole; **OMP-SUL**, omeprazole sulphone. IS= Internal standard

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Full Length Research Paper

Anxiolytic activity of an aqueous extract of *Alchornea cordifolia* (Euphorbiaceae) leaves

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***Alchornea cordifolia*, a half way growing shrub from Africa is very valued by traditional doctors in treating numerous ailments including psychical and nervous troubles. The aim of our study was to investigate the anxiolytic activity of *A. cordifolia* leaves by estimating the effect of an aqueous extract of this plant upon the spontaneous movements and the exploratory behaviour of laboratory mouse. Study of spontaneous movements (moving, straightening-up, activity duration) was done using an activemeter. To assess the exploratory behaviour, a hole-board test was used. The results showed that all spontaneous movements as rapid moving, rapid straightening-up as well as activity duration of mouse decreased after administration of *A. cordifolia* in the dose of 2500 mg/kg body weight, p.o. A reduction of exploratory behavior in *A. cordifolia* 2500 mg/kg body weight p.o-treated mice could also be noticed. Thus, the present findings suggest that *A. cordifolia* might be a potential candidate for use as an anxiolytic drug in the treatment of neuro-psychical troubles.**

Key words: *Alchornea cordifolia*, anxiolytic activity, spontaneous movement, hole-board test, mouse.

INTRODUCTION

In African countries, about 80% of the population depends on traditional medicine for their treatment, because it is readily available and affordable economic-wise. *Alchornea cordifolia* (Euphorbiaceae) is a shrub or small tree spreading from Senegal to Kenya and Tanzania and throughout Central Africa to Angola is highly valued by traditional doctors. In addition to its sedative and anti-spasmodic activities, it is also used to treat a variety of respiratory problems, genital-urinary problems, wounds, pain, and many others uses. The leaves are also taken to treat psychical and nervous troubles (Adjanohoun and Ake, 1979). The scientific importance given to this plant by many researchers has helped to highlight several properties it possess: antibacterial (Ajao et al., 1985; Lamikanra, 1990; Lamikanra et al., 1999; Sutton et al., 2000; Tona et al., 2000), anti-malarial (Banzouzi et al, 2002), anti-inflammatory (Manga et al., 2004; Osadebe and Okoye, 2003; Mavar-Manga et al., 2008), antispasmodics (Ogungbamila and Samuelsson, 1990), antioxidant (Nia et al., 2005), and anti-diarrhea (Agbor et al., 2004). However, very few studies have been conducted on the activity of this plant on the central nervous system. The aim of this study was to investigate the anxiolytic activity that *A. cordifolia* leaves possess by

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measuring its effects on behavior and exploratory activity of mice.

MATERIALS AND METHODS

Plant

Fresh and matured leaves of *A. cordifolia* (Schum. and Thonn.) Müll. Arg. (Euphorbiaceae) were collected in Abidjan along the old Bingerville road and verified by a botanist expert (Professor Ake Assi, Department of Botany, University of Abidjan).

Animal

White mouse of *Mus muculus* race, of either sexes, weighing between 20 and 27 g were used. They were fasted 18 h before the experiment with water *ad libitum*. Five homogenous weight set of 6 mice were set up.

Preparation of lyophilized aqueous extract

Shade dried leaves were crushed in a Ritscher grinder (SM 100 serial from Germany) and 50 g of the obtained powder was then macerated for 48 h at 25°C in a glass balloon containing 500 ml of distilled-water. The obtained aqueous extract was then filtered and freeze-dried with a Telstar Gryodos-80 labeled freeze-drier. The freeze-dried product was kept in a glass flask at a 4°C temperature.

Chemical substance used

Chlorpromazine (LARGACTIL ®) Laboratory EurekaSanté was used as reference standard.

The study of spontaneous movements in activemeter

The method of Pérez et al. (1998) was used for the study of spontaneous movement. The activemeter AM 1051 operates by using infrared light ray to detect movement. There are two sets of light ray, placed in two separate and independent matrices, one above and one below. The lower ray is used to detect the normal movement and the upper ray to detect if the animal rises. The sensor operates by detecting the number of times that the ray changes from "obstructive" to "non-obstructive" and vice versa. In this way more spontaneous movements can be recorded like rapid movements, rapid straightening up, and duration of all spontaneous movements. Five (5) uniform batches of six (6) mice were formed. One batch served as controls lot and received saline (10 ml/kg body weight), another batch received chlorpromazine (2 mg/kg body weight), reference chemical substance used in this study. The other three batches each received different doses of our extract (625, 1250 and 2500 mg/kg body weight.). The mice were placed one after the other in the Activity Monitor AM1051, 30 min after treatment and rapid movements, rapid straightening up, and duration of all spontaneous movements are recorded for a period of 5 min. The operation was repeated three times at intervals of 30 min.

The study of exploratory activity of mice

The method of Boissier and Simon (1967) was used for the study of exploratory activity of mice. A hole board with holes measuring 40 × 40 cm by 2.2 cm thickness and with 16 equidistant holes, 3 cm in diameter fitted with infrared transmitters and receptors. These transmitters and receivers are incorporated in miniature into walls of each hole and around the board. The board is connected to a counter which automatically detects the exploratory movements of mice. Out of the five lots, one lot was used as control and received normal saline solution (10 ml/kg body weight), another batch received reference chemical substance, that is, chlorpromazine (2 mg/kg body weight), the other three batches were administered each an increasing dose of the extract (625, 1250 and 2500 mg/kg p.o). The mice were placed one after the other in the middle of the board after 30 min of treatment and the number of holes explored within 5 min was recorded. The procedure was repeated every 30 min for 90 min. The average number of holes explored was calculated.

Statistical test

The comparison of these means was performed using Student's t test. The difference between two averages is considered significant if the calculated t is greater than or equal to t theoretical risk α = 5%.

RESULTS

The differences recorded in activities with the extract at dose 625 and 1250 mg/kg body weight is not significantly different from each other, which will not present the results obtained with the dose of 625 mg/kg body weight.

Spontaneous movements

These spontaneous movements were represented by the number of rapid movements (Figure 3) and the number of rapid straightening up (Figure 2). The duration of all spontaneous movements is as shown in Figure 1. Overall, we noted that the mean obtained for the control lot were higher than all other lots. There was a significant decrease in mean and duration of spontaneous movements of mice at 2500 mg/kg body weight of extract as compared to the control from 30 to 90 min, but still higher than the mean obtained with lot receiving chlorpromazine.

The exploratory activity

The number of holes explored in 5 min by different groups of mice is shown in Figure 4. Herein, it was noted that the mean of the control group was higher than all other lots. From 30 to 90 min, the number of explorative activities decreases. There was a significant decrease in explorative activities of mice receiving the extract at 2500 mg/kg body weight dose as compared to the control, but remain superior as compared to the mean obtained with mice receiving chlorpromazine.

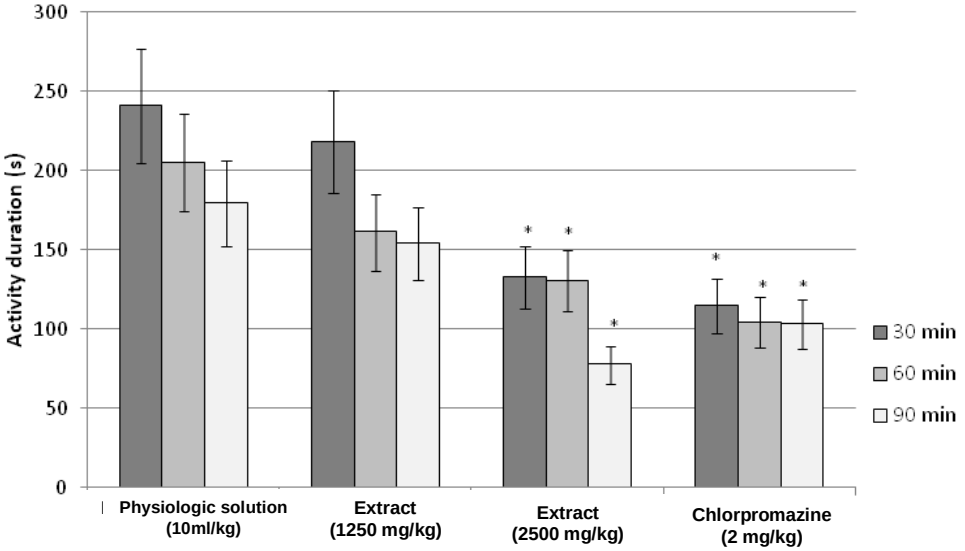

Figure 1. Effects of *Alchornea cordifolia* extract 30, 60, and 90 min after force-feeding along the activity duration. Histograms represent the rapid movements average ± SEM of 6 experiments of each lot. *Significant difference as compared to the physiological solution lot P<0.05 of *t* test of student.

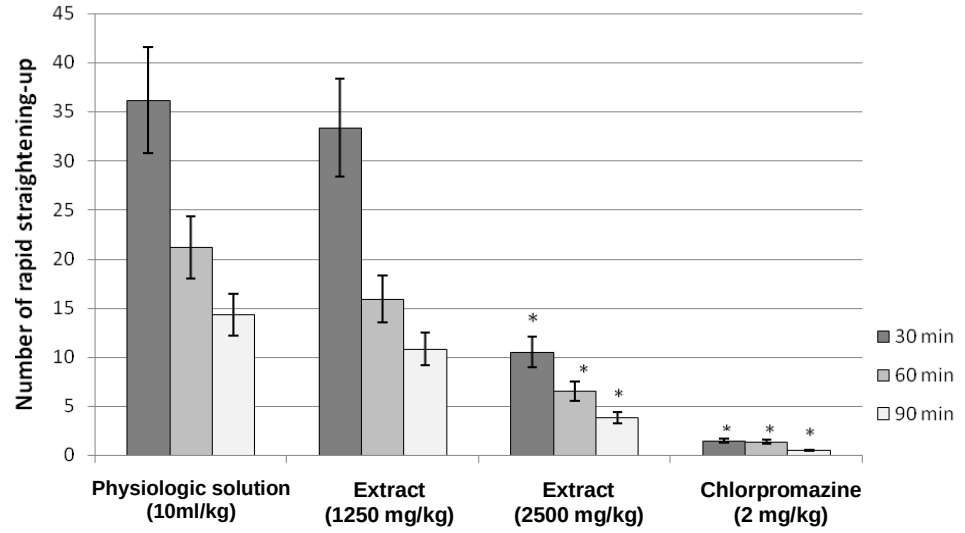

Figure 2. Effects of *Alchornea cordifolia* extract 30, 60, and 90 min after force-feeding on rapid straightening-up. Histograms represent the rapid straightening average ± SEM of 6 experiments of each lot. *Significant difference as compared to the physiological solution lot P<0.05 of *t* test of student.

DISCUSSION

Alchornea cordifolia is used in Africa traditional medicine in the treatment of various neurological and psychiatric disorders. This study is meant to highlight the importance and efficiency of traditional medicine by revealing the anxiolytic activity of *A. cordifolia* leaves. Spontaneous movements and exploratory behaviour of mice were the measures used to assess the central nervous system depression of *A. cordifolia*. The study of spontaneous movements in mice (rapid movements, rapid straightening up, duration of all spontaneous movements) using the activity meter is used to measure exploration and voluntary locomotion in a new environment and in a limited space. Spontaneous movements' particularly rapid straightening up reflect an excitement of the nervous

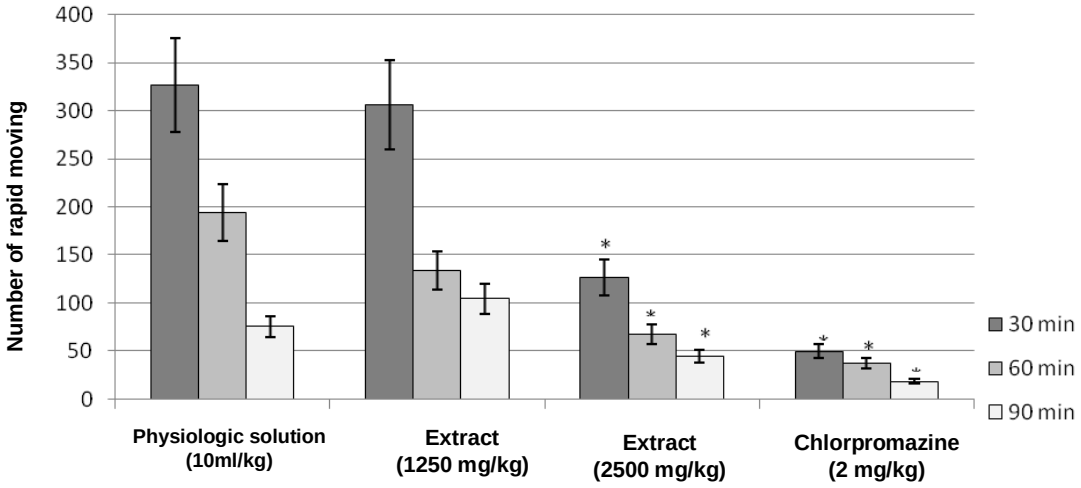

Figure 3. Effects of *Alchornea cordifolia* extract 30, 60, and 90 min after force-feeding on rapid moving. Histograms represent the durations average ± SEM of 6 experiments of each lot. *Significant difference as compared to the physiological solution lot P<0.05 of *t* test of student.

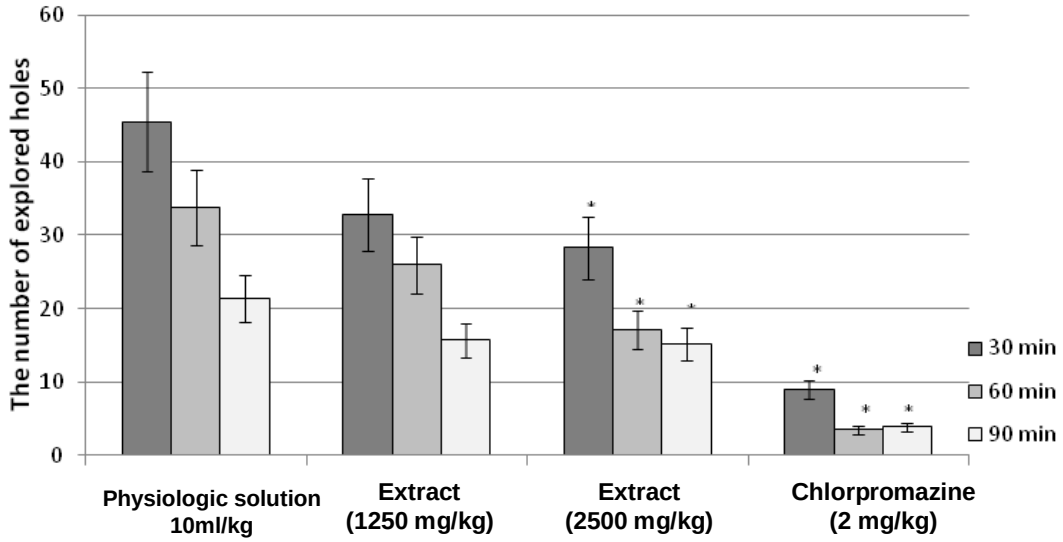

Figure 4. Effects of *Alchornea cordifolia* extract 30, 60, and 90 min after force-feeding over the number of explored holes. Histograms represent the durations average ± standard duration, n=6 for each lot. *Significant difference as compared to the physiological solution lot P<0.05 of *t* test of student.

system in mice. The decrease in spontaneous movements is the result of a decrease in the excitability of the central nervous system or central depressant effect (Mansur et al., 1971; Morais et al., 1998). In this study, all spontaneous movement in mice, rapid movement (Figure 3) rapid straightening up (Figure 2) and the duration of activity (Figure 1) were reduced as a result of *A. cordifolia* extract administration at a dose of 2500 mg/kg body weight. These effects are similar to those of chlorpromazine, a psycholeptic agent depressing the central nervous system.

This molecule is used in the treatment of agitation and aggression in the acute or chronic psychotic states. Thus *Alchornea cordifolia* could possess central nervous system depression activity. The hole board test provides a simple method to measure the response of an animal to an unfamiliar environment and is widely used to assess emotionality, anxiety and/or response to stress in animals (Takeda et al., 1998) showed that the exploration of holes by the mouse reflect changes in the emotional state of the animals. Thus, the hole board test is a measure of exploratory behavior of animal, and substance that reduces

this behavior is responsible for the anxiolytic activity (File and Wardill, 1975; Pellow et al., 1985). The ability of exploration can be seen as a sign of anxiety (Crawley, 1985) although it is difficult to dissociate from locomotive activity. Our results may reflect anxiolytic activity of *A. cordifolia* as a reduction was observed in the exploratory behavior of mice treated with the extract at 2500 mg/kg body weight similar to that observed with chlorpromazine. This fact reinforces the hypothesis of anxiolytic activity described earlier by reducing spontaneous movements measured in the activemeter. Many neurotransmitters influence spontaneous movements in mice.

These include the gamma-aminobutyric acid (GABA), opioids, dopamine receptors (Walting and Keith, 1998). *A. cordifolia* could interact with these mediators. GABA-A is the main mediator of anxiety in the central nervous system. In addition to anxiety, it is involved in other physiological functions related to behavior, and various psychological and neurological disorders such as epilepsy, depression, Parkinson's syndrome and Alzheimer's disease.

The anxiolytic properties of *A. cordifolia* observed in this study could be explained by an interaction with the metabolism of GABA-A. This hypothesis could be in agreement with the results of Pedersen et al. (2009). In fact they explained that *A. cordifolia* has an affinity for the benzodiazepine binding site on GABA-A receptors without activating or inhibitory activity demonstrated on these receptors.

Several phytochemical constituents have proved to be inhibitors of spontaneous movement in mice. These are flavonoids, which have also shown an affinity for the benzodiazepine receptor (Pedersen et al., 2008) and saponins (Wagner et al., 1983; Dubois et al., 1986). Knowing that *A. cordifolia* contains in its leaves saponins (Adjanohoun, 1994) and flavonoids (Manga et al., 2004), the anxiolytic activity observed in this study could be partly due to these phytochemical constituents.

A. cordifolia leaves could therefore have a depressing effect on the central nervous system which justifies its uses in traditional medicine in the treatment of neuropsychiatric disorders.

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