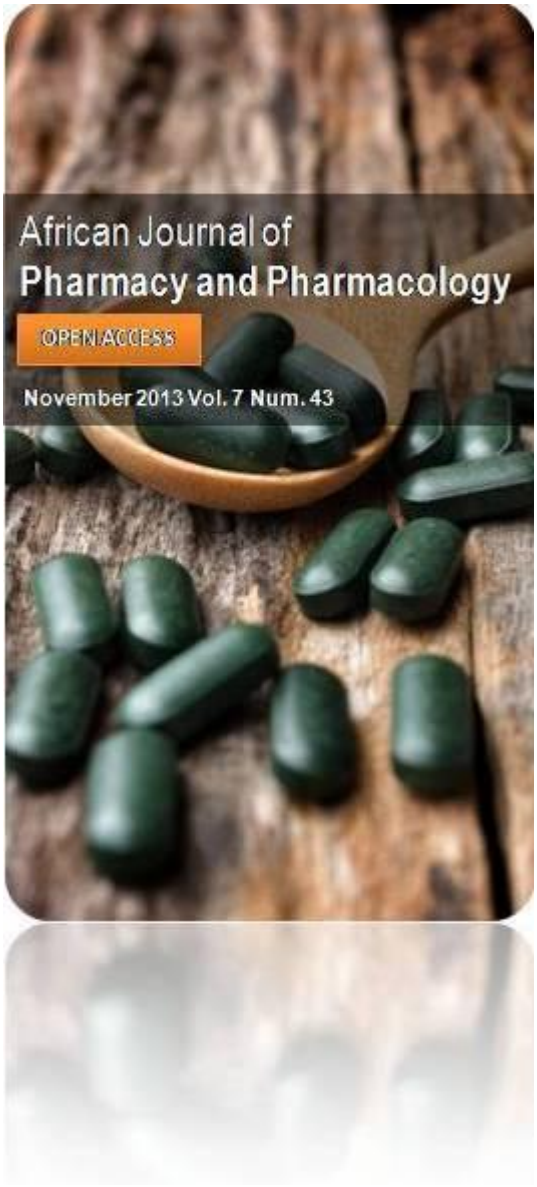

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Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University,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

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Full Length Research Paper

Construction of DNA finger printing for dry saffron

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Saffron (*Crocus sativus* L.), as an effective gynecological medicine, has been widely used in Europe and Asia for thousands of years. In recent years, saffron has been found with antispasmodic, hypolipidemic, antitumor and antidepressant-like effects. However, the counterfeit and adulterants products are extraordinarily common in the market. The aim of the study was to establish a simple, fast and reliable method to identify the genuine saffron from the counterfeits. The investigation was carried out on DNAs from ten batches of saffron from different places and ten kinds of counterfeit saffron. Twenty-five primers (eleven UBC primers, eight OPC primers, three internal transcribed spacer (ITS) primers and three primers designed from CCD4 gene) were chosen to draw the DNA fingerprint of dry saffron. The results indicated that 3 primers (UBC818, UBC859 and UBC868) were capable to draw the DNA fingerprint for dry saffron, and it was very easy to distinguish the genuine from the counterfeits. This paper presents an idea of identification of dry material to prevent the misuse of valuable materials.

Key words: Saffron; DNA fingerprinting, inter-simple sequence repeat (ISSR), internal transcribed spacer (ITS), random amplified polymorphic DNA (RAPD).

INTRODUCTION

Saffron, the dry style of *Crocus sativus* L., *Crocus Linn.*, *Iridaceae*, has been widely used as an effective gynecological medicine in Europe and Asia for thousands of years (Chinese Pharmacopoeia Committee, 2010). In addition, saffron has antispasmodic (Premkumar et al., 2003), hypolipidemic (Asdaq and Inamdar, 2010), antitumor (Abdullaev and Espinosa-Aguirre, 2004), antidepressant-like effects (Wang et al., 2010) and can improve memory and learning ability of rats (Abe and Saito, 2000). Moreover, the saffron, honored as "the golden plant" in the world, is quite expensive due to 70,000 to 200,000 fresh flowers which can only produce 1 kg dry saffron style with 370 to 470 manual labor hours (Li, 2008). Therefore, the counterfeit saffron products and adulterants, which have no pharmacological action, are extraordinarily common in the market.

The existing saffron identification and characterization are still primarily by the morphological character (Escribano et al., 2000) and the physico-chemical property (Hassan-beygy et al., 2010). Some reports indicated that different cultivated and tissue-cultured saffron had remarkable difference in essential components (Sun et al., 2004). These suggest that thin layered chromategraphy (TLC), high performance liquid chromatography (HPLC) which authenticate saffron by chemical components might not be reliable. Therefore, a reliable and sensitive method should be established to authenticate and distinguish saffron from counterfeit or adulterant saffron products. Recently, the DNA fingerprint has been widely applied in the area of phylogeny and authentication

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studies among closely related species. The amplified fragments obtained by using common markers such as inter-simple sequence repeats markers (ISSR) (Ammiraju et al., 2001), random amplified polymorphic DNA markers (RAPD) (Hadrys et al., 1992), amplified fragment length polymorphism markers (AFLP) (Vos et al., 1992), nuclear internal transcribed spacer (ITS) (Deng et al., 2007) and chloroplast DNA sequencing (Jung et al., 2005), could reflect genetic variation and genetic differentiation, and also could be used to distinguish the genuine from counterfeits. Besides, the material needed is few, so this kind of method is especially suitable for the precious material.

Therefore, in this paper, we wanted to construct the DNA fingerprint of saffron. This method together with other conventional methods mutually can provide the scientific basis for monitoring and evaluating the quality of saffron.

MATERIALS AND METHODS

The genuine and counterfeit saffron samples were collected and identified by Shenzhen Institute of Drug Control (Shenzhen, China) as shown in Table 1. All samples were washed with double distilled water and rinsed with 70% (v/v) ethanol to remove surface contaminants and then stored at -20°C until used.

Primers

A set of primers (altogether 100) used in the ISSR analysis were designed by University of British Columbia (Zietkiewi et al., 1994). 100 UBC primers were screened and 11 of them were capable of generating polymorphic profiles among saffron, its homologous garden species and two *Crocus* species. Herein, we tested utility of these 11 primers in constructing DNA fingerprint of dry saffron. Caiola et al. (2004) carried out a RAPD analysis in *Crocus sativus* L. and related *Crocus* species. The results showed that saffron and six closely related *Crocus* species can result in the genetic diversity. According to his study, 8 OPC primers were chosen and applied in this study. Considering that RAPD analysis was not very stable and reliable, we would use the OPC primers to construct DNA fingerprint of dry saffron, only if the UBC primers were unavailing. Ahrazem et al. (2010) reported the isolation and analysis of CCD4 genomic DNA regions in *C. sativus* L. So the CCD4 gene primers used to amplify the CCD4 genomic DNA regions in saffron was designed by us. All primers used in this study are shown in Table 2.

DNA extraction, amplification electrophoresis and cloning

Total DNA for study was extracted from 150 mg of dry samples using AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, America), according to the instruction of the manufacturer. DNA amplification reactions were carried out in 25 µl volume containing 2 µl (300 ng) DNA template, 2.5 µl 10 × buffer (100 mM Tris-HCl (pH 8.3); 500 mM KCl; 15 mM $MgCl_2$), 2 µL dNTPs (2.5 mM), 1 unit of Taq polymerase and 2 µl primers (10 mM, manufactured by Invitrogen in Guangzhou). A control polymerase chain reaction (PCR) tube containing all components, but no template, was run with each primer to check for contamination. PCR parameters were tried according to the electrophoresis atlas and the suitable PCR parameters for different primers were discovered. PCR amplification products were analyzed by gel electrophoresis in 1.2% (w/v) agarose in tris base, acetic acid and EDTA (TAE) 1 × and detected by staining with 0.05% (v/v) EB. DNA marker was prepared according to the manufacturer's instruction (Omega DL2000 DNA Marker, Switzerland). Gels were photographed under ultra violet (UV) radiation with Syngene genegenius-302 nm. PCR products were recovered from agarose gel using the AxyPrep DNA Gel Extraction Kit (Hangzhou, China). Purified DNA fragment was cloned into pUCm-T Easy vector (Nantong, China). Rapid Plasmid Miniprep System (Nantong, China) was then used for plasmid purification. The plasmid samples were sent to sequence by Beijing Genomics Institute (BGI) (Shenzhen, China).

RESULTS

DNA extraction

Total DNA solution was isolated from dry samples and examined by Thermo NanoDrop ND2000. The results showed that the DNA density was 148.9 ng/µl and the OD_{260}/OD_{280} was 1.77, which indicating that the purity of DNA was very high.

ISSR analysis

UBC868

As shown in Figure 1, six bands (ranging from 100 to 1000 bp) were obtained from ten batches of saffron using UBC868. The counterfeits had obvious differences with the genuine in both band number and molecular weight; except for the w4. The electrophoretogram of w4 was similar to that of the genuine, more but darker bands and different molecular weights were observed, which indicated that other materials might be added into w4.

UBC859

As shown in Figure 2, two obvious bands (varying from 500 to 750 bp) were observed from ten batches of saffron using UBC859. All counterfeits had more than 2 bands, and there was obvious difference in the brightness. The w4 had more but lower-bright bands than the saffron.

UBC818

As shown in Figure 3, two obvious bands (varying from 200 to 600 bp) were obtained from ten batches of saffron using UBC818. All the counterfeit ones had more than 2 bands, and there was obvious difference in the position. The w4 had four bands containing the two brands of true saffron.

DISCUSSION

The aim of this research was to construct the DNA

Table 1. Samples used in this study and the collection place of samples.

No.	Sample	Place for collection
1	Saffron	New Zealand
2	Saffron	Tibet, China
3	Saffron	Iran
4	Saffron	Iran
5	Saffron	New Zealand
6	Saffron	Spain
7	Saffron	Tibet, China
8	Saffron	Iran
9	Saffron	Tibet, China
10	Saffron	Spain
w1	counterfeit saffron	Yulin Herb market, China
w2	counterfeit saffron	Yulin Herb market, China
w3	counterfeit saffron	Guangzhou Herb market, China
w4	counterfeit saffron	Bozhou Herb market, China
w5	counterfeit saffron	Bozhou Herb market, China
w6	counterfeit saffron	Anguo Herb market, China
w7	counterfeit saffron	Anguo Herb market, China
w8	counterfeit saffron	Chengdu Herb market, China
w9	counterfeit saffron	Chengdu Herb market, China
w10	counterfeit saffron	Chengdu Herb market, China

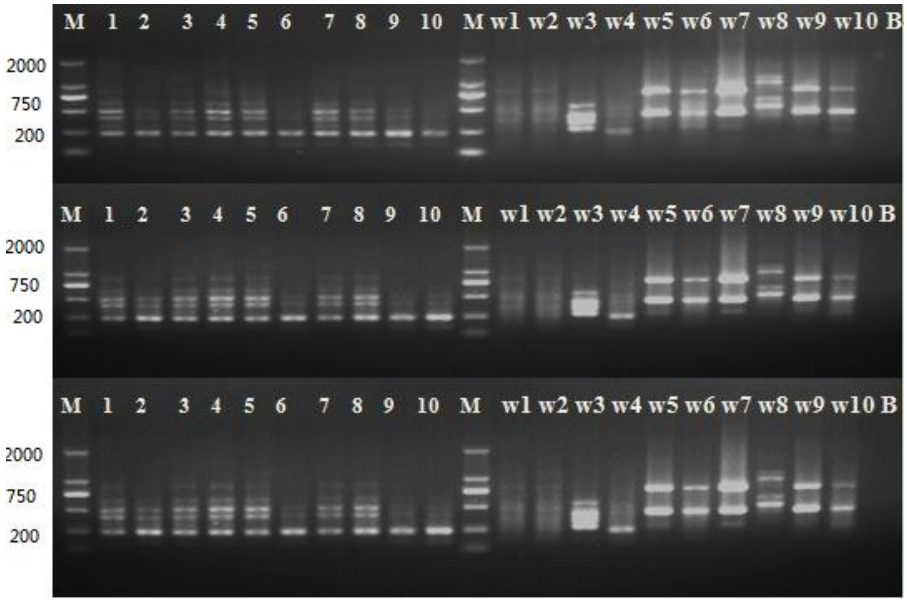

Figure 1. ISSR profiles of the UBC868. M, Marker (2000, 1000, 750, 500, 250 and 100 bp); lane 1 to 10, saffron; lane w1 to w10, the counterfeit saffron; B, Blank Finally PCR amplification was performed as follows: initial 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 50°C, 1 min at 72°C, and a final 8 min extension at 72°C.

fingerprinting of dry saffron. In this study, we showed a method using the ISSR analysis for the determination of saffron and counterfeit saffron. The experiment was carried out in triplicates, and all results obtained were reproductive. This method can even differentiate the adulterants. The investigation was carried out on DNAs,

Table 2. Primers used in this study.

No.	Category	Primer name	Sequences(5'to3')
1	UBC	UBC818	CACACACACACACACAG
2		UBC823	TCTCTCTCTCTCTCTCC
3		UBC824	TCTCTCTCTCTCTCTCG
4		UBC835	AGAGAGAGAGAGAGAGCC
5		UBC859	TGTGTGTGTGTGTGTGAC
6		UBC868	GAAGAAGAAGAAGAA
7		UBC873	GACAGACAGACAGACA
8		UBC876	GATAGATAGACAGACA
9		UBC881	GGGTGGGGTGGGGTG
10		UBC895	AGAGTTGGTAGCTCTTGATC
11		UBC899	CATGGTGTTGGTCATTGTTCCA
12	OPC	OPC01	TCCCAGCAGA
13		OPC02	GTGAGGCGTC
14		OPC05	GGCTTTAGCC
15		OPC06	CCAGAACGGA
16		OPC07	GTCCCGACGA
17		OPC08	TTTGGGTGCC
18		OPC09	CTCACCGTCC
19		OPC14	GTCGGTTGTC
20	CCD4 gene	CCD4a	CAATCTCAAGTATTAGCATTC
21		CCD4b	CACTACCCATCTCATCAAGA
22		CCD4a/b-f	CTGCTGTGACAGCAGCTCAGC
23	ITS	ITS1	AGAAGTCGTAACAAGGTTTCCGTAGG
24		ITS4	TCCTCCGCTTATTGATATGC
25		ITS5	GGAAGTAAAAGTCGTAACAAGG

DNAs. DNA extraction was very difficult from the dry materials, of which the DNA degrades mostly. We chose the AxyPrep Multisource Genomic DNA Miniprep Kit to isolate DNA. In this study, ITS, RAPD, CCD4 gene amplification and ISSR methods were employed to differentiate *C. sativus* L. from related counterfeit material. ITS region of nuclear ribosomal DNA is often employed to identification, particularly at the genomic level.

In this study, however, these regions could not be used. The result of sequencing confirmed that these bands were from fungi, comparing with the National Center for Biotechnology Information (NCBI) database. The reason would be the samples used in this study were dry materials. They were kept for a long time. In this time, these samples were preserved in the normal temperature. So it was very suitable for fungi to grow. As time went by, DNA from the saffron degraded in a great measure, but DNA from fungi was much more stable. The ITS amplification produced varied bands from all the samples and different origins of the samples grew varied fungi. So the samples had the obviously different bands. Therefore the ITS sequence was mostly from fungi. This result indicated that ITS sequence is not suitable to use in the dry materials.

RAPD method could not get stable fragments due to the short random primers and CCD4 gene amplification could not get brands because the DNA degraded too much in the dry materials. So these OPC primers and CCD4 gene primers could not be used to draw the DNA fingerprint of dry saffron.

ISSR analysis usually detects a higher level of polymorphism than RFLP or RAPD analysis (Godwin et al., 1997). ISSR, providing repeatable and stable fragments, is the most widely used primers to construct the DNA fingerprint (Powell et al., 1996). By comparison with the ISSR fingerprint, differences could be obtained between related species. Simple sequence repeat (SSR) is very abundant and conservative, and it is very stable even in dry materials of which the DNA degraded mostly (Ammiraju et al., 2001). Several studies have indicated that

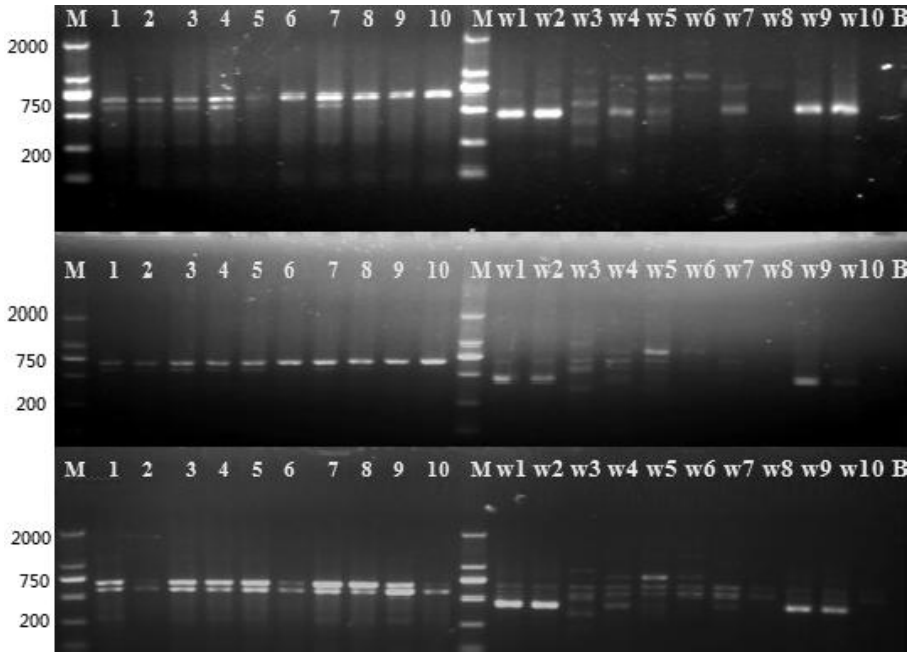

Figure 2. ISSR profiles of the UBC859. M, Marker (2000, 1000, 750, 500, 250 and 100 bp).
Lane 1 to 10, saffron; Lane w1 to w10, counterfeit saffron; B, Blank. Finally PCR amplification was performed as follows: initial 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 55°C, 1 min at 72°C, and a final 8 min extension at 72°C.

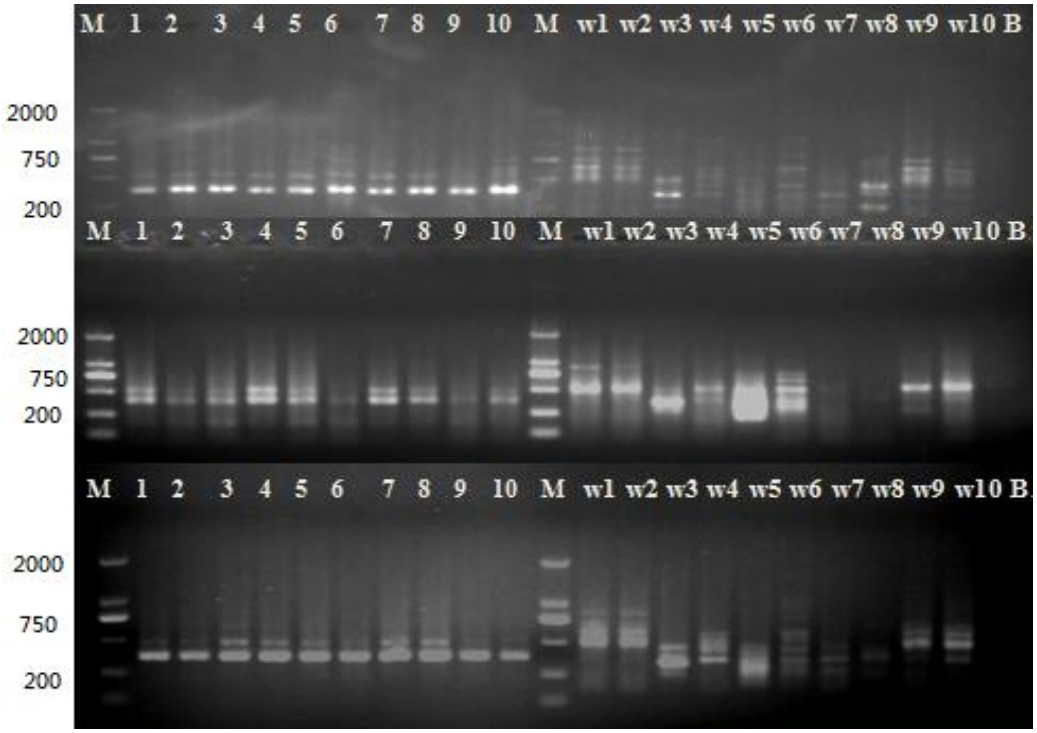

Figure 3. ISSR profiles of the UBC818. M, Marker (2000, 1000, 750, 500, 250 and 100 bp).
Lane 1 to 10, saffron; Lane w1 to w10, counterfeit saffron; B, Blank. Finally PCR amplification was performed as follows: initial 5 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 58°C, 1 min at 72°C, and a final 8 min extension at 72°C.

ISSR markers are potentially useful both for cultivar identification and phylogenetic studies. For example, ISSR was used to identify closely related *Citrus cultivars* (Fang and Roose, 1997). The ISSR fingerprint could be used to differentiate the genotypes belonging to either *Japonica* or *Indica* sub-species of cultivated rice (Blair et al., 1999). ISSR was used to authenticate the Chinese herb Huajuhong (*Exocarpium citrigrandis*) and related medicinal material (Su et al., 2010).

In our study, primers $(CA)_8G$, $(TG)_8AC$, and $(GAA)_5$ generated unique fingerprints, while $(TC)_7C$, $(TC)_7G$, $(AG)_8CC$, $(GACA)_4$, $(GATA)_2(GACA)_2$, $(GGGTG)_3$ generated no band or unstable bands. Only bands present in three replicated PCRs were considered. Some PCR bands were recovered from agarose. Then, purified DNA fragment was cloned into pUCm-T vector. The plasmid samples were sent to sequence by The Beijing Genomics Institute (Shenzhen, China). These results showed that these fragments could not be sequenced accurately. Poor sequencing results were due to either the characteristics of the primers or the abundance of the priming sites in the genome (Fang and Roose, 1997). Therefore, the ISSR primers are nonspecific to be the sequencing primers. ISSR primers are not suitable to sequence.

Conclusion

In this study, ISSR primers were employed to differentiate ten batches of saffron from ten kinds of counterfeit saffron. 3 primers (UBC868, UBC859 and UBC818) were screened from 25 primers with the utilization of PCR and electrophoresis. The DNA fingerprint of saffron was constructed to differentiate genuine saffron between counterfeit saffron. This molecular authentication method is fast and reliable. Moreover, this method together with other conventional methods mutually can prevent the misuse of these valuable herbs.

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Full Length Research Paper

Anti-inflammatory, antinociceptive and ulcerogenic properties of indomethacin tablets based on solidified reverse micellar solution (SRMS)

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The aim of the present study was to evaluate *in vivo* the anti-inflammatory, antinociceptive and ulcerogenic properties of indomethacin tablets based on solidified reverse micellar solution (SRMS). SRMS consisting of mixtures of phospholipid (Phospholipon® 90H) and triglyceride (Softisan® 154) were prepared in the ratios of 1:1, 2:1 and 1:2, respectively. SRMS based tablets containing 75 mg of indomethacin each were prepared using validated plastic mould. The physicochemical properties of the tablet formulations were studied using both official and unofficial tests. Anti-inflammatory, analgesic/antinociceptive and ulcerogenic properties of indomethacin tablets based on SRMS were studied. The results showed that the physicochemical properties of the tablet formulations were significantly affected by the composition/ratio of the lipid matrix used. The softening time in SIF ranged from 53.7 ± 0.5 to 102.6 ± 0.5 min. Results of analgesic/antinociceptive properties showed that indomethacin tablets formulated with the SRMS 1:1 had an increase in pain reaction time at 7 h significantly ($p < 0.05$) different from the results exhibited by tablets formulated with the lipid matrices, SRMS 1:2 and 2:1 and the reference, which showed a decrease in pain reaction time at 7 h. Indomethacin tablets based on SRMS had good anti-inflammatory properties and also inhibited the ulcerogenicity of indomethacin by 70 to 80%. Therefore, indomethacin tablets based on SRMS could be used for improved oral bioavailability of indomethacin and to enhance patient's compliance due to inhibition of gastric irritation effect of this drug.

Key words: Non steroidal anti-inflammatory drugs (NSAIDS), solidified reverse micellar solutions (SRMS), tablets, ulcerogenicity, antinociception, lipids.

INTRODUCTION

Efforts have recently been made to develop gastrointestinally safe non-steroidal anti-inflammatory drugs (NSAIDs) on the basis of reduced ability to interfere with the surface-active phospholipid layer in the gastrointestinal mucus. Lichtenberger et al. (1995) proposed that pre-associating nonsteroidal anti-inflammatory drugs (NSAIDs) with zwitterionic phospholipids prior to their administration should reduce the ability of the NSAIDs to associate with the phospholipids in the mucus gel, and should therefore reduce their ulcerogenicity. They

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demonstrated this to be the case by pre-associating aspirin and other NSAIDs with dipalmitoyl-phosphatidylcholine (DPPC) in evaluating their ulcerogenicity. The complexes produced significantly less damage in the gastrointestinal tract (GIT) than the parent drug. Importantly, the pre-association of aspirin with DPPC did not interfere with the effectiveness of the aspirin to reduce fever or inflammation (Wallace, 2000).

Lipid excipients are generally regarded as safe (GRAS) and have been proven to be non-toxic (Chime et al., 2012a). The rapid growth in the use of lipid-based drug delivery systems is primarily due to the diversity and versatility of pharmaceutical grade lipid excipients and their compatibility with liquid, semi-solid and solid dosage forms (Attama et al., 2009; Chime et al., 2012a). The widening availability of lipid excipients with specific characteristics offers flexibility of application with respect to improving the bioavailability of sparingly soluble drugs and manipulating their release profile (Attama and Nkemnele, 2005). Most lipids do not exert pharmacological effect and are relatively cheap and widely distributed in nature. For poorly water soluble drug molecules whose dissolution in water is likely the limiting step for overall oral absorption, the primary role of ingested lipids and their lipolytic products is to impact the drug dissolution step by forming with bile components different colloidal particles, which are able to maintain a larger quantity of hydrophobic drugs in solution via micellar solubilization (Porter et al., 2007). The primary mechanism of action which leads to improved bioavailability is usually avoidance or partial avoidance of slow dissolution process which limits the bioavailability of hydrophobic drugs from conventional solid dosage form (Pouton, 2000).

Solidified reverse micellar solution (SRMS) based carriers have been investigated and successfully employed to achieve controlled release of drugs (Umeyor et al., 2012; Schneeweis and Müller-Goymann, 2000; Friedrich and Müller-Goymann, 2003). SRMS consisting of phospholipid and solid lipid such as Softisan® 154, a completely hydrogenated palm oil transform into a lamellar mesophase after melting on contact with water. This transformation enables controlled release of solubilized drugs. SRMS also offer a high solubilization rate of different types of drugs (Friedrich and Müller-Goymann, 2003). SRMS carriers have recently been investigated as a sustained release matrix for both hydrophilic and hydrophobic NSAIDS (Chime et al., 2012b, c; Chime et al., 2013).

The objectives of the work were to formulate SRMS-based indomethacin in order to enhance the oral bioavailability of the drug and eliminate the severe gastric irritation often encountered with the use of indomethacin and to evaluate *in vivo*, the anti-inflammatory, anti-nociceptive and ulcerogenicity of the formulations.

MATERIALS AND METHODS

Chemicals

The following materials were used as procured from their suppliers without further purification: Indomethacin (Merck, Germany), Softisan® 154 (Schuppen, Condea Chemie GmbH, Germany), Phospholipon® 90H (Phospholipid GmbH, Köln, Germany), and distilled water (UNN Water Resources Management Lab. Ltd., UNN, Enugu State, Nigeria). Plastic mould used was constructed in the Faculty of Engineering, University of Nigeria, Nsukka. All other reagents and solvents were analytical grade and were used as supplied.

Preparation of solidified reverse micellar solutions (SRMS)

Mixtures of Phospholipon® 90H and Softisan® 154 (1:1, 1:2 and 2:1 w/w) were prepared by fusion. In each case the lipids were weighed, melted together and stirred at a temperature of 70°C using a magnetic stirrer, until a homogenous, transparent white melt was obtained. The homogenous mixture was stirred at room temperature until solidification (Attama et al., 2009; Chime et al., 2012).

Thermal analysis

Melting transitions and changes in heat capacity of Phospholipon® 90H, Softisan® 154, indomethacin, SRMS 1:1, 2:1 and 1:2, were determined using differential scanning calorimeter (Netzsch DSC 204 F1, Germany). About 10 mg of each sample was weighed into aluminum pan, hermetically sealed and the thermal behaviour determined in the range 20 to 500°C, at a heating rate of 10 K/min under a 20 ml/min nitrogen flux.

Validation of plastic mould

The plastic mould used for tablet production was validated by formulating bland or unloaded tablets using the lipid matrices. This test was performed in order to determine the amount of SRMS that would be used in the formulation of each tablet so as to ensure the reproducibility of the process and also to ensure the uniformity of weight and drug content of the tablets. A small amount of each of the lipid matrices, SRMS 1:1, 1:2 and 2:1 each was weighed out. This was melted at a temperature of 70°C and introduced into the wells of the plastic mould and allowed to solidify. The tablets were properly scraped and removed thereafter. The tablets were weighed and the average weight was recorded.

Preparation of indomethacin tablets based on SRMS

With reference to the average weight of the bland tablets prepared with 1:1, 1:2 and 2:1 w/w of the SRMS, the amount of indomethacin to be incorporated into each tablet was calculated. The composition of the tablets is shown in Table 1. Each of the SRMS was weighed out and placed in a crucible. This was melted at 70°C using a magnetic stirrer hot plate. The required amount of the active ingredient was weighed out and transferred quantitatively into the melted lipid matrix in the crucible with stirring until a homogenous mix was obtained (Umeyor et al., 2012). The homogenous mix was scooped into the wells of the mould with a clean stainless spoon. It was allowed to solidify, scraped and allowed to dry at room temperature. The tablets were pressed out of the plastic mould and

allowed to dry properly at room temperature.

Characterization of the tablets

Uniformity of weight

Twenty tablets were randomly selected from each batch. The tablets were weighed individually using an electronic balance (Ohaus Adventurer, China) and the individual weights recorded. The mean weight, standard deviation and percentage deviation were calculated.

Determination of surface morphology of the tablets

About 20 tablets were randomly selected and evaluated in terms of shape, colour and size. The shape and colour were determined visually by placing the tablets on a plain white sheath of paper; also the photographs of the tablets were taken. The size was determined by the use of venier caliper to determine the tablet thickness and diameter.

Softening/liquefaction time of the tablets

This test is important because the tablets will first of all soften or liquefy for a substantial amount of the drug to be released from the SRMS. The method described in the European Pharmacopoeia (Ph. Eur., 2005) was adopted. The test was carried out for each batch of the tablet using a beaker containing 250 ml of simulated intestinal fluid (SIF) (pH, 7.5) maintained at 37 ± 1°C. An inner compartment sealed at one end, containing a tablet, was tied with a thermo-resistant thread unto the clamp of a retort stand and immersed into the medium. The time taken for the tablets to soften, determined by an appreciable change in shape, was recorded.

Erosion time of tablets

A method described in the European Pharmacopoeia (Ph. Eur., 2005) was adopted. The test was carried out for each batch of the tablet using a beaker containing 500 ml of SIF (pH, 7.5) maintained at 37 ± 1°C. A thermometer was inserted into the medium to maintain the temperature. An inner compartment sealed at one end and containing 3 tablets from each batch was tied with a thermo-resistant thread unto a resort stand, and immersed into the medium. The medium was stirred at 100 rpm with a magnetic stirrer bar. The erosion time was taken as the time taken for the tablet to change in shape and erode appreciably. This test was repeated three times for each batch and the mean erosion time was determined. Erosion time is central to bioavailability, because the tablet should erode appreciably in order to enhance drug release and make the drug available for absorption.

Content of active ingredient

Beer's calibration curve was obtained at a concentration range of 0.1 to 1.0 mg% for indomethacin in SIF (pH 7.5) at a predetermined wavelength of 298 nm. Twenty tablets were randomly selected from each batch of the tablets. The tablets were weighed together and crushed in a mortar with a pestle. An amount equivalent to the average weight of the crushed tablet was weighed out in an analytical balance and dispersed in distilled water. The dispersion was heated for 30 min at 70°C using a magnetic stirrer hot plate, to enhance dispersion. This dispersion was allowed to cool, filtered and an aliquot of the filtrate was assayed using spectrophotometer (Jenway 6305 spectrophotometer, Barloworld Scientific Ltd., Essex CMB 31BWL, UK). The absorbance was recorded and the concentration of indomethacin in each tablet was calculated with reference to Beer's plot.

Hardness/crushing strength test

This test was carried out using Monsanto-Stokes hardness tester (Manesty, England). Ten tablets from each batch were randomly selected. Each tablet was placed between the jaws of the hardness tester and force was applied by adjusting the knob of tester until the tablet integrity failed. The results were recorded in kgf.

Tablet friability test

Twenty tablets were randomly selected from each batch of the tablet. The tablets were dedusted and weighed. The tablets were placed into the drum of the friabilator (Erweka GmbH, Germany) and rotated 100 times at 25 rpm for 4 min. The tablets were removed from the friabilator, dedusted again and reweighed. The friability result was expressed as loss of mass expressed as a percentage of the initial mass (BP, 2009). The percentage friability was calculated from the equation 1:

$$\text{Friability (\%)} = \frac{W_i - W_f}{W_i} \times 100 \quad (1)$$

Where, W_i and W_f are the initial weight and final weight of the tablets, respectively.

Anti-inflammatory properties

The anti-inflammatory activity of the indomethacin tablets based on SRMS was carried out using the rat paw oedema test (Winter et al., 1962). All animal experimental protocols were carried out in accordance with guidelines of the Animal Ethics Committee of the Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka and in compliance with the Federation of European Laboratory Animal Science Association and the European Community Council Directive of November, 1986 (86/609/EEC). The phlogistic agent employed in the study was fresh undiluted egg albumin (Anosike et al., 2009). Adult Wistar rats of either sex (150 to 200 g) were divided into six experimental groups of five rats per group for each drug, respectively. The rats were fasted and deprived of water for 12 h before the experiment. The deprivation of water was to ensure uniform hydration and to minimize variability in oedematous response (Winter et al., 1963). The tablet was crushed using mortar and pestle. The dose of indomethacin tablet formulation based on SRMS equivalent to 10 mg/kg of indomethacin was weighed out and dispersed in 0.5 ml of water. This was administered orally to the rats using a 1 ml syringe. The negative control group received normal saline while the positive control group received 10 mg/kg of indomethacin pure sample. Thirty minutes post treatment; oedema was induced by injection of 0.1 ml fresh undiluted egg - albumin into the sub plantar region of the right hind paw of the rats. The volumes of distilled water displaced by treated right hind paw of the rats were measured using plethysmometer before and at 30 min, 1, 2, 3, 4, 5, 6, 7 and 8 h after injection of egg albumin. Average oedema at every interval was assessed in terms of difference in volume displacement of injected paw (V_t - V_o) (Ajali and Okoye, 2009). The percent inhibition of oedema was calculated using the relationship

(Parez, 1996):

$$\% \text{ Inhibition of oedema} = 1 - \left(\frac{a - x}{b - y} \right) 100 \quad (2)$$

Where a is the mean paw volume of treated rats after egg albumin injection, x is the mean paw volume of treated rats before egg albumin injection, b is the mean paw volume of control rats after egg albumin injection and y is the mean paw volume of control rats before egg albumin injection.

Analgesic properties

Analgesic activity was tested in rats using the hot plate method described by Nkomo et al. (2010). Adult Wistar rats of either sex (120 to 205 g) were divided into six experimental groups of five rats per group. The tablet was crushed using mortar and pestle. Each indomethacin tablet formulation equivalent to 10 mg/kg of indomethacin was weighed out, dispersed in 0.5 ml of water and administered orally to the rats using a 1 ml syringe. The control groups received normal saline 5 ml/kg while the reference group received 10 mg/kg of indomethacin pure sample. Rats were placed on hot plate maintained at 55 ± 1°C and the reaction latency in seconds for licking of hind paw or jumping was recorded. Recordings were taken before treatment with the different drugs and 30, 45 min and at 1, 2, 3, 4, 5, 6 and 7 h post treatment. Results were expressed as difference between the baseline reaction latency and the reaction latency at the different time intervals (Nkomo et al., 2010).

Ulcerogenicity of the tablet formulations

The ulcerogenic potentials of the indomethacin tablet formulations based on SRMS were determined using a method described by Chung-Chin et al. (2009). The studies were carried out on healthy Wistar rats (150 to 200 g). The animals were divided into six groups of five animals each. The tablet was crushed using mortar and pestle, the required dose was weighed out and dispersed in 0.5 ml of water. The control group received normal saline, the test group received indomethacin tablets based on SRMS equivalent to 10 mg/kg of indomethacin, while the reference group received pure sample of indomethacin 10 mg/kg orally. The animals were fasted 8 h prior to a single dose of either the control or test compounds, given free access to food and water and sacrificed 17 h later. The gastric mucosa of the rats was examined under a microscope using a 4× binocular magnifier. The lesions were counted and divided into large (greater than 2 mm in diameter), small (1 to 2 mm) and punctiform (less than 1 mm). For each stomach, the severity of mucosal damage was assessed according to the following scoring system: 0 – no lesions or one punctiform lesions; 1 – two to five punctiform lesions; 2 – one to five small ulcers; 3 – more than five small ulcers or one large ulcer; 4 – more than one large ulcers.

Statistical analysis

Statistical analysis was done using statistical package for social sciences (SPSS) version 14.0 (SPSS Inc. Chicago, IL.USA). All values are expressed as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA). Differences between means were assessed by a two-tailed student's T-test. $P < 0.05$ was considered statistically significant.

RESULTS

Thermal analysis

The thermograms of the materials are shown in Figure 1 and from the results, the differential scanning calorimetry (DSC) curve of Softisan® 154 showed a narrow endothermic peak, with melting peak at temperature of 61.4°C. Phospholipon® 90H showed a curve with the melting peak at temperature of 124°C. Also, indomethacin DSC curves showed a melting peak at 162.2°C. The sharp peak showed the presence of pure crystalline indomethacin. This value is comparable to the melting temperature recorded for indomethacin in BP (2009). The DSC results of the lipid matrices showed that the structuring of Softisan® 154 with P90H generally produced matrices with low enthalpies.

Surface morphology

The results of dimensional properties of the tablets are shown in Table 2. From the results indomethacin tablets had stable dimensional properties. The tablets had uniform diameter of 1.20 cm while the thickness of the tablets ranged from 0.33 ± 0.01 for N_2 tablets to 0.35 ± 0.01 for N_3 tablets.

Softening/liquefaction time

For a substantial amount of the drug to be released from the lipid matrix, the tablets will first of all soften or liquefy. Generally from the result of softening time presented in Table 2, the indomethacin tablets prepared with the lipid matrix, SRMS 2:1 exhibited the highest softening time of 102.6 min, significantly different form tablets formulated with the lipid matrix, SRMS 1:1 and 1:2 ($p < 0.05$), respectively. This may be due to structural enhancement at 2:1 ratio which generated more compact matrix. The lipid matrix, SRMS 2:1 (Phospholipon® 90H:Softisan®154) is particularly good for sustained release oral and parenteral preparations. This is because of absolute elimination of dose dumping often encountered with sustained release dosage formulations.

Weight uniformity

The result of tablet weight uniformity test presented in Table 2 showed that indomethacin tablets formulated complied with BP specifications and their percentage deviations were significantly lower than 5% ($p < 0.05$).

Tablet friability

From the results of tablet friability test presented in Table

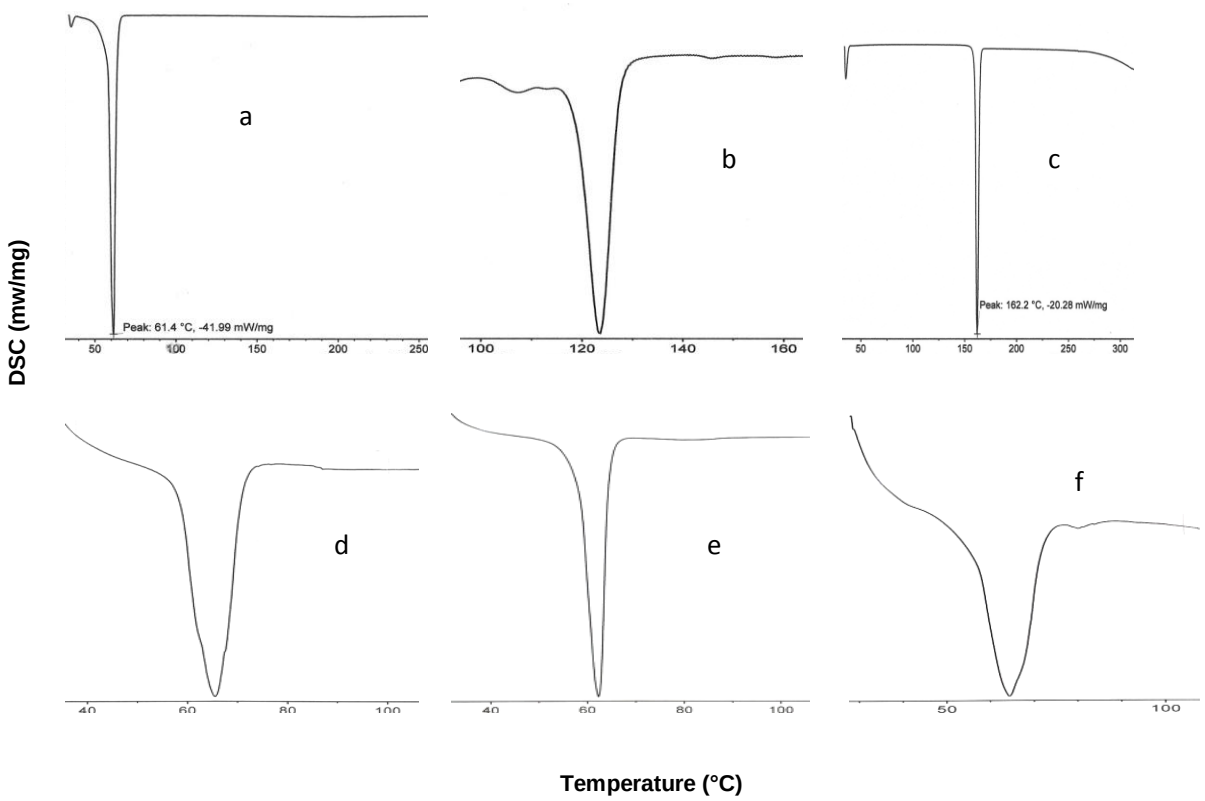

Figure 1. DSC thermograms of: (a) Softisan® 154, (b): Phospholipon® 90H, (c): Indomethacin, (d): SRMS 1:1, (e): SRMS 2:1, (f): SRMS 1:2.

Table 1. Composition of indomethacin tablets.

Batch	LM (Phospholipon 90H:Softisan® 154)	Indomethacin (mg)	Ratio of drug to lipid matrix (Drug: LM)
N_1	1:1	75.0	1:3.9
N_2	2:1	75.0	1:4.0
N_3	1:2	75.0	1:4.1

LM = lipid matrix, N_1 - N_3: Indomethacin tablets.

2, all the batches of indomethacin tablets formulated complied with BP (2009) standards for tablet friability test with friability results significantly lower than 1% ($p < 0.05$).

Crushing strength of tablets

The crushing strength test was performed on the tablets to determine the hardness profile of the tablets. From the crushing strength results presented in Table 2, all the batches of indomethacin tablets complied with the BP (2009) specification for crushing strength test of approximately 5 kgf.

Erosion time of tablets

Erosion time is important because the tablet should erode appreciably in order to enhance drug release and make the drug available for absorption. From the results of erosion time test presented in Table 2, all the tablet batches prepared with different ratios of lipid matrix passed the erosion time test. However, indomethacin tablets formulated with lipid matrix, SRMS 2:1 had the highest erosion time of 180.40 ± 1.06 min for N_2 tablets.

Content of active ingredient

The drug content of the formulated tablets were studied

Table 2. Properties of indomethacin tablets.

Batch	Diameter (cm)*	Thickness (cm)*	Weight (mg±CV)*	Hardness (kgf)[a]	Friability (%)*	Softening time (min)[a]	Erosion time (min)[a]	Drug content (mg±CV)*
N_1	1.20±0.05	0.34±0.01	369.00±0.18	5.10±0.24	0.07	64.8±0.4	134.0±2.8	75.27±0.39
N_2	1.20±0.01	0.34±0.01	369.00±0.37	5.60±0.36	0.06	102.6±0.5	180.0±1.1	75.12±1.10
N_3	1.20±0.01	0.35±0.01	379.00±0.29	5.00±0.39	0.09	53.7±0.5	124.0±0.5	75.20±0.75

*Mean for 20 tablets ± SD, [a]Mean for 10 tablets ± SD, CV: Coefficient of variation SD: Standard deviation, LM = lipid matrix, N_1 - N_3: Indomethacin tablets prepared with LM 1:1, 2:1 and 1:2, respectively.

Table 3. Result of anti–inflammatory properties of indomethacin tablets.

Batch	Paw volume (oedema) (ml) and percentage inhibition of oedema (%)								
	30 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h	8 h
N_1	0.83±0.03(24.5)	0.8±0.04 (27.3)	0.62±0.03 (27.9)	0.56±0.00 (30.0)	0.42±0.03* (44.0)	0.27±0.03* (61.4)	0.15±0.01 (78.6)	0.12±0.03 (82.6)	0.12±0.00 (82.6)
N_2	0.87±0.03 (13.0)	0.78±0.04 (29.0)	0.61±0.04 (29.0)	0.56±0.03 (30.0)	0.44±0.05* (41.3)	0.39±0.02* (44.3)	0.29±0.02 (58.6)	0.17±0.03 (75.4)	0.12±0.00 (80.0)
N_3	0.90±0.01 (10.0)	0.90±0.04 (10.0)	0.74±0.02 (14.0)	0.63±0.01 (21.3)	0.52±0.03* (30.7)	0.41±0.02* (41.4)	0.28±0.03 (60.0)	0.64±0.02 (76.8)	0.11±0.00 (82.3)
R_2	0.91±0.02 (9.0)	0.93±0.03 (15.5)	0.70±0.01 (17.6)	0.60±0.04 (25.0)	0.50±0.04* (33.3)	0.36±0.05* (48.6)	0.19±0.02 (73.4)	0.12±0.03 (82.6)	0.10±0.00 (83.2)
O	1.0 ±0.02	1.10±0.01	0.86±0.05	0.80±0.07	0.75±0.01	0.70±0.01	0.70±0.05	0.69±0.03	0.62 ±0.04

*Reduction in oedema significant at $p < 0.05$ compared to control. Values of oedema shown are mean ± SD (n = 5). Values in parenthesis are percent inhibition of oedema. N_1 to N_3: indomethacin tablets, R_2: pure indomethacin, O: control.

in order to determine whether they complied with BP (2009) standards, and to determine if the drug was lost either by physical or chemical treatments. From the results of drug content presented in Table 2, all the tablet batches complied with the BP (2009) standard for assay of active ingredient. All the tablets were within the range of 90 to 110% of the average value.

Anti–inflammatory properties

The results of anti-inflammatory properties of indomethacin tablets formulations presented in Table 3 and Figure 3 show that indomethacin tablets prepared with varying ratios of lipid matrix had significant anti-inflammatory properties comparable to the reference drug and varied significantly from the control from 1 to 8 h ($p < 0.05$). At 30 min, indomethacin tablets formulated with lipid matrices, SRMS 1:1, 2:1 and 1:2 (N_1, N_2 and N_3) inhibited the oedema by 24.5, 10 and 13%, respectively. At 8 h, the indomethacin tablets prepared with the lipid matrices, SRMS 1:1, 1:2 and 2:1 showed percentage oedema inhibition of 82.6, 82.3 and 80%, respectively as shown in Figure 3. The results are comparable to the effect of the reference drug, indomethacin pure sample which exhibited 83.2% oedema inhibition at 8 h.

Results of analgesic/antinociceptive properties

The results of analgesic properties presented in Table 4 showed that indomethacin tablets formulated with the lipid matrix, SRMS 1:1 had an increase in pain reaction time at 7 h significantly ($p < 0.05$) different from the results exhibited by tablets formulated with the lipid matrices, SRMS 1:2 and 2:1, which showed a decrease in pain reaction time at 7 h as shown in Figure 4. The tablet formulations varied significantly ($p < 0.05$) from the control (normal saline). However, the tablets formulated with the lipid matrices, SRMS 2:1 and 1:2 (N_2 and N_3) showed maximum analgesic effect

Table 4. Analgesic/antinociceptive properties of indomethacin tablets.

Group	Pain reaction time (s)								
	30 min	45 min	1 h	2 h	3 h	4 h	5 h	6 h	7 h
1:1 (N_1)	3.83±1.16	5.04±0.51	6.41±0.70	7.62±0.56*	9.84±1.70*	12.01±1.05*	14.46±1.41*	16.02±1.22*	16.46±0.72*
2:1 (N_2)	3.26±1.36	4.50±1.66	5.86±1.69	8.05±1.88*	11.75±2.50*	13.84±2.16*	17.35±1.48*	19.21±1.10*	14.41±1.12*
1:2 (N_3)	2.84±1.21	5.37±0.67	6.46±1.01	8.03±1.77*	9.76±1.75*	11.69±1.59*	13.77±1.25*	16.34±1.15*	15.44±0.56
(R_2) Ref.	3.82±1.12	4.86±0.67	6.40±1.52	9.10±1.14*	11.09±1.16*	13.49±1.08*	16.01±1.22*	18.83±1.40*	14.92±2.44
(O) Cont.	2.71±0.66	2.77±0.89	2.76±0.90	3.55±0.41	3.07±0.50	3.33±0.49	4.01±0.93	3.70±1.10	3.74±0.91

*Significant at $p < 0.05$ compared to control. Values shown are mean ± SD (n = 5), N_1 to N_3: indomethacin tablets, R_2: pure indomethacin, O: normal saline.

at 6 h.

Ulcerogenicity studies

Ulcerogenicity of indomethacin tablet formulations was studied in order to determine the effect of these formulations on the GIT. Indomethacin tablet formulations based on SRMS reduced the ulcerogenicity of the indomethacin by 70 to 80% as shown in Table 5.

DISCUSSION

The DSC measurements were carried out in order to determine the melting points of indomethacin, Softisan®, Phospholipon® 90H, SRMS 1:1, 2:1 and 1:2. The narrow melting peak of Softisan®154 indicated that it is a high purity lipid. The DSC thermogram of Phospholipon® 90H revealed that it consists entirely of stable form because of the sharp melting peak seen. Thermograms of the lipid matrices showed that (Figure 2d, e and l) SRMS 1:1, 2:1 and 1:2 generated imperfect matrices (due to distortion of crystal arrangement of individual lipids after melting and solidification), which may have created numerous spaces for drug localization (Umeyor et al., 2012; Chime et al., 2012). That was the reason for low enthalpies they exhibited. The varied fatty acid contents of these lipids may have interacted in such a manner as to partly disorder the crystal arrangement of the individual lipids (Sanna et al., 2004; Jaspart et al., 2005; Attama and Muller-Goymann, 2007; El-Kamel et al., 2007; Umeyor et al., 2012). Reduction in enthalpy generally suggests less crystallinity of lipid matrices (Umeyor et al., 2012; Attama et al., 2006).

The dimensional properties of indomethacin tablets based on SRMS showed that the tablets were smooth and spherical with no form of depressions or cracks seen in any of the batches as shown in Figure 2. The nature of drug contributed to the smooth and uniform surface seen in the tablets. Indomethacin solubilized in the lipid matrix and gave light yellow tablets. The low standard deviation of the dimensional properties confirmed the reproducibility of the method of production and reliability of this formulation. Weight uniformity test was performed on the tablets so as to determine its compliance with BP specifications (2009). Variation in weight of tablets causes variation in drug content which will also affect the bioavailability of the drug. The low coefficient of variation of tablet weight uniformity confirms the reproducibility of the formulation. The results showed that batch N_3 formulated with lipid matrix 1:2 (Phospholipon® 90H: Softisan® 154) exhibited higher mean weight than other batches of the tablets. This may be due to increase in density of the lipid matrix with increased Softisan® 154 ratio. The results of tablets friability showed that the tablets can withstand handling, packaging and transportation without affecting the integrity of the products. The results of crushing strength test also showed that the mechanical properties of the tablet will not be compromised during long term storage. The erosion time of the tablets studied in SIF (pH 7.5) showed that the formulations showed properties as sustained release tablets. Sustained release preparation and enteric coated tablets are expected to disintegrate or erode appreciably in SIF within 2 h (Ofoefule, 2002). However, batch N_2 had erosion time of up to 180 min; this may be due to the lipophilicity of the drug in the lipid matrix and formation of more compact matrix seen in lipid matrix, SRMS 2:1. Therefore, these results showed that indomethacin tablets based on SRMS could have good sustained release contents of the tablets studied showed that the drug was

Table 5. Result of ulcerogenicity of indomethacin tablets.

Group	Ulcer score	Percentage ulcer inhibition (%)	Ulcer diameter (mm)
1:1 (N_1)	0.80±0.90*	80.00	Lesion<1
2:1 (N_2)	1.20±0.58*	70.00	Lesion<1
1:2 (N_3)	1.00±0.71*	75.00	Lesion<1
Reference (R_1)	4.00±0.52*	0.00	Lesion>2
Control (O)	0.00±0.00	100.00	No lesion

Values shown are mean ± SD. (n = 5). *Significantly different from control at $p < 0.05$, N_1 to N_3: indomethacin tablets, R_1: pure indomethacin, O: normal saline.

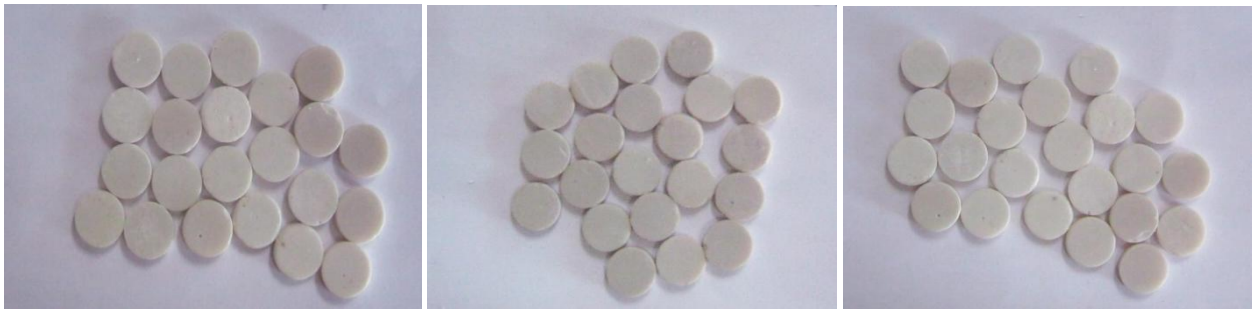

Figure 2. Indomethacin tablets formulated with lipid matrix ratio (SRMS) 1:1, 2:1 and 1:2 (N_1, N_2, and N_3), respectively.

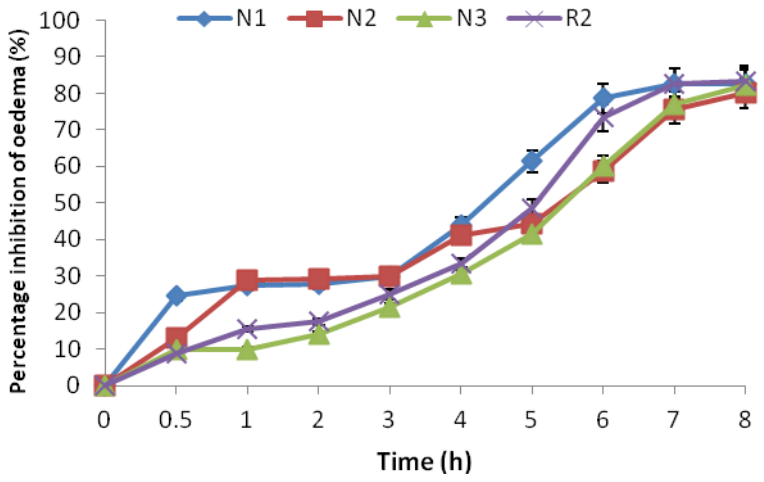

Figure 3. Plot of percentage oedema inhibition against time for indomethacin tablets formulated with SRMS 1:1, 2:1 and 1:2, respectively (N_1, N_2 and N_3) and reference drug (R_2- indomethacin pure sample, 10 mg/kg).

not lost either by physical or chemical means. The low coefficient of variation obtained in the study attests to the reproducibility and reliability of the formulation process.

The anti-inflammatory properties of the indomethacin tablets based on SRMS showed that the formulations exhibited good anti-inflammatory properties. However, the ratio of phospholipids affected the anti-inflammatory properties. Indomethacin tablets formulated with lipid matrix, SRMS 2:1 (N_2), containing higher amount of phospholipid showed lower anti-inflammatory properties

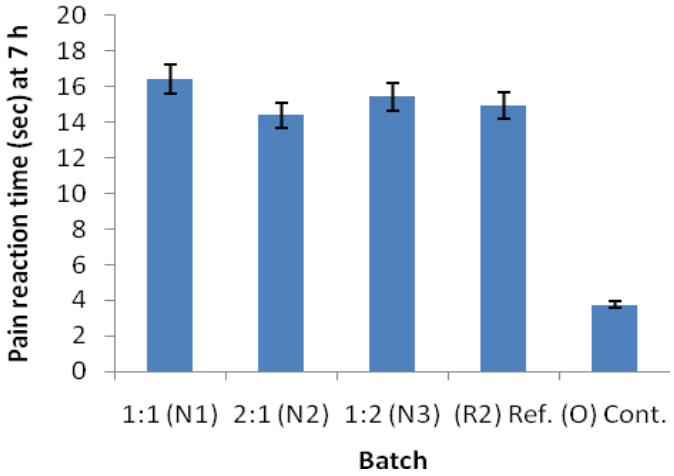

Figure 4. Antinociceptive properties of indomethacin tablet formulations at 7 h. N_1 to N_3: indomethacin tablets, R_2: pure indomethacin, O: normal saline.

from 6 to 8 h significantly different from other batches ($p < 0.05$). This may be due to the hardness, high softening and erosion time exhibited by these batches.

The antinociceptive/analgesic properties of indomethacin tablets showed that the formulations exhibited higher analgesic properties than the reference drug, due to enhanced oral absorption of indomethacin in the presence of lipids. The tablets formulated with the lipid matrices, SRMS 2:1 and 1:2 (N_2 and N_3) showed faster maximum analgesic effect than other formulations. This may be due to the form in which the drug was administered; the tablet was crushed before administration. This may have affected some of the mechanical properties of the tablets for example, the hardness, softening and erosion time of tablets. Therefore, indomethacin tablets prepared with varying ratios of lipid matrix showed good analgesic/anti-nociceptive properties. The results also revealed that the indomethacin tablets based on SRMS may have sustained release properties in addition to other properties.

The ulcer inhibition properties of the indomethacin tablets based on SRMS showed that the SRMS inhibited the ulcerogenic potentials of the highly ulcerogenic indomethacin. Also, the ratios of phospholipid used in the formulations had significant effect on the result as shown in Table 5. The N_1 tablets, formulated with the lipid matrix, SRMS 1:1 exhibited up to 80% ulcer inhibition. The formulations therefore, showed good gastro-protective potentials. The result is in agreement with the work done by Lichtenberger et al. (1995), who proposed that pre-associating NSAIDs with zwitterionic phospholipids prior to their administration should reduce the ability of the NSAIDs to associate with the phospholipids in the mucus gel, and should therefore reduce their ulcerogenicity.

Conclusion

Solidified reverse micellar solutions consisting of phospholipid and triglyceride presented good matrices for the delivery of indomethacin. The results of the *in vitro* properties of the tablets showed that the tablets had good physicochemical properties that complied with specifications. The results of the *in vivo* studies showed that SRMS inhibited the ulcerogenicity of the highly ulcerogenic indomethacin. The formulations also exhibited good anti-inflammatory and antinociceptive/ analgesic effect. Therefore, incorporating indomethacin into the SRMS enhanced the *in vivo* properties of indomethacin due to enhance absorption caused by the presence of the lipids. Indomethacin tablets based on SRMS have advantages over the commercial indomethacin which include: low cost of ingredients, low cost of technologies, little or no ulcerogenicity and better control of pain and inflammation. Further research into this field of study is highly encouraged in order to effectively scale up all its aspects and finally make this product available in the market.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Relationship assessment among content of aminoglycoside antibiotic components by HPLC and C13-NMR and their microbiological assay potencies

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Neomycin and gentamicin are broad-spectrum complexes of aminoglycoside antibiotics constituted by neomycin B and C and gentamicin C1, C1a, C2, C2a and C2b. Despite the similar chemical structures of aminoglycosides, they may present differences in biological activities. In this work evaluation of how each component of neomycin and gentamicin complexes contributes to total potencies and establishment of a relationship between chromatographic results of contents and microbiological assay results of potencies were carried out. The fractions of neomycin B, neomycin C, gentamicin C1, gentamicin C1a and the mixture of gentamicin C2, C2a and C2b were isolated by chromatographic method and then their potencies were estimated by microbiological assay. According to the results, neomycin C was 50% as active as neomycin B. There were no differences among the relative potencies of gentamicin components. It was discovered that there was a linear relationship between the chromatographic results of contents and microbiological results of potencies for both neomycin and gentamicin components. Chromatographic methods allow us to determine the composition of the mixture and the presence of possible impurities not detected by microbiological assay. However, chromatographic methods have limitations that should be kept in mind.

Key words: Aminoglycosides, microbiological assay, liquid chromatography, nuclear magnetic resonance, neomycin, gentamicin.

INTRODUCTION

Aminoglycosides are antibiotics employed in the treatment of infections caused by Gram-negative bacteria (Stead, 2000; Chambers, 2006). Their clinical use is limited due to their nephrotoxic and ototoxic effects (Stead, 2000; Chambers, 2006) and due to the emergence of resistant microorganisms that produce enzymes that modify aminoglycosides (Mingeot-Leclercq, 1999). Aminoglycosides are not absorbed by the gastrointestinal tract and for this reason they are administered via intravenous or intramuscular (Stead, 2000; Chambers, 2006) route.

The aminoglycoside antibiotics inhibit protein synthesis through interactions with the 30S subunit of the ribosome (Stead, 2000; Chambers, 2006; Benveniste and Davies, 1973). When this interaction does not inhibit the protein synthesis, it promotes read errors or early termination, disturbing the chain elongation (Mingeot-Leclercq, 1999). Chemically aminoglycosides are aminosugars linked by glycosidic bonds to an aminocyclitol ring (Stead, 2000; Chambers, 2006). They can be divided into two groups, according to the position of sugar in relation to deoxystreptamine ring. The 1,2-disubstituted compounds include neomycin and paromomycin and the 1,3-disubstituted compounds include gentamicin, tobramycin

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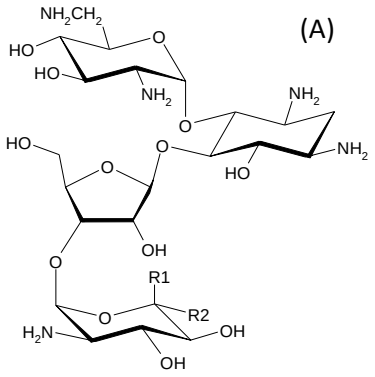

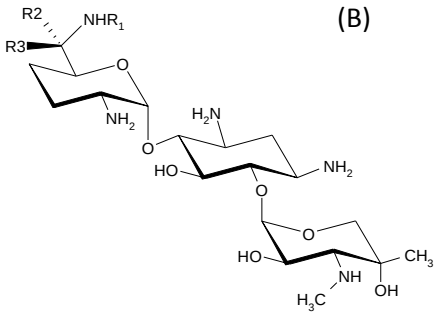

(A)	R1	R2
Neomycin B	CH_2NH_2	H
Neomycin C	H	CH_2NH_2

(B)	R1	R2	R3
Gentamicin C_1	CH_3	H	CH_3
Gentamicin C_{1a}	H	H	H
Gentamicin C_2	H	H	CH_3
Gentamicin C_{2a}	H	CH_3	H
Gentamicin C_{2b}	CH_3	H	H

Figure 1. Chemical structures of neomycin (A) and gentamicin (B) (Stead, 2000).

and kanamycin (Benveniste and Davies, 1973). The number and location of amine groups present in the sugars bonded to the deoxystreptamine ring affects the biological activity of aminoglycosides (Benveniste and Davies, 1973).

Neomycin is a broad-spectrum complex of aminoglycoside antibiotics produced by *Streptomyces fradiae* and consisting of two main components: neomycin B and C (Figure 1) (Sodolski, 1972; Adams et al., 1998). Neomycin B, main component, has increased activity in relation to neomycin (C) (Robertson et al., 1971). Commercial preparations available as sulfate salt are constituted by mixtures of neomycin B and neomycin C range from a ratio of about 50: 50 to 95: 5 (Sokolski, 1972). Despites neomycin components having similar chemical structures, they may present differences in biological activities (Sokolski, 1972).

Gentamicin is a broad-spectrum complex of aminoglycoside antibiotics produced by *Micromonospora purpurea* and consisting of five main components: gentamicin C1, gentamicin C1a, gentamicin C2, gentamicin C2a and gentamicin C2b (Figure 1) (Oden et al., 1972; Kraisintu et al., 1982). Though gentamicin components have similar chemical structures, they may present differences in biological activities (Oden et al., 1972).

The current method described in pharmaceutical compendiums for determination of the potencies of gentamicin and neomycin is the agar diffusion microbiological assay (United States Pharmacopeia, 2012; Farmacopéia Brasileira, 2010). The activity (potency) of antibiotics can be demonstrated under adequate conditions through their inhibitory effect over microbial growth (Cooper, 1963; Hewitt, 1977; Pinto et al., 2010). These methods have been employed for the evaluation of the potency of several antibiotic classes, including the aminoglycosides (United States Pharmacopeia, 2012; Farmacopéia Brasileira, 2010; Lourenço and Pinto, 2009; Lourenço et al., 2011). The agar diffusion microbiological assay is a simple method that allows the use of several experimental designs (Cooper, 1963; Hewitt, 1977; Lourenço and Pinto, 2009), but it is time consuming when compared to chemical methods. In this context, the use of rapid microbiological methods is increasingly the object of research, with the goal of obtaining reliable results in time to allow quick decision-making. The use of triphenyltetrazolium chloride as a viability redox indicator can be used with the purpose of reducing the incubation time of diffusion in an agar medium (Yamamoto and Pinto, 1996). Another feature is the conventional turbidimetric method (United States Pharmacopeia, 2012; Farmacopéia Brasileira, 2010) or a turbidimetric assay using a microplate kinetic-reading system (Lourenço and Pinto, 2011; Botelho et al., 2013).

The chemical characteristics of high polarity, solubility in water, low volatility and absence of chromophore or fluorophore groups make more difficult the development of methods for analysis of aminoglycosides (Isoherranem and Soback, 1999; Stead, 2000). The lack of chromophore groups in the chemical structure of aminoglycosides makes necessary the use of derivatization reagents that allow UV (Stead, 2000; Frutos et al., 2000) or fluorescence detection (Stead, 2000; Wang et al., 2000). In the last decades, a great number of methods of high-performance liquid chromatography have been used

used in the evaluation of aminoglycosides. These methods employed normal or reverse phase liquid chromatography with pre-column or post-column derivatization and UV or fluorescence detection (Kraisintu et al., 1982; Wiegand and Coobes, 1983; Albracht, 1987; Fabre et al., 1989; Caturla and Cusido, 1992; Sweeney and Coleman, 1998; Zhang et al., 2007; Antunes et al., 2011) or direct detection by evaporative light scattering (Clarot et al., 2004; Clarot et al., 2005; Megoulas and Koupparis, 2005), mass spectroscopy (Bogialli et al., 2005; Grahek and Kralj, 2009), pulsed amperometric (Polta et al., 1985), chemiluminescence (Serrano and Silva, 2006) and charged aerosol detectors (Joseph and Rustum, 2010). Ion-exchange or ion-pair chromatography with electrochemical detection have been also employed in the analysis of aminoglycosides (Adams et al., 1996, 1998; Manyanga et al., 2007; Hanko and Rohrer, 2007; Hanko and Rohrer, 2010).

Studies show that samples consisting of mixtures of neomycin B and neomycin C in different ratios differ in potency determined by microbiological assay (Sokolski, 1972; Adams et al., 1998). This situation also occurs in relation to mixtures of gentamicin C1, gentamicin C1a, gentamicin C2 in different proportions (Oden et al., 1972; Kraisintu et al., 1982). Chromatographic methods may be employed for determination of composition and purity of Aminoglycosides; however they do not allow assessing total potency, which is only possible by microbiological assay. Thus, despite the advantages inherent to the chemical methods, the adoption of the chromatographic method as an alternative to the microbiological assay will only be possible if correlation is demonstrated between these methods, based on the relative activity of antibiotic components.

The aim of this work is to evaluate how each component of neomycin and gentamicin complexes contributes to total potencies and to establish a relationship among chromatographic results of contents and microbiological assay results of potencies.

METHODOLOGY

Reagents and chemical reference standards

Neomycin sulfate (Batch M0I253) and gentamycin sulfate (Batch M1J001) reference standards were supplied by United States Pharmacopeia. Chemical standards of neomycin B, neomycin C, gentamicin C1, gentamicin C1a and mixture and gentamicin C2 (C2, C2a and C2b) were prepared in our lab and purified using high performance liquid chromatography (HPLC). Acetonitrile HPLC grade and methanol HPLC grade were supplied by Carlo Erba. Potassium phosphate bibasic and potassium phosphate monobasic were supplied by JT Baker. Antibiotic medium 1 and antibiotic medium 11 were supplied by Difco/BD.

Characterization of neomycin sulfate and gentamicin sulfate samples

Samples of neomycin sulfate were tested for identity of neomycin (by thin layer chromatography - TLC) and sulfate (by chemical reaction for sulfates), loss on drying (60°C in vacuum for 3 h/ specification: not more than 8%), ignition (specification: not more than 1%), content of sulfate (by barium chloride titration/ specification: between 27 and 31%), content of neomycin B and neomycin C (by high performance liquid chromatography - HPLC) and potency (by microbiological assay). Samples of gentamicin sulfate were tested for identity of gentamicin (by infrared spectroscopy and TLC) and sulfate (by chemical reaction for sulfates), loss on drying (110°C in vacuum for 3 h/ specification: not more than 18%), content of water (by Karl Fischer - KF and thermogravimetric analysis – TGA/specification: not more than 15%), ignition (specification: not more than 1%), content of sulfate (by barium chloride titration/specification: between 27 and 31%), content of gentamicin C1, gentamicin C1a, gentamicin C2, gentamicin C2a and gentamicin C2b (by HPLC and carbon nuclear magnetic resonance – C13-NMR) and potency (by microbiological assay).

Chromatographic content and purification

A liquid chromatograph (Accela, Thermo Corporation) equipped with a quaternary pump, auto-sample, and refractive index detector was used to perform content determination and isolation of neomycin and gentamicin components. The chromatograph was equipped with a Zorbax C18 (Thermo Corporation) chromatographic column with 30 mm of length, 4.1 mm of diameter and 5 µm of particle size was employed. An analytical balance (Shimadzu, AUY220) was used to measure the weight of neomycin and gentamicin reference standards and samples. A mixture of acetonitrile and water with trifluoracetic acid was employed as mobile phase. Neomycin and gentamicin samples and reference standards were diluted in mobile phase, and aliquots of 10 µl were injected in the chromatograph. The content of each component of neomycin (neomycin B and neomycin C) and gentamicin (gentamicin C1, gentamicin C1a and mixture of gentamicin C2, C2a and C2b) were calculated based on the peak area response of each component. The fractions of neomycin B, neomycin C, gentamicin C1, gentamicin C1a and the mixture of gentamicin C2, C2a and C2b were collected and then their potencies were estimated by microbiological assay.

Microbiological assay for potency

Cultures of *Staphylococcus epidermidis* (ATCC 12228) were cultivated on antibiotic medium 1 (beef extract 1.5 g/L, yeast extract 3.0 g/L, pancreatic digest of casein 4.0 g/L, peptone 6.0 g/L, dextrose 1.0 g/L and agar 15.0 g/L, pH from 6.5 to 6.6) at 37 ± 1°C for 24 h. The growth was suspended in 0.9% sodium chloride sterile solution and diluted to obtain a suspension with 25 ± 2% transmittance at 580 nm. Portions of 1% of this suspension were added to 100 ml antibiotic medium 11 (beef extract 1.5 g/L, yeast extract 3.0 g/L, pancreatic digest of casein 4.0 g/L, peptone 6.0 g/L, dextrose 1.0 g/L and agar 15.0 g/L, pH from 7.9 to 8.0) and used as inoculated layer. Twenty-one milliliters of antibiotic medium 11 was poured into 90 mm × 20 mm Petri dishes as the base layer. After solidification, portions of 4 ml of the inoculated medium were poured onto the base layer. Six stainless steel cylinders were placed on the surface of the inoculated medium and aliquots of 100 µl of neomycin (or gentamicin) reference standard and each fraction of neomycin (of gentamicin). After incubation at 37 ± 1°C for 24 h, the inhibition zone diameters were measured and the potencies calculated according to 3 × 1 experimental design, as described by Lourenço and Pinto (2009).

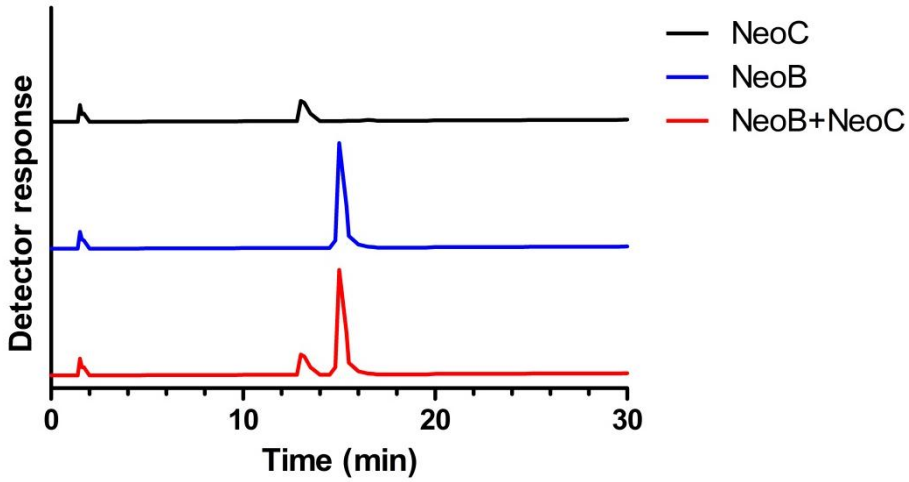

Figure 2. Chromatogram of neomycin sulfate complex (—), neomycin B fraction (—), and neomycin C fraction (—).

RESULTS AND DISCUSSION

The fractions of neomycin B and neomycin C were collected after chromatographic isolation. After collected, the fractions were diluted (when necessary) to obtain a proper concentration of neomycin. The amounts of neomycin B and neomycin C in each fraction were estimated based on calculated area response factor for the standard and fractions runs. This provided some insight into the potency in each fraction. The amounts of neomycin B and neomycin C were estimated based on area ratio for neomycin B and neomycin C peaks (Figure 2). We assumed that both neomycin B and neomycin C have the same response factor, due to similarity of its chemical structures. However, we expect that neomycin C potency is lower than neomycin B potency. According to our results, neomycin C is 50% as active as neomycin B against *S. epidermidis* (ATCC 12228).

According to the results of the tested sample, the content of neomycin C is low and therefore it does not affect the total potency of neomycin sulfate complex in a significant way (Figure 3). As expected, neomycin B contributes more significantly to the total potency of neomycin sulfate complex. The content of neomycin B and neomycin C by chromatographic method was found to be about 87 ± 2% (95% of confidence level, n = 4) and 9 ± 2% (95% of confidence level, n = 4), respectively. According to microbiological assay potencies, neomycin B contributes with 93 ± 22% (95% of confidence level, n = 4) and neomycin C with 4 ± 2% (95% of confidence level, n = 4) of total potency.

The plot of results based on liquid chromatography content and results of microbiological assay potency shows acceptable linearity, as presented in Figure 4. Correlation coefficient found was 0.823, slope was 0.8 ± 0.2 (95% of confidence level, expected to be 1) and interception was 2.3 ± 3.5 (95% of confidence level, expected to be 0). It suggests that there is a direct relationship between the content of neomycin B estimated by chromatographic method and the microbiological assay potency. However, in samples that contain higher amounts of neomycin C, the total potency of neomycin sulfate complex may be affected due to the lower potency of neomycin C with respect to neomycin B.

The fractions of gentamicin C1, gentamicin C1a and mixture of gentamicin C2, C2a and C2b were collected after chromatographic isolation. After collected, the fractions were diluted (when necessary) to obtain a proper concentration of gentamicin. The amounts of gentamicin C1, gentamicin C1a and mixture of gentamicin C2, C2a and C2b in each fraction were estimated based on calculated area response factor for the standard and fractions runs. This provided some insight into the potency in each fraction. The amounts of gentamicin components were estimated based on area ratio for each peak. We assumed that all components have the same response factor, due to similarity of its chemical structures. Chromatography method does not allow us to estimate individual contents of gentamicin C2, gentamicin C2a and gentamicin C2b (Figure 5). However, we estimated the content of these components by a carbon nuclear magnetic resonance (C13-NMR) method (Figure 6), using a 1,4-dioxane standard for quantification of gentamicin components. According to C13-NMR and thermogravimetric analysis (TGA) (Figure 7), we estimated the composition of gentamicin sulfate complex (Table 1).

According to the results of the tested sample, the contents of gentamicin C1, gentamicin C1a and mixture of gentamicin C2, C2a and C2b are almost equal (about one-third on each component). According to the results

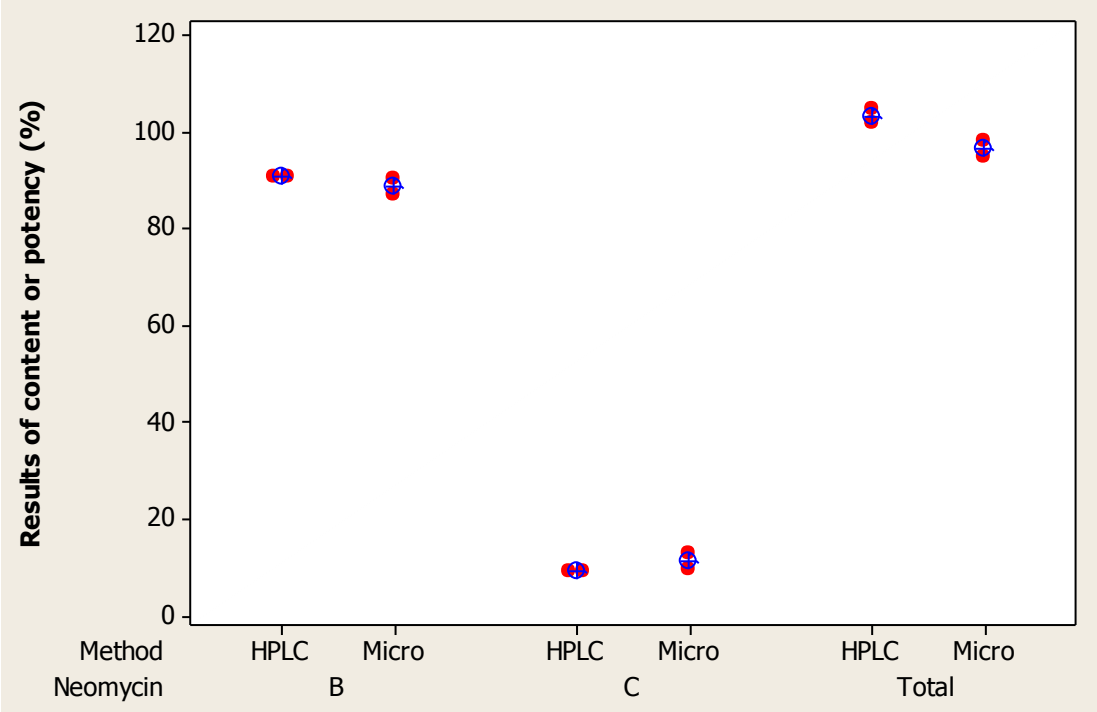

Figure 3. Plot of the results of the contents of neomycin components (neomycin B and neomycin C) estimated by HPLC (%) and their potencies by microbiological assay (%). ( ) individual values and ( ) mean values.

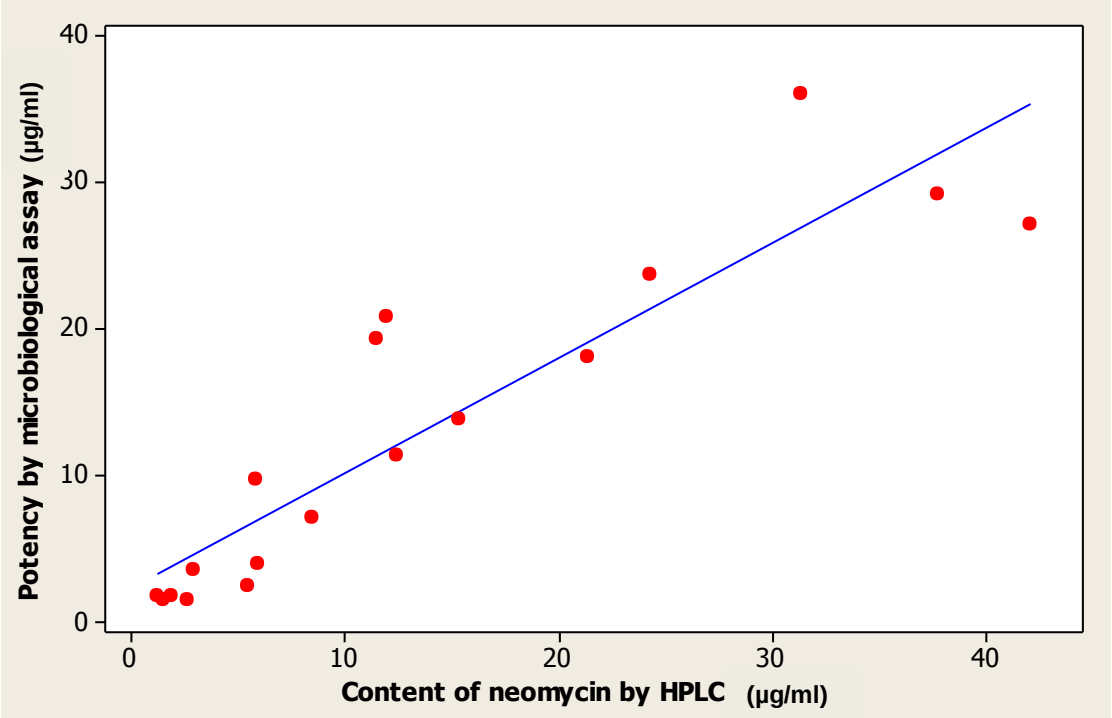

Figure 4. Relationship among the contents of neomycin components (neomycin B and neomycin C) estimated by HPLC (µg/ml) and their potencies by microbiological assay (µg/ml).

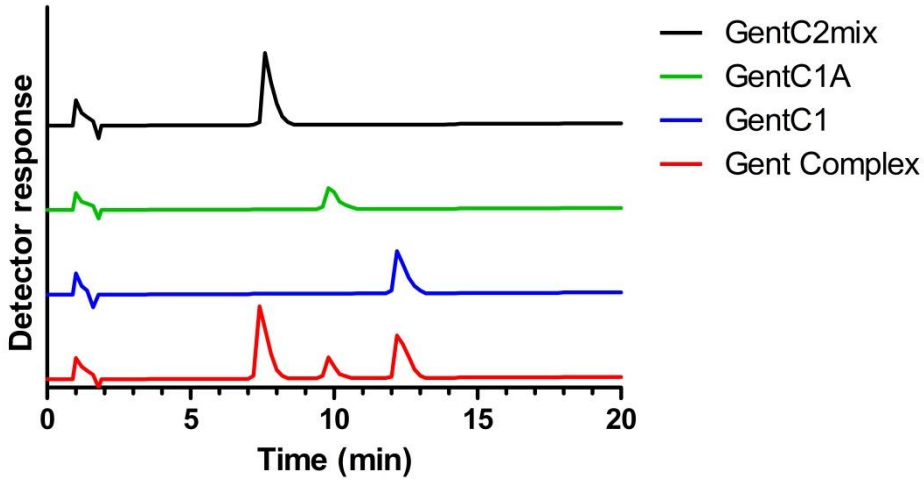

Figure 5. Chromatogram of gentamicin sulfate complex (—), gentamicin C1 fraction (—), gentamicin C1A fraction (—), and gentamicin C2mix fraction (—).

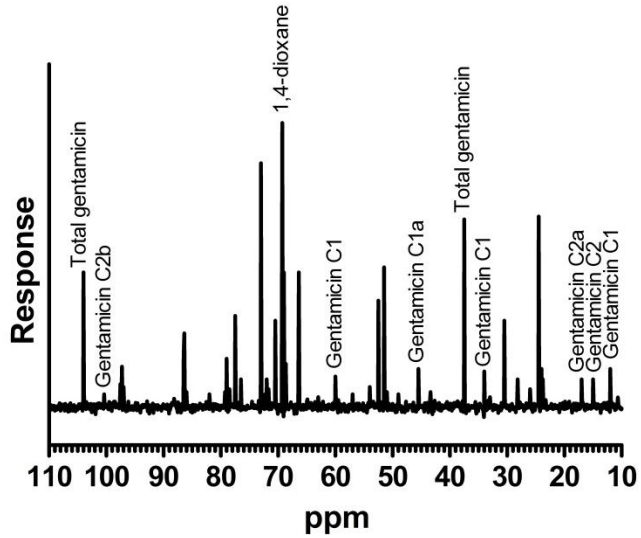

Figure 6. C13-NMR identification of gentamicin C1, gentamicin C1a, gentamicin C2, gentamicin C2a and gentamicin C2b using 1,4-dioxane standard.

of microbiological assay potencies of fraction, all components of gentamicin have similar relative potencies, which means that their contributions to total potency of gentamicin sulfate complex was directly related to their contents (Figure 8). The contents of gentamicin C1, gentamicin C1a and mixture of gentamicin C2, C2a and C2b by chromatographic method were found to be about 33 ± 4% (95% of confidence level, n = 3), 24 ± 9% (95% of confidence level, n = 3) and 46 ± 9% (95% of confidence level, n = 3), respectively. According to microbiological assay potencies, gentamicin C1 contributes with 28 ± 15% (95% of confidence level, n = 3), gentamicin C1a contributes with 28 ± 9% (95% of confidence level, n = 3) and mixture of gentamicin C2, C2a and C2b contributes with 43 ± 7% (95% of confidence level, n = 3) of total potency.

The plot of results based on liquid chromatography content and results of microbiological assay potency shows acceptable linearity, as presented in Figure 9. Correlation coefficient found was 0.980, slope was 0.9 ± 0.1 (95% of confidence level, expect to be 1) and interception was 1.1 ± 8.1 (95% of confidence level, expect to be 0). It suggests that there is a direct relationship between the content of gentamicin components estimated

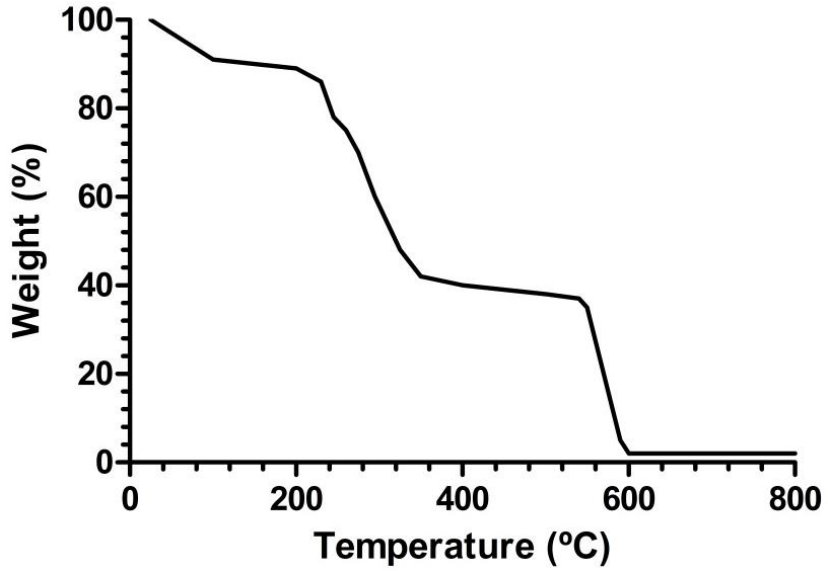

Figure 7. Thermo-gravimetric analysis of gentamicin sulfate using nitrogen and heat rate of 5°C/min.

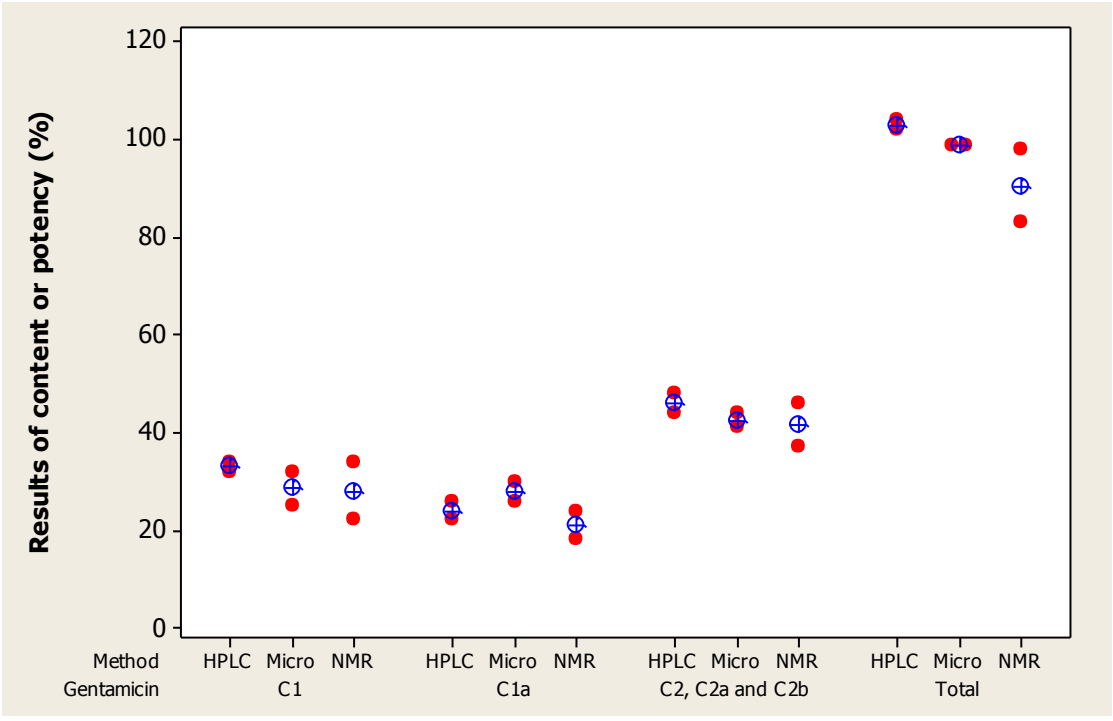

Figure 8. Plot of the results of the contents of gentamicin components (gentamicin C1, gentamicin C1a and mix of gentamicin C2, C2a and C2b) estimated by HPLC (%) and NMR (%) and their potencies by microbiological assay (%). ( ) individual values and ( ) mean values.

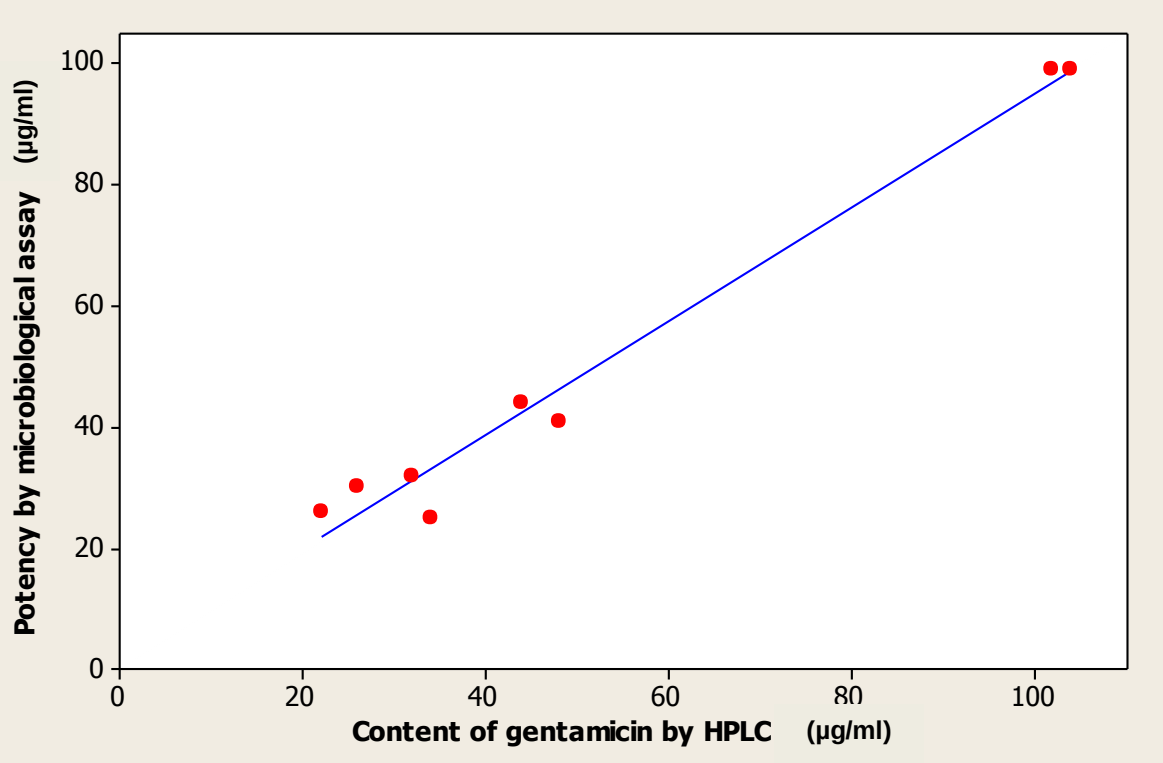

Figure 9. Relationship among the contents of gentamicin components (gentamicin C1, gentamicin C1a and mix of gentamicin C2, C2a and C2b) estimated by HPLC (µg/ml) and their potencies by microbiological assay (µg/ml).

Table 1. Composition of gentamicin sulfate complex based on TGA and C13-NMR results.

Component	Result (%)
Water content	10.8-12.9%[1]
Sulfate content	32.1-34.7[1]
Total gentamicin content	49.3-51.3[1,2]
Gentamicin C1	21.8-34.2[2]
Gentamicin C1a	18.2-24.7[2]
Gentamicin C2	15.9-34.3[2]
Gentamicin C2a	10.6-11.5[2]
Gentamicin C2b	1.0-9.6[2]
Other substances (impurities)	1.7-2.8[1,2]
Total	98.7-99.1[1,2]

[1]TGA and [2]C13-NMR.

method and the microbiological assay potency. Based on the chemical structure of gentamicin C2, gentamicin C2a and gentamicin C2b, we expect that there are no differences in their microbiological assay potencies. However, we were not able to estimate their relative potencies.

Conclusion

Based on the results, it can be concluded that the chromatographic methods may be employed as an alternative in the analysis of aminoglycosides. However, the analyst should keep in mind the limitations of chromategraphic methods with regard to the relationship between content of each component and the total potency of aminoglycosides. Only the microbiological assay method is able to determine the total potency of aminoglycoside. On the other hand, the chromatographic method offers the advantage of the possibility of determining the composition of the mixture and the presence of possible impurities not detected by microbiological assay.

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Full Length Research Paper

Dynamics of drug resistance development in HIV-positive Ugandan mother-child pairs during 18 months after nevirapine single-dose exposure for PMTCT

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Single-dosed nevirapine (NVP), which frequently selects for resistant virus, is still often applied in resource-limited settings to prevent vertical human immunodeficiency virus (HIV) transmission. We followed-up 83 NVP-exposed HIV-positive mothers and newborns between delivery and 18 months postpartum, testing for vertical transmission and for common NVP-selected resistance mutations through highly sensitive allele-specific polymerase chain reaction (PCR). Ten infants turned seropositive within 18 months; 9 mother-child-pairs were available for resistance testing. Mutations were detected in plasma virus of 7/9 (78%) mothers and 4/9 (44%) infants. Resistant virus predominantly emerged at 2 to 8 weeks after NVP-exposure. NVP resistant HIV-1 variants did not persist longer in infants than in their mothers; however, the success of non-nucleoside reverse transcriptase inhibitors (NNRTI)-containing treatment might be limited for HIV-infected infants if initiated within 6 months after NVP exposure.

Key words: Human immunodeficiency virus (HIV), preventing mother-to-child transmission (PMTCT), nevirapine (NVP), resistance, antiretroviral treatment (ART) initiation, paediatric ART.

INTRODUCTION

Single-dosed nevirapine (sdNVP) for human immunodeficiency virus (HIV)-infected women and their newborns has been a standard regimen for prevention of mother-to-child transmission of HIV (PMTCT) in endemic Sub-Saharan Africa since 2001 (Guay et al., 1999). Although the current World Health Organization (WHO) PMTCT guidelines recommend a triple antiretroviral regimen (WHO, 2006, 2010), sdNVP is still offered as a minimal intervention in many resource-limited countries. In Uganda, 58% of all PMTCT clients still received sdNVP in 2010 (UNAIDS, 2010). However, exposure to NVP seems to have a negative impact on the virologic response to a subsequent NVP-containing antiretroviral treatment (ART) if started within 6 months of exposure (Lockman et al., 2007; Stringer et al., 2010). SdNVP frequently selects for resistance mutations, conferring cross resistance to non-nucleoside reverse transcriptase inhibitors (NNRTI), as verified for mothers (Arrive et al.,

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2007) and infants (Eshleman et al., 2001, 2005; Martinson et al., 2007).

Even the presence of minor drug resistant variants was reported to reduce the efficacy of subsequent treatment in adults (Lecossier et al., 2005; Coovadia et al., 2009; Rowley et al., 2010; Li et al., 2011) and children (MacLeod et al., 2010; Sigaloff et al., 2011).

In the absence of drug-selective pressure, resistant virus populations fade over time (Eshleman et al., 2001, 2005; Loubser et al., 2006). However, previous studies showed persistence of minor resistant variants for a period of more than a year in NVP-exposed women (Flys et al., 2005, 2007), and a 12-months NVP-free interval after sdNVP exposure has been recommended to ensure unrestricted treatment response to non-nucleoside reverse transcriptase inhibitors (NNRTI)-containing ART (Stringer et al., 2010). At the same time, it has not been clearly answered yet whether such a time interval is also required for children. Most data regarding the presence of NVP-resistant virus populations in infants is based on results of the less sensitive Sanger sequencing method (Martinson et al., 2007) or on samples taken 4 to 12 weeks after birth or after treatment failure (Eshleman et al., 2001; MacLeod et al., 2010; Sigaloff et al., 2011). So far, the emergence and persistence of minor resistant HIV-variants in infants following sdNVP has not been assessed in tight time intervals during the first year of life or beyond.

In the present study, we monitored the presence of NVP resistance mutations for a period of 18 months in HIV of Ugandan mothers and their vertically infected infants after sdNVP exposure. Therefore, a highly sensitive allele-specific real-time PCR (ASPCR) assay with detection limits of <1% was applied to detect the NVP-selected resistance mutations K103N and Y181C in the reverse transcriptase (Hauser et al., 2012). The aim of the study was to determine the time of emergence and persistence of resistance mutations in plasma virus of infants compared to their mothers within 18 months after birth.

METHODOLOGY

Study population

During 2003 to 2005, we observed 90 HIV-1-infected, treatment-naïve mothers and their newborns who participated in a PMTCT program in Fort Portal District Hospital, Western-Uganda. In accordance with national guidelines, mothers received 200 mg NVP at the onset of labour; newborns received 2 mg/kg NVP syrup within the first 72 h after birth (HIVNET012 protocol). Blood samples of mothers and babies were taken at birth, week 2 to 4, week 6 to 8, and months 3, 6, 12 and 18. All women had given informed consent. The presence of NVP was confirmed in 83/90 maternal delivery blood samples. These 83 samples were the basis for the present investigation, as well as for other studies (Kunz et al., 2009; Hauser et al., 2011; Pilger et al., 2011). The 83 HIV-positive women gave birth to 86 newborns (80 single and 3 twin births); 74 (86%) infants were exclusively breastfed and 12 (14%) received replacement feeding. The study was approved by the National Council of Science and Technology of Uganda and by the Ethical Committee of Charite-Universitätsmedizin Berlin, Germany.

Tests applied to determine HIV status and resistance mutations

Since maternal HIV-1 antibodies are transferred to the fetus through the placenta during pregnancy, HIV-PCR has to be performed for diagnosis of HIV-1 infections in early paediatric blood samples. The maternal antibodies in infants usually disappear 18 months after birth, and uninfected infants revert to HIV-seronegative status (Gulia et al., 2007). In the present study, only those paediatric specimens taken 18 months after birth were tested in two commercial HIV-1 antibody tests (Murex HIV-1.2.0, Abbott GmbH & Co. KG, Wiesbaden, Germany and Gene Screen HIV1/2 version 2, BioRad Laboratories, Munich, Germany). For reactive and indifferent HIV-1 antibody test results, as well as for samples available only before the age of 18 months (in case of death or loss to follow-up of the child), HIV-PCR was performed using the “outer” PCR of ASPCR (Hauser et al., 2012). In case of a positive PCR-result, all samples of the infected child were tested by PCR to determine the time of vertical transmission. Per our definition, positive PCR-results for samples taken at birth indicated “*in-utero* transmission” (Bryson et al., 1992), a negative PCR-result for a sample taken at birth followed by positive PCR-results for samples taken at week 2 to 4 indicated “intrapartum transmission” (via late *in utero* transmission or early breastfeeding), and a positive PCR-result in the sample taken at week 6 (or later), but negative for samples taken earlier, indicated “postpartum transmission” (via late breastfeeding).

Since subtypes A and D are the predominant HIV-1 subtypes circulating in Uganda (Gale et al., 2006), plasma samples of HIV-positive infants and their mothers were investigated for the most common NVP-selected resistance mutations K103N (AAC and AAT codons) and Y181C (TGT codon), using the subtype A and D-specific ASPCR with detection limits < 1% (Hauser et al., 2012). If maternal delivery sample (baseline sample; assumed to contain HIV-1 wild-type only; Church et al., 2007) was not available, a wild-type DNA-standard was used to determine the proportion of resistant variants in the total viral population. In case of HIV-negative or lacking newborn birth sample, the corresponding maternal sample was used. Proportions of K103N mutants encoded either by codon AAC or AAT were summarized. Sensitivity of ASPCR assays for detection of drug-resistant HIV-1 depends also on the input viral load. In order to avoid false-positive results, we established a threshold considering the respective viral load of any given sample (Hauser et al., 2012). If the calculated proportion of drug-resistant HIV-1 was below the calculated theoretical threshold, it was considered to be false-positive and presence of HIV-1 wild type was assumed.

Population-based sequencing was conducted using the Viroseq HIV-1 Genotyping System version 2.0 (Abbott, Wiesbaden, Germany) and the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). HIV-1 subtyping was performed using the REGA HIV subtyping tool.

RESULTS

Eight out of 86 infants were tested HIV-positive at 6 to 8 weeks after delivery or earlier (transmission rate 9.3%). In total, ten children were infected within the study period of

18 months (transmission rate 11.6%). One vertically infected newborn was lost to follow-up, so nine HIV-positive mother-child pairs were included into the resistance analysis. Delivery samples were available from seven mother-child pairs. From all mother-child-pairs, we obtained 3 to 6 follow-up samples from the time span between 2 to 4 weeks and 18 months postpartum. According to our definition, three babies were infected *in utero*, one intrapartum and three postpartum. For two infants, time of infection could not be identified, since samples of birth and/or 2 to 4 weeks after were lacking. Mother-child pairs were infected with HIV-1 subtypes A1 (n = 4), D (n = 3), G (n = 1) and K (n = 1). One child each had died by month 6, month 12, and month 18.

For resistance testing by ASPCR, a mean of 4 maternal and 3 infant follow-up samples were investigated for K103N and Y181C mutations. The dynamics of resistance development in all nine mother-child-pairs are shown in detail in Table 1. In 7/9 mothers and 4/9 infants, NVP-resistant HIV-1 variants could be identified during the study period. In six out of these seven mothers, HIV-resistance emerged 2 to 8 weeks after delivery and persisted for at least six months in all but one woman. In two of these women, resistant HIV-1 was still detectable in the 12 months samples in low (0.05%) and high (100%) proportions. For 4/7 (57%) mothers, resistant variants were identified as minority (< 5%) only. NVP-resistant HIV-variants in infants were also present in the 2 to 8 week postpartum samples of 3/4 newborns. In one infant, viral resistance emerged later (3 months postpartum) and in very low proportions (0.06%) only. For 2/4 infants (50%) resistant HIV-1 were detected in low proportions (< 5%). High proportions of resistant variants (18.7 and 24.0%, respectively) in early follow up samples (week 2 to 4) were identified in two out of three *in utero* infected newborns.

In contrast, in the three postpartum-infected infants, no resistant virus was identified, although their mothers carried resistant HIV-1 during the observation period: In one of these mothers, resistant variants were present in high proportions during the first twelve months postpartum, whereas in two mothers, resistant virus variants were reduced to low or undetectable proportions. Persistence of resistant variants in infants was observed for a maximum of six months (n = 1). NVP-resistance mutations were observed in 3 of 6 subtype D, and in 5 of 8 subtype A infected mothers and infants, while drug-resistant variants in proportions above 5% of the total viral population were identified in 3 of 3 subtype D and in 1 of 5 subtype A infected mothers and infants.

DISCUSSION

In the present study, the 6 to 8 week postnatal HIV-1 transmission rate after sdNVP intervention for mother and newborn was 9.3%, hence comparable to published data (15%; Guay et al., 1999). Investigations on the emergence of NVP-resistant HIV-1 in this mother-child-cohort revealed the presence of at least one drug-resistant variant (K103N and/or Y181C) in 44% of infants and 78% of women during the observation period. These frequencies are consistent with previously published data, showing NVP-resistant HIV-variants after sdNVP exposure in 46% of infants at week 4 to 12 (Eshleman et al., 2001; Martinson et al., 2007) and 87% of women at week 6 to 8 (Loubser et al., 2006; Flys et al., 2007).

Due to the long half-life of NVP, HIV is exposed to slowly decreasing NVP levels over weeks providing resistance-selective conditions (Kunz et al., 2009). For mothers with established HIV-1 infection, emergence of NVP-resistance mutations 1 to 6 weeks after exposure is well documented (Hauser et al., 2011) and seems to occur in infected newborns as well: presence of drug resistant variants 2 to 8 weeks postnatally could be detected in three out of four infants. The highest proportions of drug resistant variants 2 to 8 weeks after birth were identified in infants infected during pregnancy. Since vertical HIV-transmission in those cases took place prior to intrapartum NVP-exposure, high proportions of resistant variants were likely to be selected in the infant itself. Viremia in infants infected *in utero* under NVP-selective pressure increases the development of resistant HIV-variants, fosters proviral integration and thus the archiving and persistence of NVP-resistance mutations (Ghosn et al., 2006).

In the postpartum-infected infants of the present study (n = 3), no resistant virus was identified, although NVP-resistance was detected in plasma virus of their mothers (Table 1). While in two mothers, resistant viral variants had faded to low proportions (no.9: 0.05% K103N) or wild-type virus only (no.8: K103K and Y181Y) at the estimated time of transmission (assumed between last HIV negative and first positive sample of the child), the third mother (no.7) displayed high proportions of resistant plasma virus (< 100% Y181C) in all follow-up samples. Nevertheless, transmission of resistant virus from this mother to her child could not be documented. Hence, the breast milk of this mother is assumed to have carried wild-type virus (K103K and Y181Y) only. Studies on distribution of resistant HIV-variants in plasma and breast milk have shown that different resistance patterns and proportions are present in respective body compartments (Pilger et al., 2011; Raisler et al., 2005).

Half of the mothers and infants displayed resistant HIV-variants in low proportions (< 5% of the total HIV-population). The presence of minor NVP-resistant variants is reported to be correlated with virologic failure for mothers (Lecossier et al., 2005; Coovadia et al., 2009; Rowley et al., 2010; Li et al., 2011) and for NVP-exposed infants (MacLeod et al., 2010). According to this so-called “Mashi” study, which observed 26 NVP-exposed infants,

Table 1. Drug resistant HIV-variants in plasma of mother-child pairs detected by ASPCR.

No.	Transmission	Sub-type		Delivery		Week 2-4		Week 6-8		Month 3		Month 6		Month 12		Month 18	
				K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C	K103N	Y181C
1	IU	K	Child	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	Child died	
		K	Mother	<ld	<ld	2.2	1.1	1.7	<ld	<ld	<ld	0.12	<ld	<ld	<ld	–	
2	IU	A1	Child	<ld	<ld	17.8	18.7	3.6	1.3	–		–		–		<ld	<ld
		A1	Mother	<ld	<ld	–		<ld	1.1	<ld	<ld	<ld	3.2	–		<ld	<ld
3	IU	D	Child	<ld	<ld	24.0	4.8	0.8	<ld	10.7	<ld	47.6	<ld	<ld	<ld	<ld	<ld
		D	Mother	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld
4	IP	A1	Child	O		<ld	<ld	<ld	<ld	0.06	<ld	<ld	<ld	Child died		n.a.	
		A1	Mother	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	–		–		–	
5	§	G	Child	–		–		0.02	<ld	0.03	<ld	Child died		n.a.		n.a.	
		G	Mother	–		–		9.3	<ld	54.8	<ld	7.3	<ld	–		–	
6	§	A1	Child	–		–		<ld	<ld	<ld	<ld	<ld	<ld	–		<ld	<ld
		A1	Mother	–		–		–		1.2	<ld	0.4	<ld	–		<ld	<ld
7	PP	D	Child	O		O		<ld	<ld	<ld	<ld	<ld	<ld	–		–	
		D	Mother	<ld	<ld	3.3	100	60.9	100	1.4	100	<ld	100	<ld	100	–	
8	PP	D	Child	O		O		O		O		O		–		<ld	<ld
		D	Mother	<ld	<ld	0.9	8.4	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld	<ld
9	PP	A1	Child	O		O		O		O		O		O		<ld	<ld
		A1	Mother	<ld	<ld	0.05	<ld	<ld	<ld	-		<ld	<ld	0.05	<ld	<ld	<ld

<ld = <limit of detection; – = no sample available; O = HIV PCR negativ; IU = in-utero transmission; IP = intrapartal transmission; PP = postpartal transmission; § = time of transmission unknown.

virologic failure is also correlated with an early initiation of therapy of less than seven months after NVP-exposure. Higher rates of NVP-associated resistance mutations in HIV-1 subtype D strains as compared to subtype A strains have been observed in other studies (Eshleman et al., 2005). While in our study the emergence of NVP-resistance was observed slightly more frequently in NVP-SD exposed mothers and infants infected with HIV-1 subtype A during the observation period, the presence of drug-resistant variants reaching higher proportions (above 5%) in the total viral population was more frequent in HIV-1 subtype D infections as compared to subtype A. However, statistical significance could not be calculated due to the small sample size.

In our study, HIV resistance mutations persisted longer in mothers (12 months) than in infants (six months). Since the presence of resistant variants may expand to become the predominant population under drug selection pressure (Lee et al., 2005),

a minimum of a 12-months interval between sdNVP and NNRTI-containing treatment is recommended for the mothers (Stringer et al., 2010). However, applying a time interval to sdNVP-exposed children is much more challenging: our data (despite the very small sample size) and data of Persaud et al. (2011) (26% of children with NVP-resistant HIV six months after sdNVP exposure) suggest that the start of therapy within seven months cannot be recommended. Rather, the fact that resistant HIV-1 variants could be archived in the cellular reservoir of antenatally infected infants (Ghosn et al., 2006; Persaud et al., 2007, 2011) indicates the need of extended intervals in children. On the other hand, HIV-disease progression in children is much faster than in adults, and consequently, 85% of infants are in need of ART within the first six months of life (Mphatswe et al., 2007; Violari et al., 2007). Therefore, recommendations for an interval longer than 7 months before ART initiation in sdNVP exposed infants are not realistic.

The small sample size of seropositive NVP-exposed mother-infant-pairs is a limitation of the study. In rural settings like our study area in Uganda, loss to follow-up of pregnant, HIV-positive women is a common problem, especially over long time periods (Lubega et al., 2013). At the same time, due to transmission rates at 10 to 15% for sdNVP, even with a reasonable number of HIV-positive pregnant women, the number of infected infants will always be limited. Accordingly, most comparable studies focussing on HIV-infected mother-infant-pairs are also based on small sample sizes (Kiptoo et al., 2008; Delaugerre et al., 2009; Permar et al., 2013). On the other hand, our study is the first one to follow up HIV-infected NVP-exposed mother-infant-pairs until 18 months postpartum to analyse the dynamics of resistance development. Thus, regardless of the small cohort, our findings are of high interest especially when considering that even today, many PMTCT clients in Uganda and also in other countries still receive only sdNVP for prophylaxis, despite current recommendations for combination regimens.

Conclusion

Due to the limited study panel, any conclusion can only be drawn with great caution. Bearing this in mind, the results from this study show that the use of sdNVP and extended NVP for newborns in PMTCT interventions in Sub-Saharan Africa could implicate a large risk of resistance development in case the infant became vertically infected. Since resistance testing prior to the start of ART can not be performed in many resource-limited settings, NNRTIs should be replaced by other antiretroviral drugs if ART is initiated in women within the first twelve months after sdNVP intake. At the same time, a seven months-interval between NVP exposure and ART initiation seems to be sufficient to prevent a reduced NNRTI-containing treatment response in infants.

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Full Length Research Paper

In vivo trypanocidal effect of aqueous root extracts of *securidaca longepedunculata* and its phytochemical analysis

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The aqueous root extracts of *Securidaca longepedunculata* (Fresen, polygalacaea) were used to treat trypanosomiasis in this experiment. 25 Wister albino rats were inoculated with *Trypanosoma brucei*. Its trypanocidal activity was assessed through daily examination of blood samples, clinical and haematological changes at intervals, and possible deaths were among the parameters which were carefully monitored. The treatment involved a therapeutic dose of diminazene aceturate (3.5 mg/kg), a combination of sub-therapeutic dose of diminazene (1.75 mg/kg) and sub-therapeutic dose of the extract, oral infusion of 200 and 100 mg/kg of the extract, respectively for 7 days. In all rats treated with diminazene and the extract, there was a significant decrease ($p < 0.05$) in parasitemia even though those that received the extract alone relapsed. And there was a significant increase ($p < 0.05$) in haematological values as well. Hence, these findings provide a possible, cheap and available alternative to the existing but costly trypanocides additionally, due to phytochemical data revealed.

Key words: *Securidaca longepedunculata,* trypanosomiasis, diminazene aceturate, parasitemia, *Trypanosoma brucei,* Human African trypanosomiasis.

INTRODUCTION

African trypanosomiasis causes sleeping sickness in people and “nagana” (depressed and low in spirits) in cattle. Two sub-species of trypanosomes infect humans: (1) *Trypanosoma brucei gambiense*, which causes the more chronic form of the disease; and (2) *Trypanosoma brucei rhodesiense*, which is responsible for the more acute form. Accurate statistics for Human African trypanosomiasis (HAT) are not available, but it is estimated that there are currently 300,000 to 500,000 cases with 50,000 deaths annually (Fairlamb, 2003). HAT or sleeping sickness is a major public health problem in 36 Sub-saharan African countries and is caused by *T. b. gambiense* and *T. b. rhodesiense*. About 25,000 new cases of the disease are being reported annually, and around 50 million people are classed as at risk of contracting the disease.

Until now, the only effective drug for treatment of advanced HAT is the trypanocidal melarsoprol. The mortality rate of melarsoprol treated patients is reported to be 1 to 5% (Bouteille et al., 1998; World Health Organization (WHO), 2007). The search for alternative treatment against African trypanosomiasis remains elusive and effective treatment is beset with problems of drug resistance and toxicity (Onyeyili and Egwu, 1995; Gutterridge, 1985; Aldhous, 1994). The four drugs (suramin, pentamidine, melarsoprol and eflornithine) are

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currently available to treat trypanosomiasis (Kuzoe, 1993), with only melarsoprol and eflornithine being effective against the meningoencephalitis that develops in the late stages of the disease. In addition to emerging cases of drug resistance, all four drugs require lengthy, paranteral administration and all but eflornithine have severe toxic side effects (Onyeyili and Egwu, 1995; Gutterridge, 1985) thus, underscoring the urgent need to develop more effective and safer trypanocidal drugs.

Atawodi et al. (2002, 2003) recently, investigated *in vitro* trypanocidal activity of some plants that are commonly used traditionally to treat trypanosomiasis in Nigeria. One of such plants is *Securidaca longepedunculata* which was reported to have *in vitro* activity against *T. congolens* and *T. brucei* organisms. This *in vitro* claim was however cofirmed by Aderbauer et al. (2008). "The root of *S. longepedunculata* Fresen (Polygalaceae) and the extract of *Guiera senegalensis* J. F. Gmel (Combretaceae) were able to reduce parasitemia in mice, experimentally infected with *T. brucei brucei* by 48 and 42% at the dose of 150 mg/kg b.w. intra peritoneal, two times daily for three days". Still confirmed in animals injected with *T. evansi* and *T. brucei*, and also giving an excellent result, were studies carried out by Ameh et al. (2007) and Yusuf et al. (2008), respectively. All the more, further work needs to be done in order to ascertain/establish the efficacy, phytochemistry and possibly fractionate the structures of *Securidaca longependuculata* plant extract. In addition to these works is presented in this publication, a report on systematic *in vivo* assessment of aqueous root extracts of *S. longepedunculata* trypanocidal activity using *T. brucei* in Wister albino rats.

MATERIALS AND METHODS

Plant

S. longepedunculata roots were collected from Zuru town, in Kebbi State, North-western Nigeria. The plant was confirmed by a botanist in Usmanu Danfodio University Sokoto, (UDUS), Northern Nigeria, where a voucher specimen was deposited for reference purposes. The roots of *S. longepedunculata* plant were harvested and carefully air-dried at room temperature (to prevent fungal or bacterial growth) in a laboratory to a constant weight. The dried materials were pounded with a pestle and mortar and sieved to fine powder. This work was carried out at the Department of Veterinary Pharmacology and Physiology, UDUS between September and October, 2007.

Preparation of plant extract

S. longepedunculata powder (500 g) was weighed and macerated with 1,500 ml of distilled water and heated to boiling point. The mixture was filtered using Whitman filter paper. The filtrate obtained was further concentrated in an oven (Gallenkamp oven BS size three) at 50°C. The concentrate then was preserved in a refrigerator pending further experiments. The percentage yield was calculated using this formula:

$$\text{Percentage yield} = \frac{\text{Weight of oven dried crude extract}}{\text{Weight of powdered extract}} \times 100$$

Experimental animals

Wister albino rats of both sexes were purchased for this research work and housed in metallic cages in groups of fives, and were fed with animal feed and water. They were clinically examined and confirmed to be free of trypanosomes and other micro protozoa organisms.

Trypanosome stock

The *T. brucei* organisms (Basa strains) used for this studies were obtained from an experimentally infected rat previously inoculated with the parasite from the Department of Biochemistry, Ahmadu Bello University (ABU), Zaria, Northern Nigeria. The organisms were maintained by sub-passaging into healthy wister albino rats every 5 to 7 days through intra peritoneal injection of 0.2 ml/kg blood solution made in phosphate buffered solution (PBS) to contain approximately 10^6 to10^7 infected red cells (David et al., 2004; Peter and Anatoli, 1998). Parasitemia was confirmed in the infected rats after 48 h of infection with *T. brucei*. All the experimental rats were inoculated through intra peritoneal (i.p) routes. The parasitemia was checked daily with an electronic microscope (model no.0602279) of 400 magnification using wet blot film method from the blood collected through the tails of the animals.

Acute toxicity test

A limit dose of 3000 mg/kg b.w. of *S. longepedunculata* extract (SLE) was used. Animals were dosed one at a time and observed at least once during the first 30 min after dosing, periodically during the first 4 h and thereafter for a total of 14 days. At the expiration of the initial 48 h, four additional animals were sequentially dosed and observed just as described earlier. This is in accordance with Organisation for Economic Co-operation and Development (OECD) guidelines 425 (2000) and interagency research animal committee (IRAC) (2004) recommendations.

Administration of the plant extract

A standard protocol was drawn up in accordance with the Good Laboratory Practice (GLP) regulations of the World Health Organization (WHO Document, 1998). Thirty healthy albino rats were randomly selected for this study and divided into 6 groups of 5 rats each, and treated as follows after confirming parasitemia:

1. Group A: Infected with *T. brucei* and treated with diminazene aceturate once 3.5 mg/kg b.w. therapeutic dose (i.p.)
2. Group B: Infected with *T. brucei* and treated with sub-therapeutic dose of diminazene aceturate (1.75 mg/kg b.w.) once and 100 mg/kg b.w. of SLE once daily for 7 days orally.
3. Group C: Infected with *T. brucei* and treated with 200 mg/kg b.w. of SLE once daily for 7 days orally.
3. Group D: Infected with *T. brucei* also treated with 100 mg/kg b.w. of the root extract (SLE) once daily for 7 days orally.
4. Group E: Is a negative control group that is infected with *T. brucei* but no treatment.

5. Group F: Is a positive control group, not infected and no treatment.

Phytochemical screening of the experimental plant

The phytochemical tests that were carried out included; qualitative screening to identify saponins, (including saponin glycosides), volatile oils, triterpenoids, steroids, alkaloids, tannins, glycosides, flavonoids and anthraquinones in the test material using extract residue. Also, the quantitative test to estimate the quantity of saponins, alkaloids and volatile oils in a known weight of the powdered form of the test material using standard procedures as described by Trease and Evans (1989), El-Olemmy et al. (1994) and Harbone (1993) were carried out.

Assessment of therapeutic activity

The criteria used in the assessment of the trypanocidal effect of the various agents included the examination of blood specimens daily for degree of parasites, clinical changes at daily intervals following treatment, possible death, and also haematological changes. In this case, blood was collected from the heart by cardiac puncture of the animals using ethyldiaminetetraacetic acid (EDTA) as an anticoagulant. Packed cell volume (PCV), red blood cell count (RBC), white blood cell (WBC) and haemoglobin (Hb) count was measured in all cases at the pre-inoculation stage, at the peak of parasitemia and at post treatment stage. PCV was determined by microhaematocrit method. WBC and RBC were done using the improved neubauer haemocytometer, respectively (Ajagbonna and Adebayo, 2002).

Statistical analysis

Results are presented as mean ± standard deviation (SD) and test of significance between the mean parameters is done using analysis of variance (ANOVA), and significance is considered as $p < 0.05$ (google.com).

RESULTS

Trypanocidal efficacy

In response to both the therapeutic and sub therapeutic doses to the drugs, test shows that parasitemia decreased significantly ($p < 0.05$) after one to two days in rats (groups A and B) treated with therapeutic dose of diminazene (3.5 mg/kg) alone as well as in combination regimen of SLE (100 and 1.75 mg/kg of diminazene, no trypanosomes were detected in the blood samples of these rats (groups A and B). Interestingly, from the second day post treatment, the animals remained parasite free for the next forty days of observation. The result also shows that in all animals treated with *securidaca* alone (200 and 100 mg/kg) groups C and D parasitemia decreased gradually until the 7th day of post treatment observation when no parasites were detectable in their blood samples.

Microscopy after the second free day however, revealed trypanosomes in the blood of the first two of the rats and then later on the others. Thereafter, parasitemia re-established in these rats and by the 27th day all rats in groups C and D were dead, apparently from re-emergent parasitemia. There was no significant decrease ($p < 0.01$) in the response to treatment with either 200 or 100 mg/kg of SLE but relapse parasitemia set faster in group D than in group C.

Toxicity results

No death records or any sign of toxic effect were observed in the rats given 3000 mg/kg of SLE and this means that the median lethal dose (LD_{50}) for the oral administration of the extract was therefore greater 3000 mg/kg. Table 2 shows that PCV (%) and mean WBC count ($10^3/mm^3$) increased from 31.0 ± 0.6 and 6.3 ± 0.22 at parasitemia to 40.0 ± 0.30 and 10.7 ± 0.1, respectively in animals treated with the combination of diminazene and SLE. The RBC and Hb count followed the same patterns of improvement.

Phytochemical screening

Table 3 revealed some chemical constituents of the plant including alkaloids (0.30), volatile oils (1.25), flavonoids (0.34), while terpenoids, tannins and steroids were in lesser amounts.

DISCUSSION

The clinical signs of pale mucous membrane, anorexia, weakness and emaciation in this study are characteristics and typical of trypanosomiasis in animals. These findings are in agreement with Ezeokonkwo and Agu (2003, 2004) and Anene (2006). The results of this work show that all the fractions exhibited mild to moderate trypanocidal activity *in vivo* but did not clear the parasitaemia completely. Furthermore, all the fractions of *S. longepedunculata* dose dependently showed no significant changes in the liver parameters (Table 1) but enhanced a quicker recovery from haematological depression caused by parasitaemia (Table 3), which is in agreement with the findings of Ameh et al. (2007), Ajagbonna et al. (2005) and Asuzu and Chineme (1990). That the result shows development of parasitemia within 4 to 5 days (Table 1) is not surprising since earlier reports (Anene et al., 2006., Ameh et al., 2007; Ajagbonna et al., 2005; Onyeyili and Onwualu,1999) are in agreement with it.

The treatment in all groups commenced on day 4 (at peak parasitemia) and a corresponding significant decrease in parasitemia was observed ($p < 0.05$). The parasites totally disappeared from the blood stream two

Table 1. Parasitemia per day of observation in different groups of rats with *Trypanosoma brucei* infection.

Treatment	1	2	3	4	5	6	7	8	9	10
Groups (rats)	**13**	**15**	**17**	**19**	**21**	**25**	**30**	**35**	**40**	**45**
A	0/5	1/5	3/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5
	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
B	0/5	3/5	4/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5
	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
C	0/5	3/5	4/5	5/5	5/5	5/5	3/5	2/5	1/5	1/5
	0/5	3/5	4/5	5/5	5/5	5/5	3/3	2/2	1/1	0
D	0/5	2/5	4/5	5/5	5/5	5/5	3/5	3/5	2/5	1/5
	0/5	1/5	2/5	4/5	5/5	5/5	5/5	5/5	0	0
E	0/5	3/5	4/5	5/5	5/5	5/5	5/5	4/4	4/4	3/3
	2/2	1/1	0	0	0	0	0	0	0	0
F	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

0/5 = No parasitemia. 0 = Dead rats. SLE = *Securidaca longepedunculata* extract.

Table 2. Comparison of changes in the haematological indices in the different treatment groups of rats with *Trpanosoma brucei.*

Haematological indices	A	B	C	D	E	F
PCV (%)	*39.0±02	*40.0±0.3	36.3±0.2	35.0±0.3	*31.0±0.6	46.8±1
RBC (10^6/mm^3)	*5.10±0.1	*5.9±0.1	4.8±0.1	4.8±0.1	*4.1±1.5	6.7±0.0
WBC ($10^3$$mm^3$)	*9.9±0.3	*10.7±0.1	8.6±0.1	8.0±0.4	*6.3±0.22	12.1±0.6
Hb (gm/100ml)	*15.8±0.3	*16.4±0.2	13.9±0.1	13.7±0.3	*10.9±0.1	18.8±0.8

Results are presented as mean ± SD, *P<0.01. * = Significant haematological recovery (p< 0.05) when compared to control E and F.

Table 3. Phytochemical results.

Constituent	Percentage yield
Triterpenoids	+ + +
Steroids	+ +
Volatile oils	(1.25)
Alkaloids	+++ (0.30)
Tannins	+ + +
Glycosides	-
Flavonoids	+ + (0.34)

+ + = Constituents in moderate concentrations, + + + = Constituents in high concentrations.

decrease in parasitemia was observed ($p < 0.05$). The parasites totally disappeared from the blood stream two days post treatment in groups A and B, and six days post treatment in groups C and D. This treatment regimen result is in agreement with Ameh et al. (2007), Ajagbonna et al. (2005) and Asuzu and Chineme (1990). Meanwhile, there was progressive increase in parasitemia in the infected control up to 11 days post inoculation when they eventually died (Table 1). It is noteworthy that all groups treated with extract alone (C and D) developed relapse parasitemia two days after clearance and eventually died after days 25 and 30, respectively. This could be associated to the crude nature of the plant material, thus responsible for the low content of the active ingredient (Barakat et al., 2013).

Table 2 presents that combination therapy offered the best result in terms of enhancing a quicker recovery from haematological depression caused by parasitemia. The PCV recovery was 40.0 ± 0.3 from 31.0 ± 0.6, the RBC

also recovered as 5.9 ± 0.1 from 4.1 ± 1.5, as well as WBC 10.7 ± 0.1 from 6.3 ± 0.22 and Hb 16.4 ± 0.2 from 10.9 ± 0.1, respectively, in addition to the good result of its treatment regimen (Table 1). This is also in agreement with Sammy et al. (2013), Ameh et al. (2007), Ajagbonna et al. (2005) and Asuzu and Chineme (1990).

In the present study, *S. longepedunculata* a recently discovered plant in the treatment of trypanosomiasis has been shown to possess an *in vivo* trypanosomal activity. Other plants have also been reported to possess *in vivo* activities against *T. brucei* (Asuzu and Chineme, 1990; Nok et al., 1993). Although, the mechanism for the *in vivo* anti-trypanosomal activity observed is not known, it is suggestive that since the phytochemical results of SLE indicate the presence of volatile oils, alkaloids, flavonois, terpenoids and steroids; these substances may be attributed to the trypanocidal activity observed in this study (El-olemy et al., 1994; Steenkamp et al., 2013). Thus, this plant promises to be a readily available, affordable and effective alternative trypanocide. However, more work is required to establish the structures of the active compound in *S. longepedunculata* so as to ascertain if this compound is the same as other trypanocidal compounds found in other members of the genus.

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Short Communication

Contribution to the quality of teaching learning in veterinary pharmacology: Intramuscular general anesthesia in sheep applies to the teaching of veterinary pharmacology and surgery

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The current research was conducted with the objective of developing a practical laboratory of Veterinary Pharmacology course, specifically for the purpose of evolving a practical part of the theory in the chapter of general anesthetics. The practice of the evaluation of drugs acting on the central nervous system in this discipline has difficulty with the choice of experimental model, because sometimes there are no laboratory animals such as rats, mice or rabbits; however, it is possible to use larger animals such as sheep. In this paper, we demonstrated that sheep is a suitable experimental model for demonstrating the action of drugs that produce sedation and anesthesia. The effects by the action of xylazine, ketamine and atropine can be produced in about 60 min which corresponds to time allotted in a teaching practice. Therefore, the authors recommended other sister colleges in Ethiopia and elsewhere in the world to use sheep as experimental model for demonstration of general anesthesia for their students.

Key words: Anesthetics, drugs, central nervous system, sheep.

INTRODUCTION

The key to successful surgery and a successful practice is working toward better implementation of anesthesia, from drug delivery to monitoring. Safe and effective anesthesia provides an opportunity for better surgery, and faster and more comfortable recovery (Grubb et al., 2010). Veterinary Pharmacology is delivered for third year students in College of Veterinary Medicine, Mekelle University. The course has got both theory and practical parts. One of those practical parts is demonstration of general anesthesia in laboratory animals so as to prepare the students for the upcoming courses like Veterinary Surgery. However, the assessment of drugs acting on the central nervous system in this discipline has difficulty with the choice of experimental model, because there are no laboratory animals such as rats, mice or rabbits; nevertheless, it is possible to use larger animals such as sheep.

The sheep can be an experimental model for the evaluation of drugs that act on the central nervous system during the available time for laboratory practice. The objective of this paper is to develop adequate anesthetic technique that can fit with the allotted time for laboratory

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practice in our college using sheep as experimental model.

MATERIALS AND METHODS

Study area

The present study was conducted in Veterinary Pharmacology Laboratory at College of Veterinary Medicine, Mekelle University, Kelamino Campus. Kelamino is located in Mekelle city. Mekelle is situated approximately 783 km north of Addis Ababa at an altitude of 2,000 meter above sea level. The mean annual rainfall of the study area is 628.8 mm. The annual minimum and maximum temperatures are 11.8 and 29.94°C, respectively (Bureau of Planning and Economic Development (BoPED), 1998).

Drugs and instruments used for the study

Drugs and instruments used in the current study were the following. Xylazine: bulbs (1 ml equivalent to 23.32 mg); Ketamine: bulbs (1 ml equivalent to 50 mg); atropine ampoules (1 ml equivalent to 1 mg), Digital Thermometer (range 32 to 42°C), endotracheal tubes, stethoscope (YUYUE), 1 ml syringes, cotton and disinfectant: iodine tincture (2%).

Experimental animals

The experimental animals were male sheep. The animals were identified by their numbers: Sheep No.1, 2, 3 and 4. The body weights of the animals ranged from 17 to 23 kg.

Administration of drugs

The drugs were administered in the inside of the inner thigh intramuscularly based on their body weight. The injection site was disinfected by 2% tincture of iodine and the drugs were administered as follows: Sheep No. 1 and 2 received xylazine at the dose rate of 0.3 mg/kg. Sheep No. 3 and 4 received xylazine at the dose rate of 0.3 mg/kg, atropine 0.2 mg/kg and after 15 min, ketamine at the dose rate of 15 mg/kg was administered. Atropine was used for the reduction of salivary and bronchial secretions (Walter et al., 2008). Sheep No.3 and 4 were intubated using endotrachial tubes so as to avoid potential obstruction of the upper airway (Taylor, 1991).

Measurement of parameters

The sheep were controlled all the time before and after the administration of the drugs. The actions of the drugs on the central nerves system such as sedation, cutaneous insensibility and unconsciousness were recorded after the administration of the drugs. The Ramsay sedation scale was used to assess the level of sedation after administration of the drugs (Ramsay et al., 1974). Heart rates, respiratory rates, rectal temperatures and rumen motility were recorded before and after the application of the drugs (Grubb et al., 2010). Heart rate and respiratory rate were determined with the help of stethoscope and stop watch in the left and right lateral sides of the thoracic cavity, respectively. Rectal temperature was recorded using a digital thermometer. Ruminal motility was recorded by placing hand firmly behind the last rib in the depression of paralumbar fossa in the left side for 2 min.

RESULTS

In sheep No. 1 and 2, symptoms like sedation, motor in coordination with visible muscle relaxation, cutaneous insensibility and increase salivary secretion were observed after 15 min of drug administration. These symptoms disappeared in 45 to 60 min. In sheep No. 3 and 4, sedation followed by drowsiness, ataxia, relaxation of the limbs and unconsciousness with slight salivation were observed in less than 3 min after the administration of the drugs. After 60 min, the animals recovered from unconsciousness but maintained some degree of cutaneous insensibility and ataxia, and muscle relaxation with difficulty for walk. All animals at the end of the experiment recovered; any case of complication or death was not present. Table 1 depicts the physiological parameters results obtained in the experimental sheep before and after administration of the drugs.

DISCUSSION

Sheep No. 1 and 2 showed physiological parameters in normal range for the species prior to the administration of xylazine (Mendoza et al., 2010). However, there was decrease in body temperature after the administration of the drug and this could be explained by blocking of the hypothalamic thermoregulatory center by xylazine (Walter, 2008). The decrease in heart rate could be attributed to inhibition of the release of the neurotransmitter noradrenalin. The increase in respiratory rate could also be attributed to the activation of alpha 2 adrenergic receptors and their implication for the relaxation of the bronchial smooth muscles (Schwartz and Clark, 1998; Kastner, 2006; Walter, 2008). After application of the drug, sheep No. 1 and 2 exhibited effects of xylazine action like sedation, analgesia and muscle relaxation. Xylazine is an alpha 2 adrenergic receptor agonist. Alpha-2 agonists inhibit noradrenalin release and P nociceptive release. The locus ceruleus is particularly rich in alpha-2 receptors, and is involved in the sedation. Muscle relaxation might be attributed to the action of xylazine and inhibition of transmission of nerve impulses in intraneuronal level. Inhibition of motility of rumen could be explained due to the inhibition of release of the neurotransmitter acetylcholine exerted by xylazine (Schwartz and Clark, 1998; Johnston, 2005; Kastner, 2006; Walter, 2008).

Similarly, sheep No. 3 and 4 showed physiological parameters in normal range for the species before administration of xylazine, atropine and ketamine (Mendoza et al., 2010). The decrease in temperature, heart rate, the increase in respiratory rate and inhibition of ruminal motility

Table 1. Recorded physiological parameters in the four experimental sheep before and after administration of the drugs.

Parameter	Sheep No. 1 and 2		Sheep No. 3 and 4	
	Before inj.	After Inj.	Before Inj.	After Inj.
Ave temp (°C)	39	38	39.5	38.5
Ave. RR (per min)	14	24	18	26
Ave. HR (per min)	75	60	76	70
Ave. RM (per 2 min)	2	NP	2	NP

Ave. Tem = average temperature, Ave. HR = average heart rate, Ave. RM = average runimanl motility, Ave. RR = average respiratory rate, Before inj. = before injection, After inj. = after injection, NP= not perceptible for more than 2 min.

could be explained similar to sheep No. 1 and 2. The result obtained from sheep No. 3 and 4 showed synergism between xylazine and ketamine. Atropine action decreased the salivary secretion when compared to sheep treated only with xylazine.

In addition, ketamine produced a dissociative anesthesia due to inhibition of excitatory neurotransmitter acetylcholine and excitatory neurotransmitter glutamate (Edmonds et al., 1995). Ketamine also exerted agonist action on Mu and gamma opioid receptors (Sarton et al., 2001). In addition, ketamine produced depression of the thalamocortical portion of the brain and increases the activity of the limbic system which causes analgesia and sleep although the eyes often remain open with a slow nystagmic gaze along with preservation of the corneal and light reflexes (Boothe, 2005; Anonymous, 2009; Ozkan et al., 2010). In the case of these sheep, the anesthetic state was maintained around 60 min, followed by rapid recovery which coincides with the reported effects for ketamine xylazine mixture (Caracuel et al., 1992).

Conclusion

The sheep is a suitable experimental model for demonstrating the action of drugs that produce sedation and anesthesia as part of teaching practices in Veterinary Pharmacology and Veterinary Surgery. The effects by the action of xylazine, ketamine and atropine can be produced in about 60 min, which is the average duration of laboratory practices in Veterinary Pharmacology and other specialties. Hence, this experience can be transferred to other Ethiopian universities that do not have laboratory animal facilities.

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Short Communication

In vivo radio imaging studies on designed swelling gastro retentive drug delivery system

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The objective of the study was to correlate the results of the *in vitro* release of a previously reported swelling gastro retentive drug delivery system with the *in vivo* radio imaging in rabbits. Tablets containing metformin hydrochloride evaluated for *in vitro* drug release profile showed drug release up to 14 h. Tablets containing barium sulfate was administered to rabbits after overnight fast. Radio imaging study showed that swelling matrix tablets remains in the upper small intestine of rabbit for the period of 10 h and it correlates with *ex vivo* retention time of 10 h.

Key words: Swelling matrix tablets, x-ray imaging, *ex vivo-in vivo* correlation.

INTRODUCTION

In the previous published article we reported the formulation and *in vitro* evaluation of swelling gastro retentive drug delivery system using novel polymer hypromellose (HPMC) K200M. We have reported the promising potential of HPMC K200M in the formulating swelling drug delivery system which showed *ex vivo* retention time of 10 h, depending on the *in vitro* swelling and desired *in vitro* drug release (Ige and Gattani, 2011).

Swelling devices (swelling GRDF) after being swallowed, swell to optimum size, which prevents their passage through pylorus. A dramatic swelling time frame which exists over seconds to few hours has been reported for some polymers in solid dosage form. It may swell quickly in the gastric contents and can be retained in the stomach until the size reduced, for example by erosion. Such enlarged dosage form should not block the pylorus, and size reduction should be gradual to prolong its residency in stomach (Timmermans and Moes, 1994; Munday et al., 1998; Klausner et al., 2003; Streubel et al., 2006). Swelling GRDF explores a number of applications for drugs having poor bioavailability because of narrow absorption window and absorption in the upper part of gastro intestinal tract only. It retains the dosage form at the site of absorption and thus enhances the bio-availability (Sriamornsak et al., 2007).

Previously, we have reported that the *in vitro* retention time of 10 h can be achieved using HPMC K200M as matrix tablets for swelling gastroretentive drug delivery system. *In vitro* evaluation of swelling system was carried out by using nited States Pharmacopeia (USP) dissolution 2 apparatus (paddle method) in 0.1 N HCl (pH 1.2) and phosphate buffer (pH 6.8) dissolution media (900 ml) at 100 rpm and the temperature was maintained at 37 ± 0.5°C (Aly and Megwa, 1989; Gohel and Panchal, 2002). We used New Zealand White strain rabbits since this strain has been used previously for an *in vivo* radio imaging study to assess the performance of the mucoadhesive gastro retentive drug delivery system.

In the present investigation, our previously reported *in vitro* data have been correlated with the *in vivo* radio imaging study conducted in rabbits. Here, we observed a 14 h sustained in *in vitro* drug release (using a type 2

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dissolution test apparatus with 100 rpm) which was then confirmed using a radio imaging technique to establish an *ex vivo-in vivo* correlation.

METHODOLOGY

Metformin hydrochloride and HPMC K200M was supplied as gift sample by Aurobindo Pharmaceuticals Ltd, Hyderabad, India. All other materials like $BaSO_4$ of extra pure quality for x-ray diagnosis, microcrystalline cellulose (MCC), polyvinylpyrrolidone (PVP) K30 and magnesium stearate used were of reagent grade and used without further purification.

Preparation of swelling matrix tablets of metformin hydrochloride

Sustained release swelling matrix tablets each containing metformin hydrochloride was designed using software (two factor-optimal) Design Expert version 8.0.1.0 (Stat-Ease Inc Minneapolis, MN). Swelling matrix tablets were manufactured by conventional wet granulation method. Here, the drug is replaced by $BaSO_4$ for x-ray diagnosis. PVP K30 10% was prepared with isopropyl alcohol (IPA) and used as binder. Talc and magnesium stearate was used as lubricants. $BaSO_4$ (500 mg), HPMC K100M and HPMC K200M (1:3), lubricant and binder were mixed to form a damp mass. It was passed through 20# (mesh) sieve and the granules were air dried for 30 min and compressed into matrix tablet by tablet Minipress machine (Rimek, Mumbai, India) with 5 mm diameter tooling.

In vivo radio imaging study in rabbits

The protocol for *in vivo* study was approved by the Institutional Animal Ethical Committee (IAEC) of R C Patel Institute of Pharmaceutical Education and Research, Shirpur and is in accordance with guidance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India. In order to evaluate the *in vivo* residence time of the swelling matrix tablets the formulation batch F8 was selected for *in vivo* x-ray imaging (Sakkinen et al., 2003; Tucker et al., 1981). Six adult male New Zealand white strain rabbits of three months age and weighing approximately 2.0 to 2.5 kg were used for this study. The rabbits were fasted overnight before the start of the study. The tablets excluding drug and containing 30% $BaSO_4$ as well as swelling polymer HPMC K200M, HPMC K100M and PVP K30 10% binder were manufactured by method as described in the preparation using 5 mm tooling set. The tablet was administered through plastic tubing followed by flushing of 25 to 30 ml of water. During the entire study, the rabbits had free access to water only. Photomicrographs (Wipro Ge Dx300 with horizontal x-ray system, Wipro GE medical system, Pune-04, India) were taken at 0, 2, 4, 6, 8 and 10 h.

Ex vivo-in vivo correlation

As previously stated, the aim of the work described here was to correlate the results of the previously reported *in vitro* drug release study with the *in vivo* radio imaging technique to check for a difference in the retention time, if any, and to establish the findings of the *in vitro* study. The experimental results are expressed for three determinations. Statistical evaluation of the data was done using analysis of variance (ANOVA). The evaluation of data was used to assess the significance of differences.

RESULTS AND DISCUSSION

The formulation F2 containing HPMC K100M and HPMC K200M in the ratio of 1:3 exhibits highest percent cumulative drug release. The rate of drug release from the optimized swelling matrix tablets was slower than the remaining formulations of the tablets. Percent swelling and *in vitro* drug release tend to increase together according to observed positive correlation coefficient of 0.874 and p-value less than 0.05, indicating a direct relationship between the two variables (Ige and Gattani, 2011). On the basis of *in vitro* drug release and percent swelling it may conclude that polymer swelling plays an important role in pattern and amount of drug release from the tablets. This might be due to the gel forming ability of HPMC at a high concentration which retards the rate of drug release from the tablets.

In vivo radio imaging study in rabbits

Formulations of swelling matrix tablets of formulation code F8 have shown the good *in vitro* swelling ability and *ex vivo* retention time in this study. Hence it was selected for *in vivo* x-ray imaging study to establish the product performance (residence time in stomach) in rabbits (n = 6). Photomicrographs were taken immediately after 0, 2, 4, 6, 8 and 10 h and are shown in Figure 1. The presence of tablet in the upper small intestine can be clearly noticed and it remains in the stomach not being subjected to disintegration in rabbits. *In vivo* x-ray imaging study clearly indicated that the prepared swelling matrix tablets of metformin hydrochloride retained up to 10 h in upper part of small intestine of the rabbit and hence they had good *in vivo* residence time in the stomach of rabbit. Photomicrographs was taken immediately after administration of the tablets and revealed the nature and position of the tablet up to 10 h.

Ex vivo-in vivo correlation

This novel single unit swelling gastro retentive dosage form with sufficient *in vitro* retention and *in vivo* residence time in upper small intestine up to 10 h could be fascinating for enhancement of bioavailability and the stomach specific delivery of metformin hydrochloride for the effective management of type 2 diabetes mellitus.

Conclusion

This modified swelling drug delivery system could be a

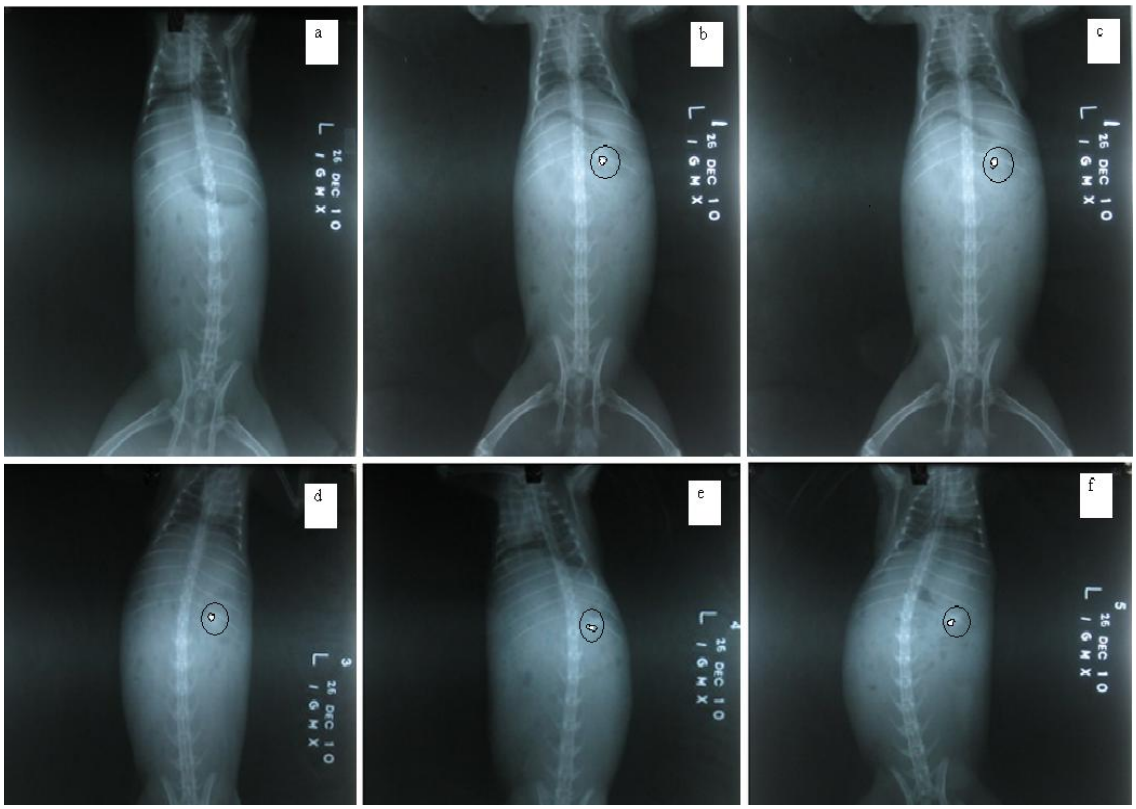

Figure 1. Photo micrographic images of swelling matrix tablet of formulation code F8 after 0 h (a) 2 h (b), 4 h (c), 6 h (d),8 h (e) and 10 h (f) in the upper small intestinal region of the rabbit.

valuable tool for achieving the desired retention time, as a good correlation between *in vitro* studies and *in vivo* radio imaging studies was observed. The *in vitro* percent swelling, *ex vivo* mucoadhesion strength and *in vivo* radio imaging results in rabbits (n = 6) were consistent and reproducible. However, bioavailability studies are awaited to confirm the efficacy of the present swelling drug delivery system.

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