sciendo

Eur. Pharm. J. 2021, 68(2), 1-7.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2020-0014

EUROPEAN PHARMACEUTICAL JOURNAL

The Effect of Different Extracts of Beetroots as Antioxidant and Anti-Anaemia On Phenylhydrazine-Induced Rats

Original Paper

Ni Putu Ermi Hikmawanti, Lusi Putri Dwita✉, Dimas W. Wisnunanda, Fanny Farista

Faculty of Pharmacy and Sciences, Universitas Muhammadiyah Prof. DR. HAMKA, Jakarta Timur, DKI Jakarta, Indonesia

Received 19 June, 2020, accepted 1 February, 2021

Abstract **Aim:** evaluate antioxidant and anti-anaemia activity of dichloromethane, hydroethanolic, and alkaloids-free hydroethanolic extracts of beetroot (*Beta vulgaris* (L.) subsp. *vulgaris*) on phenylhydrazine-induced rats. **Methods:** Male rats were divided into five groups: normal control group, negative control group, dichloromethane extract group, hydroethanolic extract group, and alkaloids-free hydroethanolic extract group. All groups were induced with phenylhydrazine (30 mg.Kg^{-1} BW) for three days, except for the normal control group. After induction, each treatment group received each extract (200 mg.Kg^{-1} BW) for 21 days. The haematology parameters (haemoglobin levels, the number of erythrocytes, and haematocrit levels) were measured using Haematology Analyzer, and the antioxidant activity was measured through MDA level parameters in rats. Data were analysed using one-way ANOVA and then continued with the Tukey test. **Results:** The results showed that the hydroethanolic extract of beetroot increased the percentage of erythrocytes (33.5%), haemoglobin (25%), and haematocrit (24.4%) to the negative control group, which was comparable to the normal control group ($p > 0.05$). In addition, the best antioxidant activity was shown in the hydroethanolic extract of beetroot, which is comparable to the normal group ($p > 0.05$). **Conclusion:** The beetroot hydroethanolic crude extract could be potentially produced in a natural pharmaceutical product as a beneficial resource within anti-anaemia and antioxidant activities.

Keywords *Anti-anaemia – Antioxidant – Beta vulgaris – Beetroot – Phenylhydrazine*

INTRODUCTION

Anaemia is a condition when the number of red blood cells or haemoglobin concentration in the body is lower than normal. Anaemia is a problem that can occur at any age. WHO estimates that 42% of children less than five years of age and 40% of pregnant women worldwide are anaemic. In Indonesia, anaemia's prevalence in women of reproductive age is 27.85% and in children under five years is 36.78% (World Health Organization, 2020). The main cause of anaemia is lack of nutrition (inadequate supply of iron), drug toxicity, blood loss, genetic, or pathological diseases (Chaddha & Mittal, 2016).

Anaemia due to iron deficiency has a significant impact on human health. However, this rarely directly causes death. Erythrocytes require large amounts of iron to produce heme and haemoglobin. Iron deficiency results in decreased formation of red blood cells, which causes anaemia. Based on the research by Jaiswal et al. (2014), the ethanol extract of beetroot contains high iron, folic acid, and vitamin C. These contents are presumed related to the extract hematinic effect on phenylhydrazine-induced rats. Beetroot also contains iron, magnesium, potassium, manganese, copper, sodium, calcium, zinc (Odoh & Okoro, 2013), and bioactive compounds such as betalains, alkaloids, flavonoids, terpenoids, steroids, glycosides, and saponins (Hadipour et al., 2020). These contents have benefits in improving the haematopoiesis process.

Flavonoids are polyphenolic secondary metabolic compounds. These compounds have a protective effect against membranes lipo-peroxidative damage caused by free radicals (Nijveldt et al., 2001). Flavonoid-rich extracts effectively overcome the effects of phenylhydrazine induced malondialdehyde (MDA) damage to red blood cells membrane glycerol back-bone and peroxidation of phospholipids (Ologundudu et al., 2009; Chaddha & Mittal,

* *E-mail: lusi_putridwita@uhamka.ac.id*

2016). On the other hand, tannins were centred on tannic acid, and other hydrolysable tannins are responsible for reductions in feed intake related to the antinutritional effects. It could bind with protein and red blood cells (Chung et al., 1998), affecting haemoglobin levels. Tannins could also inhibit iron absorption. Therefore, most pregnant women who consume the source of tannins like tea go through anaemia (Shah et al., 2020), and it is recommended that tannin-rich foods are not consumed together with meals (Santos-Buelga & Scalbert, 2000). Alkaloids from *Datura stramonium sp.* can reduce levels of erythrocytes, haemoglobin, and haematocrit by interfering with the process of erythropoiesis and causing the process of destruction of blood cells (Benouadah et al., 2016). Quinoline group alkaloids are used as antimalarial drugs by providing a binding effect to heme, a product related to haemoglobin, making the heme-quinoline conjugate toxic and interferes with haemoglobin activity (Heinrich et al., 2012).

Extracting solvent is one of the factors that influence the extraction results. The solvent's polarity is an aspect that underlies the selection of the extracting solvent (Tiwari et al., 2011). The hydroalcoholic extract contains phenolics, flavonoids, alkaloids, carbohydrates, glycosides, and tannins in beetroot (Ahmad et al., 2013). Alkaloids in plant material can be removed by acidification using citric acid. The residue extracted with 70% ethanol still contains flavonoid compounds as in the crude extract (Widiyanti et al., 2016). Meanwhile, tannins are slightly soluble in dichloromethane. This study aims to evaluate the effects of anti-anaemia and antioxidants in different extracts include dichloromethane extract (DEB), hydroethanolic extract (HEB), and alkaloids-free hydroethanolic extract (AFHEB) of beetroots. The difference in chemical composition contained in each extract obtained is expected to indicate differences in anti-anaemia and antioxidant activities. Thus, this study can be used as a reference to optimize the development of beetroots extract as a supplement to overcome anaemia conditions.

MATERIALS AND METHODS

Collection of Plant Material

Fresh beetroots were obtained from Indonesian Peasant Stores. The plant grows on a farm in Lembang, West Java, Indonesia. It was harvested at the age of 2 to 3 months beet plants. The plant was authenticated in Herbarium Bogoriense, Biology Research Center, Indonesian Institute of Sciences, Cibinong, Indonesia.

Preparation of The Extracts

The plant material was air-dried at room temperature and then grounded to powder. Afterward, the dried powder of beetroot was divided into two parts. The first part, namely releasing-alkaloid beetroot powder. The dried powder (820.0 g) was added with a weak citric acid solution to the formed water-soluble alkaloids salt (Widiyanti et al., 2016). Releasing-alkaloid beetroot powder was then extracted using hydroethanolic (ethanol: water, 70:30) for 3´24 h in a macerator. The filtrate obtained was labelled as an alkaloids-free hydroethanolic extract of beetroot (AFHEB)). Whereas the second part, beetroot powder (820.0 g) was extracted separately using hydroethanolic or dichloromethane in the same way as the previous procedure. The filtrates were then labelled as hydroethanolic extract (HEB) and dichloromethane extract (DEB) of beetroots, respectively. Each filtrate was evaporated using a vacuum rotary evaporator N-1200 BS series (EYELA, Shanghai, China) at 50°C. The percentage of yield of each extract was calculated. Each extract's physicochemical characteristics, such as water content, total-ash content, and loss on drying, were performed according to the Indonesian Herb Pharmacopoeia (Ministry of Health Republic of Indonesia, 2008) and WHO guidelines (World Health Organization, 2011).

Phytochemical screening of the extracts

Secondary metabolites such as alkaloid, phenolic, flavonoid, triterpenoids, steroids, and saponin in the extracts were identified qualitatively. The chemical reagents used were Dragendorff, Mayer, and Bouchardat reagents for alkaloids detection; $FeCl_3$ reagent for phenolics detection, $AlCl_3$ reagent for flavonoids detection; Liebermann-Burchard reagent for triterpenoids/steroids detection and gelatine reagent for tannins detection (Hanani, 2015; Harborne, 1987; Ministry of Health Republic of Indonesia, 2008).

Determination of Total Flavonoids Content (TFC)

Total flavonoid content was evaluated using the colorimetric method described by Chang et al. (2002). Briefly, one mL of extract (10000 ppm in methanol) was added with 3 mL of methanol, 0.2 mL of $AlCl_3$, and 0.2 mL of sodium acetate 1 M and distilled water up to 10 mL. The solution was mixed and then incubated for 30 minutes. The absorbance was measured at 415 nm against methanol blank with a Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan). Quercetin was used as the standard with concentration ranging from 5–19 ppm for the construction of a calibration curve, and the concentrations are expressed as quercetin equivalents (mg QE.g^{-1} extract). The test was performed in triplicate, and the results were expressed as mean ± SD.

Determination of Total Iron (Fe) Content

Extract (0.5 g) was put into the vessel, then added HNO_3 and H_2O_2 30%. Then the destruction process is carried out and allowed to stand at room temperature. After this, the solution was transferred to a 50.0 mL flask and distilled water was

added to the boundary mark. The solution was put into a test tube, and the test solution is measured using ICP-OES. This test was conducted at the Regional Health Laboratory, DKI Jakarta, Indonesia.

Preparation of Animals

The Health Research Ethics Committee of the Faculty of Medicine, Universitas Indonesia, approved the experimental design, with ethical approval number: KET-552/UN2.F1/ETIK/PPM.00.02/2019. The design used includes Randomized Design. Twenty-five male rats aged 2–3 months, 150–250 g, were obtained from Research Animal Breeder, Bekasi, Indonesia. The animals are divided into five groups, where each group consisted of four animals. Before treatment, the animals were acclimatized for seven days. At this stage, the animals were given standard drinks and feed.

Experimental Design

Animals are grouped into (1) Normal Control Group: No treatment; (2) Negative Control Group: received Na-CMC 0.5%; (3) Extract Group I: received DEB; (4) Extract Group II: received HEB; (5) Extract Group III: received AFHEB, at a dose of 200 $mg.Kg^{-1}$ BW. All groups (except the normal control group) were induced with phenylhydrazine hydrochloride (Fisher Scientific Company, New Jersey, USA) (30 $mg.Kg^{-1}$ BW i.p once a day for three days) to induce anaemic conditions, then were given the test substance once a day orally for 21 days. On the 22nd day, the animals were injected with ketamine intramuscularly at a dose of 40 $mg.Kg^{-1}$ BW. The blood sample was taken through the orbital sinus and collected in the EDTA tube.

Anti-anaemia Activity Assay

The haematology examinations (haemoglobin levels, the number of erythrocytes, and haematocrit levels) of blood serum were performed at Primate Research Center, Bogor Agricultural University, Bogor, Indonesia using Automated Haematology Analyzer MEK-6450K (Nihon Kohden, Tomioka, Japan).

Antioxidant Activity Assay

One mL blood serum was put into the test tube, 0.5 mL TCA 20% was added to it, then centrifuged at 3000 rpm for 10 minutes, and the supernatant was collected**.** One mL of supernatant was added with 1 mL of TBA 0.67% into a tube, then placed in a water bath at a temperature of 95–100^{o}C for 10 minutes, then cooled with running water. Tetraethoxypropane (TEP) was used as a standard with a concentration of 0.6–2.6 $nmol.mL^{-1}$. The absorbance was measured at 532 nm using a Spectrophotometer UV-Vis Seri UV-1601 (Shimadzu, Kyoto, Japan).

Table 1. The Result of Beetroot Extraction.

Type of Extract	Characteristics		
	Percentage of Yield (%, w/w)	Loss of Drying (%, w/w)	Ash Content (%, w/w)
DEB	1.92	2.48	2.16
HEB	34.52	9.89	9.66
AFHEB	24.55	13.27	19.75

Note: DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

Table 2. Phytochemical Screening Results of Beetroot Extracts.

Compounds	DEB	HEB	AFHEB
Alkaloids	+	+	-
Flavonoids	+	+	+
Tannin	-	+	+
Phenolic	+	+	+
Saponin	-	+	+
Steroids	-	-	-
Triterpenoids	-	-	-

Note: (+) = detected; (-) = not detected, DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

Data Analysis

The data were presented in mean and standard deviation. Kruskal Wallis and Mann Whitney were used to evaluate statistical significance.

RESULTS

Characteristics of the beetroot extracts include a percentage of yield, loss of drying, and ash content shown in **Table 1.** Based on **Table 1**, HEB contains more chemical compounds than other extracts (AFHEB > DEB).

Phytochemical screening aims to identify the presence of chemical substances in the sample qualitatively. Based on the results in **Table 2**, it can be concluded that phenolic, flavonoids, tannins, alkaloids, and saponin compounds are present in extracts. Meanwhile, saponins compounds are only found in HEB. HEB contains high levels of flavonoids and iron (Fe) compared to other extracts (AFHEB > DEB) (**Table 3**).

Based on **Table 4,** the erythrocyte, haemoglobin, and haematocrit levels of beetroot extract groups showed a significant difference compared to the negative control group ($p < 0.05$). HEB group showed the best result, where all the blood parameters comparable to normal control group. Beetroot extract also showed activity as an antioxidant,

shown by reduced MDA levels (**Figure 1**). The best antioxidant activity also showed by HEB, comparable to the normal control ($p = 0.352 > 0.05$).

DISCUSSION

The selection of the suitable organic solvent affects the success in solid-liquid extraction. It is usually done based on the dielectric constant (ε) characteristic of the extracting solvent, which is closely related to its polarity index (Katritzky et al., 2004). Water has a dielectric constant of 78.30, while ethanol is 24.30 (Khoddami et al., 2013) and dichloromethane is 9.1 (Saeker et al., 2006). The polarity index values for water, ethanol, and dichloromethane were 9.1, 5.2, and 3.7, respectively (Snyder, 1974). It shows that water is more polar than the other two solvents (water> ethanol> dichloromethane). The principle of 'like dissolved like' is defined as the condition where a phytochemical substance is dissolved in a similar polarity solvent (Wakeel et al., 2019). In other words, a polar solvent will be able to dissolve a polar substance, and a nonpolar solvent can dissolve a nonpolar substance. Some solvents might have similar polarity index values but could attract compounds of different quantities (Khoddami et al., 2013). For example, in the use of absolute ethanol extracting solvents or ethanol-aqueous (with variations of ethanol concentration in them). This difference can occur due to the differences in each solvent's ability to form chemical bonds with the bioactive metabolites contained in the plant matrix (Huaman-Castilla et al., 2019). Other things that can also affect plant chemical constituents' acquisition are the bioactive compounds' chemical structure, the extraction time, and the temperature used (Khoddami et al., 2013). The difference in the type and polarity of the extracting solvent can cause differences in the quality, quantity, toxicity, bioactivity, and safety of the extract produced (Eloff, 1998).

In this study, the extracted beetroot activity was tested using a variety of solvents as anti-anaemia and antioxidants. A previous study by Jaiswal et al. (2014) reported that ethanol extract of beetroot (200 $mg.Kg^{-1}$ BW) extracted in a Soxhlet apparatus effectively increases the levels of haemoglobin and erythrocytes. Vitamins (such as Vitamin C and folic acid) and minerals (one of them is iron) in beetroots are active ingredients responsible for these activities. Since some of the compounds, such as tannins and alkaloids could interfere with the hematopoietic activity, in this research, we try to optimize the result by evaluating the anti-anaemic activity in dichloromethane extract (DEB), hydroethanolic extract (HEB), and alkaloids-free hydroethanolic extract (AFHEB) of beetroots.

The yields of different extracts of beetroots increased in the following order: DEB < AFHEB < HEB. The highest yield was found in HEB (34.52%) followed by AFHEB (24.55%) > DEB (1.92%). It shows that polar compounds dominate in beetroots. It might also contain non-secondary metabolite polar compounds that also dissolve during the extraction process, such as carbohydrates (which are composed of fibres and sugar) (Neha et al., 2018), proteins, essential and non-essential amino acids, and other compounds (Hadipour et al., 2020). Beetroot contains bioactive compounds such as phenolics (epicatechin, catechin hydrate, vanillic acid, p-coumaric acid, protocatechuic, caffeic acid, syringic acid, proline, dehydro vomifoliol, 4-hydroxybenzoic acid, chlorogenic acid, and ferulic acid, etc.), flavonoids (betagarin, betavulgarin, cochliophilin A, dihydroisorhamnetinas, 2,5-dihydroxy 6,7-methylenedioxyisoflavone, 3,5 dihydroxy-6,7-methylenedioxyflavanone, 5-hydroxy-6,7 methylenedioxyflavone, rutin, quercetin, and 40-hydroxy-5-methoxy-6,7- methylenedioxy flavanone), saponins (betavulgarosides (I, II, III, IV, V, VI, VII, VIII, IX, X), hederagenin, akebonoic acid and gypsogenin), betalains (betacyanin

Table 3. Flavonoids and Iron Contents of Beetroot Extracts.

Type of Extract	Iron (Fe) content ($mg.Kg^{-1}$)	Flavonoids Total Content ($mgQE.g^{-1}$)
DEB	6.52	1.66
HEB	20.43	8.35
AFHEB	11.30	6.07

Note: DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

Table 4. The Anti-anaemia Activity of Beetroot Extracts.

Groups	Blood Parameters		
	Erythrocytes ($x10^6 \mu L$)	Haemoglobin ($g.dL^{-1}$)	Haematocrit (%)
Normal Control	7.00 ± 0.78^a	16.78 ± 0.37^a	50.55 ± 1.22^a
Negative Control	4.73 ± 0.95^b	12.48 ± 0.48^b	37.82 ± 1.49^b
DEB	6.31 ± 0.52^a	14.94 ± 0.46^c	45.18 ± 1.48^c
HEB	7.12 ± 0.61^a	16.66 ± 0.39^a	50.04 ± 1.22^a
AFHEB	6.96 ± 0.55^a	15.46 ± 0.31^c	46.54 ± 0.86^c
Normal Level Value	5.0–12.0	11.1–18.0	36.0–52.0

Note: Different letters in the same column show significant differences ($p < 0.05$). DEB = Dichloromethane Extract of Beetroot; HEB = Hydroethanolic Extract of Beetroot; and AFHEB = Alkaloids-Free Hydroethanolic Extract of Beetroot

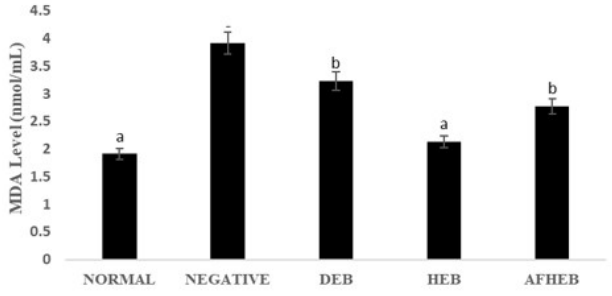

Figure 1. MDA levels after treatment for 21 days.

and betaxanthin), alkaloids (calystegine B1, calystegine B2, calystegine C1, calystegine B3 and ipomine), triterpenoids/ steroids (beta-amyrin acetate, boehmerylacetate and friedelin), volatile compounds (pyridine and 4-picolene), and so on (Hadipour et al., 2020).

Tiwari et al. (2011) explained that 70% ethanol as an extraction solvent was able to dissolve phenolic compounds (include flavonoids) better than absolute ethanol. Ethanol dissolves polyphenolic compounds, tannins, flavonoids (flavonols), triterpenoids, steroids, and alkaloids. In comparison, DEB showed the least number of phytochemicals. Dichloromethane (CH_2Cl_2) is a solvent with a medium polarity level. The polarity of dichloromethane due to its electronegative Cl atoms (Houghton & Raman, 1998). Dichloromethane is specially used for the selective extraction of only terpenoids (Tiwari et al., 2011) but is still able to dissolve alkaloids and aglycones (include flavonoids) (Houghton & Raman, 1998). Dichloromethane has a low ability to dissolve tannin compounds. Therefore, in the phytochemical screening evaluation (Table 1), tannins were not detected in DEB. Meanwhile, the AFHEB contains tannin but no alkaloids. Both total flavonoid and iron levels in the AFHEB are lower than in the HEB (**Table 3**). In this research, the procedure for removing alkaloids from the dried powder of beetroot not only results in the loss of alkaloids but also other compounds including flavonoids, iron or might be the pigment compounds of beetroot. Betalains are 'chromoalkaloids' found in plants of the order Caryophyllales (except in the Caryophyllaceae and Molluginaceae families) (Wink, 2010). Betalains are a natural nitrogen-containing pigment compounds that have two structural groups, viz. the red-violet betacyanins (betanin, prebetanin, isobetanin and neobetanin) and the yellow to orange betaxanthins (vulgaxanthin-1, vulaxanthin-2, and indicaxanthin) (Hadipour et al., 2020). These compounds are indole-derived (Ninfali et al., 2017; de Oliveira et al., 2020) that can be water or alcohol-extracted and stable at pH from 2 to 6 (Azeredo, 2009). Halwani et al. (2018) reported that red pigment compounds (betanine and vulgaxanthin-1) were found in high amounts in water extract and citric acid extract of beetroot in low pH conditions. The presence of this compound can result in a false positive reaction in alkaloid identification using Dragendorff and Mayer precipitated reagents. It is known that these two reagents can also react with nitrogen-containing compounds other than alkaloids (Evans, 2009). We recommend that when identifying alkaloids with these reagents, the test solution must be free from compounds with nitrogen atoms (such as proteins, amino acids, or betalains).

The results showed that phenylhydrazine induction for three days in the negative control group reduced the number of erythrocytes by 32.4%. Phenylhydrazine induces oxyhaemoglobin into methaemoglobin and produces hydrogen peroxide. This hydrogen peroxide causes lipid peroxidation in the red blood cell membrane resulting in lysis of the blood cells, leads to the condition known as haemolytic anaemia (Singh et al., 2014). In this study, the increase in Fe levels and antioxidant activity was in line with the increase in anti-anaemia activity. The DEB and AFHEB showed less potential activities antioxidant (**Figure 1.**) and lower Fe content than the HEB. The same trend was shown in the anti-anaemia activity (**Table 4**). Iron levels influence haemoglobin production in the body. Iron is an essential component in the formation of heme molecules. The body needs nearly 30 mg of iron each day in the erythropoiesis process (the formation of erythrocytes in bone marrow), and only 1–2 mg can be absorbed (Lesjak & Srai, 2019). As the flavonoid-rich plant, administration of beetroot prevents further oxidation on the red blood cells, therefore prevent further damage to the cells (Chaddha & Mittal, 2016). The pigment in beetroot also acts as an antioxidant. Research showed that beetroot betalains could reduce MDA levels in male mice (Clifford et al., 2015). Red pigment betacyanin is also a powerful antioxidant and protects against several types of cancer (Neha et al., 2018). Betacyanins have better antiradical activity in vitro due to their hydroxyl group position than betaxanthin (Azeredo, 2009).

Polyphenols (including phenolic acids, flavonoids, lignans, and stilbenes) are a source of natural antioxidants that can be obtained from medicinal plants. Flavonoids (including flavonols, flavanones, catechins, flavones, anthocyanidins, and isoflavonoids) are found in vegetables and fruits. Flavonoids have also been studied to improve haematological parameters, increase iron levels in the spleen tissue, and ferroportin expression in iron deficiency anaemia (Mazhar et al., 2017). In addition, secondary metabolites such as saponins, phenolics, and glycosides might also be responsible for the acclaimed anti-anaemic potential of plants used in traditional medicine (Gbadamosi et al., 2012).

This research shows that the content of tannins and alkaloids in the beetroot ethanol extract does not affect its activity as anti-anaemia. However, it is crucial to quantitatively measure the presence of tannins and alkaloids in each extract to support definite conclusions regarding their connection to anti-anaemia activity in beetroots.

CONCLUSION

The hydroethanolic extract of beetroot has good anti-anaemia and antioxidant activities. Based on this study, the chemical constituents of the extract plays an important role in its pharmacological activity. The appropriate extraction methods of beetroot can produce an extract as a source of raw materials with maximum chemical content to achieve the intended pharmacological activity.

ACKNOWLEDGMENT

The authors thank to the Lembaga Penelitian dan Pengembangan (Lemlitbang) Universitas Muhammadiyah Prof. DR. HAMKA, Indonesia for internal research fund Batch 1 2019 in the Scientific Basic Research scheme.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

References

[1] Ahmad, A., Ansari, S. H., Ahamad, J., & Naquvi, K. J. (2013). Pharmacognostic specifications of roots of Beta vulgaris cultivated in India. *Asian Journal of Biomedical and Pharmaceutical Sciences*, *3*(26), 5–10.

[2] Azeredo, H. M. C. (2009). Betalains: properties, sources, applications, and stability - a review. *International Journal of Food Science and Technology*, *44*(12), 2365–2376. https://doi.org/10.1111/j.1365-2621.2007.01668.x

[3] Benouadah, Z., Mahdeb, N., & Bouzidi, A. (2016). Evaluation of acute and sub-acute toxicity of alkaloids from Datura stramonium sp. In mice. *International Journal of Pharmacognosy and Phytochemical Research*, *8*(11), 1759–1766.

[4] Chaddha, S., & Mittal, A. (2016). Role of Flavonoids in the Treatment of Hemolytic Anaemia-a review. *European Journal of Pharmaceutical and Medical Research*, *3*(5), 212–216. Retrieved from www.ejpmr.com

[5] Chang, C.-C., Yang, M.-H., Wen, H.-M., & Chern, J.-C. (2002). Estimation of Total Flavonoid Content in Propolis by Two Complementary Colorimetric Methods. *Journal of Food and Drug Analysis*, *10*(3), 178–182.

[6] Clifford, T., Howatson, G., West, D. J., & Stevenson, E. J. (2015). The Potential Benefits of Red Beetroot Supplementation in Health and Disease. *Nutrients*, *7*, 2801–2822. https://doi.org/10.3390/nu7042801

[7] de Oliveira, S. P. A., do Nascimento, H. M. A., Sampaio, K. B., & de Souza, E. L. (2020). A review on bioactive compounds of beet (Beta vulgaris L. subsp. vulgaris) with special emphasis on their beneficial effects on gut microbiota and gastrointestinal health. *Critical Reviews in Food Science and Nutrition*, 1–12. https://doi.org/10.1080/10408398.2020.1768510

[8] Eloff, J. N. (1998). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology*, *60*(1), 1–8. https://doi.org/10.1016/S0378-8741(97)00123-2

[9] Evans, W. C. (2009). *Trease and Evans Pharmacognosy* (16th ed.). Toronto: Elsevier.

[10] Gbadamosi, I. T., Moody, J. O., & Yekini, A. O. (2012). Nutritional Composition of Ten Ethnobotanicals Used for the Treatment of Anaemia in Southwest Nigeria. *European Journal of Medicinal Plants*, *2*(2), 140–150. https://doi.org/10.9734/ejmp/2012/1025

[11] Hadipour, E., Taleghani, A., Tayarani-Najaran, N., & Tayarani-Najaran, Z. (2020). Biological effects of red beetroot and betalains: A review. *Phytotherapy Research*, *34*(8), 1847–1867. https://doi.org/10.1002/ptr.6653

[12] Halwani, A. F., Sindi, H. A., & Jambi, H. A. (2018). Characterization of physical properties of red beet pigments. *Journal of Biochemical Technology*, *9*(2), 16–20.

[13] Hanani, E. (2015). *Analisis Fitokimia (In Bahasa)*. Jakarta: Buku Kedokteran EGC.

[14] Harborne, J. B. (1987). *Metode Fitokimia: Penentuan Cara Modern Menganalisis Tumbuhan (In Bahasa)* (K. Padmawinata & I. Soediro, Eds.). Bandung: ITB Press.

[15] Heinrich, M., Barnes, J., Gibbons, S., & Williamson, E. M. (2012). *Fundamentals of Pharmacognosy and Phytotherapy* (2nd Ed.). London: Elsevier.

[16] Houghton, P. J., & Raman, A. (1998). *Laboratory Handbook for the Fractionation of Natural Extracts*. London: Springer Sciences Bussines Media.

[17] Huaman-Castilla, N. L., Martínez-Cifuentes, M., Camilo, C., Pedreschi, F., Mariotti-Celis, M., & Pérez-Correa, J. R. (2019). The impact of temperature and ethanol concentration on the global recovery of specific polyphenols in an integrated HPLE/RP process on Carménère pomace extracts. *Molecules*, *24*(17), 1–16. https://doi.org/10.3390/molecules24173145

[18] Jaiswal, A., Ganeshpurkar, A., Awasthi, A., Bansal, D., & Dubey, N. (2014). Protective effects of beetroot extract against phenyl hydrazine induced anemia in rats. *Pharmacognosy Journal*, *6*(5), 1–4. https://doi.org/10.5530/pj.2014.5.1

[19] Katritzky, A. R., Fara, D. C., Yang, H., Tämm, K., Tamm, T., & Karelson, M. (2004). Quantitative Measures of Solvent Polarity. *Chemical Reviews*, *104*(1), 175–198. https://doi.org/10.1021/cr020750m

[20] Khoddami, A., Wilkes, M. A., & Roberts, T. H. (2013). Techniques for analysis of plant phenolic compounds. *Molecules*, *18*(2), 2328–2375.

[21] Lesjak, M., & Srai, S. K. S. (2019). Role of dietary flavonoids in iron homeostasis. *Pharmaceuticals*, *12*(119), 1–21. https://doi.org/10.3390/ph12030119

[22] Mazhar, M., Faizi, S., Gul, A., Kabir, N., & Simjee, S. U. (2017). Effects of naturally occurring flavonoids on ferroportin expression in the spleen in iron deficiency anemia: In vivo. *RSC Advances*, *7*(38), 23238–23245.

[23] Ministry of Health Republic of Indonesia. (2008). *Farmakope Herbal Indonesia (Indonesian Herb Pharmacopoeia)* (1st Ed.). Jakarta: Ministry of Health Republic of Indonesia.

[24] Neha, P., Jain, S. K., Jain, N. K., Jain, H. K., & Mittal, H. K. (2018). Chemical and functional properties of Beetroot (Beta vulgaris L.) for product development: A review. *International Journal of Chemical Studies*, *6*(3), 3190–3194.

[25] Nijveldt, R. J., van Nood, E., van Hoorn, D. E. C., Boelens, P. G., van Norren, K., & van Leeuwen, P. A. M. (2001). Flavonoids: A review of probable mechanisms of action and potential applications. *The American Journal of Clinical Nutrition*, *74*, 418–425.

[26] Ninfali, P., Antonini, E., Frati, A., & Scarpa, E. (2017). C-Glycosyl Flavonoids from Beta vulgaris Cicla and Betalains from Beta vulgaris rubra: Antioxidant, Anticancer and Antiinflammatory Activities-A Review. *Phytotherapy Research*, *31*(6), 871–884. https://doi.org/10.1002/ptr.5819

[27] Odoh, U. E., & Okoro, E. C. (2013). Quantitative phytochemical, proximate/nutritive composition analysis of beta vulgaris linnaeus (chenopodiaceae). *International Jpurnal of Current Research*, *5*(12), 3723–3728.

[28] Ologundudu, A., Ologundudu, A. O., Ololade, I. A., & Obi, F. O. (2009). The effect of hibiscus anthocyanins on 2, 4-dinitrophenylhydrazine-induced hepatotoxicity in rabbits. *African Journal of Biochemistry Research*, *3*(4), 140–144.

[29] Saeker, S. D., Latif, Z., & Gray, A. L. (Eds.). (2006). *Methods in Biotechnology: Natural Products Isolation* (2nd ed.). Totowa, New Jersey: Humana Press.

[30] Santos-Buelga, C., & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture*, *80*, 1094–1117. https://doi.org/https://doi.org/10.1002/(SICI)1097-0010(20000515)80:7<1094::AID-JSFA569>3.0.CO;2-1

[31] Shah, T., Warsi, J., & Laghari, Z. (2020). Tea drinking and its co-occurrence with anemia in pregnant females. *Rawal Medical Journal*, *45*(1), 163–167.

[32] Singh et al. (2014). Molecular Mechanism of Phenylhydrazine Induced Haematotoxicity : A Review. *Molecular Mechanism of Phenylhydrazine Induced Haematotoxicity : A Review*, *2*(3), 390–394.

[33] Snyder, L. R. (1974). Classification of the solvent properties of common liquids. *Journal of Chromatography*, *92*, 223–230.

[34] Tiwari, P., Kumar, B., Kaur, M., Kaur, G., & Kaur, H. (2011). Phytochemical screening and Extraction: A Review. *Internationale Pharmaceutica Sciencia*, *1*(1), 98–106.

[35] Wakeel, A., Jan, S. A., Ullah, I., Shinwari, Z. K., & Xu, M. (2019). Solvent polarity mediates phytochemical yield and antioxidant capacity of Isatis tinctoria. *PeerJ*, *7*(e7857), 1–19. https://doi.org/10.7717/peerj.7857

[36] Widiyanti, P., Prajogo, B., & Hikmawanti, N. P. E. (2016). Cytotoxicity of Justicia Gendarussa Burm F. Leaf Extracts on Molt-4 Cell. *Indonesian Journal of Tropical and Infectious Disease*, *6*(1), 24–28.

[37] Wink, M. (Ed.). (2010). *Biochemistry of Plant Secondary Metabolism* (2nd Ed.). New Delhi, India: Wiley-Black Well.

[38] World Health Organization. (2011). *Quality control methods for herbal materials*. Retrieved from https://apps.who.int/iris/handle/10665/44479

[39] World Health Organization. (2020). Anaemia. Retrieved from Anaemia website: https://www.who.int/health-topics/anaemia#tab=tab_1

sciendo

Eur. Pharm. J. 2021, 68(2), 8-15.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2020-0016

EUROPEAN PHARMACEUTICAL JOURNAL

Non-Absorbable Oral Gentamicin Sulphate: Biopharmaceutical and Dosage Form Evaluation

Original Paper

Nwakile Dozie C.[1], Dozie-Nwakile O.C.[2], Okoye E.I.[1], Umeyor C.E.[1], Uronnachi E.C.[1], Uchendu I.K.[2]✉, Attama A.A.[2], Okore V.C.[2]

[1]*Nnamdi Azikiwe University, Awka, Nigeria*
[2]*University of Nigeria, Enugu, Nigeria*

Received 20 July, 2020, accepted 7 February, 2021

Abstract Gentamicin sulphate is an antibiotic belonging to the aminoglycosides and to class III of the Biopharmaceutical Classification System (BCS). Gentamicin sulphate is highly water soluble, but has very low intestinal permeability. The wide use is because of its broad spectrum of activity. In the current study, the suitability of administering gentamicin sulphate orally for local action against susceptible gastrointestinal tract (GIT) bacteria was investigated. The possibility of the drug escaping into the systemic circulation even in the presence of some permeation enhancers was ascertained. Representatives of potential GIT bacteria pathogens were evaluated for their susceptibility to the drug at concentrations obtainable in the GIT using standard microbiological methods. Dose levels that will inhibit these potential bacteria pathogens were also established, as well as the frequency of their administration. Different batches of oral capsules of 250 mg gentamicin sulphate were formulated and their release profiles ascertained using standard methods. The results showed that the selected representatives of the GIT potential pathogenic bacteria were all susceptible to gentamicin sulphate. The drug at its plausible dosage levels of 14.28 mg/kg (1,000 mg/70 kg), 10.71 mg/kg (750 mg/70 kg) and 7.14 mg/kg (500 mg/70 kg) did not cross the mucosal barrier into the systemic circulation even in the presence of some permeation enhancers. The drug's frequency of administration were found to be on 8-hourly bases. Gentamicin sulphate (250 mg) granules formulated with polyethylene glycol (PEG 4000) as granulating aid were quick drying because the granules were not hygroscopic. The formulated gentamicin sulphate capsule batch released enough concentration of the drug that inhibits the test organism within 2 min of dissolution. The above stated doses are acceptable in the dosage form design; it is possible to formulate non-absorbable oral gentamicin sulphate dosage form for local activity in the GIT using existing conventional solid dosage formulating equipment.

Keywords *Oral dosage form – gentamicin sulphate – enterobacteria – permeation enhancers – mucosal barrier – systemic circulation*

INTRODUCTION

Gentamicin sulphate is an antibiotic belonging to the aminoglycosides and to class III of the Biopharmaceutical Classification System (BCS) (Pignatello *et al*., 2016; Baasov *et al*., 2015; Ito *et al*., 2005). Gentamicin sulphate is highly water soluble, but has very low intestinal permeability. The drug has been used extensively through parenteral administration in the treatment of wide range of susceptible bacterial infections. The wide use is because of its broad spectrum of activity. Conventionally, gentamicin sulphate is administered parentally (Imamura and Adams, 2003; Labiris *et al*., 1999). The drug has also been presented for dermal topical applications (Imamura and Adams, 2003; Ipsen *et al*., 1991; Nnamani *et al*., 2013; Pick*et al*., 1997; Ravis *et al*., 2013) and as inhalations (Labiris *et al*., 1999; Aquino *et al*., 2012; Goldman *et al*., 1990; Lim *et al*., 2002). It has been evaluated for rectal local activity (Fix *et al*., 1983) as well as implants (Krasko *et al*., 2007; Soriano *et al*., 2000).

As noted earlier, the low intestinal permeability makes oral administration, hence formulation, very difficult. However, in veterinary practice, oral gentamicin sulphate has been recommended in weaning swine for the control and treatment of colibacillosis caused by strains of E. coli sensitive to gentamicin, and for the control and treatment of swine dysentery associated with Treponema hyodysenteriae (Legal Information Institute, 2016). It has also been evaluated in dogs in combination with labrasol to enhance its intestinal mucosa permeability (Rama Prasad *et al*, 2003).

* *E-mail: ikenna.uchendu@unn.edu.ng*

In humans, oral administration of gentamicin sulphate has been included in the treatment trial of necrotizing enterocolitis, a condition of death of tissue in the intestine, often occurring mostly in premature or sick babies (Gephart *et al*, 2012; Shah and Sinn, 2012). This is a form of localised drug action in which the gentamicin sulphate acts on the diseased tissues. The widespread use of gentamicin sulphate through the oral route in humans to achieve systemic therapeutic concentration has been limited by low intestinal permeability. Early researches have centred on the incorporation of permeation enhancers to achieve intestinal absorption and systemic circulation. Verma *et al* (2014) in a review enumerated some of these permeation enhancers. Attempts have been made to formulate the drug for oral administration by incorporating some of these intestinal mucosa penetration/permeation enhancers to achieve systemic circulation (Anilkumar *et al*., 2011; Rama Prasad *et al*., 2003; Shaikh *et al*., 2012). Umeyor *et al.* (2016) have achieved improved systemic circulation of orally administered gentamicin sulphate by surface modified self-nano-emulsifying formulations (SNEFs). However, the drug has not been formulated into dosage form(s) for local activity in the gastrointestinal tract (GIT). An unaided formulation of the drug administered orally will invariably accumulate in the gut, and the drugs' broad spectrum antibacterial activity could be used for local action against bacteria pathogens inhabiting the GIT. Representative enterobacteria, including, but not limited to, *E. coli, S. typhi*, *K. pneumonia, S. aureus* etc., have been known to cause diseases in the GIT (Walker *et al*., 2014).

Gentamicin sulphate powder is well known for its hygroscopic nature. Presenting the drug powder into granules is hindered by atmospheric moisture absorption. Due to the drug powder's hygroscopic nature, wet granulation and direct compression into tablet/capsule dosage forms become impossible. Preliminary investigation had shown that it is possible to use polyethylene glycol 4000 (PEG 4000) as a granulating aid to hold the powder particles together.

In this research, therefore, gentamicin sulphate was evaluated for suitability in the treatment of GIT localised susceptible bacterial infections. The gentamicin sulphate powder was granulated using PEG 4000 as the granulating aid. The granules were formed into different batches of capsules of 250 mg and the release profile of the formed capsules ascertained.

MATERIALS AND METHODS

Selection of susceptible bacteria for the *in vitro* permeability study

The bacteria used were obtained from cultures maintained at the Medical Laboratory Department of University of Nigeria Teaching Hospital (UNTH) Ituku-Ozala, Enugu State, Nigeria. Twenty (20) ml of molten Mueller–Hinton agar was poured into sterile Petri dishes, and standardised concentrations (McFarland 0.5) of overnight cultures of the test isolates were inoculated aseptically on the agar plates. The mixtures were gently rocked and allowed to solidify. Holes of diameter 5 mm were bored at the centre of the agar plates using a sterile metal cork-borer. A 20 µl of 4 µg/ml gentamicin sulphate reconstituted using rat sera and sterile water in the ratio of 1:1 was poured in each hole under aseptic condition. The plates were kept at tropical room temperature (27°C) for 1 h to allow for diffusion. The plates were then incubated at 37°C for 24 h, and the inhibition zones diameters (IZD) measured. The sample that showed the highest IZD (*S. aureus*), as shown in Table 1, was chosen for the *in vitro* permeability study.

Table 1. Formula for the production of different batches of gentamicin sulphate oral capsules.

		Quantity (mg)	
Ingredients	**Batch A**	**Batch B**	**Batch C**
Gentamicin sulphate	250	250	250
Carb-o-sil®	15	15	15
N-starch	30	30	30
Ac-di-sol®	5	5	5
PEG 4000	50	---	---
PVP	---	50	---
Gelatine powder	---	---	50

(--- no excipient)

Establishment of the baseline gentamicin sulphate dose level

Albino rats (Wister strain) weighing 150–250 g were randomly selected and used for the experiment. Doses of gentamicin sulphate as indicated in Table 2 were administered orally to the rats, and blood collected from the rats through the retro-orbital venous plexus at intervals of 0, 30, 60, 120 and 180 min. The blood was centrifuged, and sera collected from each time interval was introduced into holes bored on solidified 20 ml of molten Mueller–Hinton agar seeded with the *S. aureus*. The plates were allowed to stand for 1 h for sera diffusion and later incubated at 37°C for 24 h. The highest drug level that did not show activity was selected for combination with the permeation enhancers.

Table 2. Inhibition zone diameters (IZDs) of some bacteria for selection for the in vitro permeability study.

		Time (h)		
	0.5	**1**	**2**	**3**
Organism		IZD (mm)		
B. subtilis	13.3±0.6	7.0±0.0	10.0±0.0	3.6±0.6
K. pneumonia	8.0±0.0	5.0±0.0	2.0±0.0	0.0±0.0
*S. aureus**	20.0±0.0	14.0±0.0	15.7±0.6	4.0±0.0
E. coli	6.0±0.0	4.0±0.0	3.3±0.6	0.0±0.0
S. typhi	8.0±0.0	3.0±0.0	3.0±0.0	0.0±0.0

**significant at $p < 0.05$*

Table 3. Baseline gentamicin sulphate dose level using inhibition zone diameter (IZDs) (mm) produced by serum drug level of oral gentamicin sulphate against Staphylococcus aureus.

		Dose (mg/kg)	
Time (h)	**400**	**500**	**1,000**
0.0	0.0	0.0	0.0
0.5	0.0	7.3±0.6	10.3±0.6
1.0	0.0	8.7±1.2	11.3±0.6
2.0	0.0	8.2±1.2	12.3±0.6
3.0	0.0	7.3±0.6	13.0±0.0

Evaluation of permeability enhancement using the bioassay method

Albino rats (Wister strain) weighing 150–250 g were randomly selected and used for the experiment. Gentamicin sulphate alone was administered orally at 400 and 500 mg/kg. These are, respectively, the maximum concentration that cannot appear in the blood and the minimum concentration that can appear in the blood after oral administration of the drug (Table 2). Blood samples were collected from the rats through the retro-orbital venous plexus at 0, 30, 60, 120 and 180 min. The blood was allowed to coagulate, and sera collected from each sample were introduced into holes bored on solidified 20 ml of molten Mueller–Hinton agar seeded with the *S. aureus*. The plates were incubated at 37°C for 24 h after diffusion and the IZDs measured. More so, the solution of the drug was constituted using distilled water and thoroughly mixed with the respective quantities of the permeation enhancers as shown in the Table 3. Dose levels of gentamicin sulphate, 400 and 500 mg/kg, admixed with each permeation enhancer respectively were given orally to the rats. Blood sample was withdrawn and treated as above. The respective IZDs were measured planimetrically.

In vitro assessment of orally administered gentamicin sulphate against selected enterobacteria

Some pathogenic bacteria known to inhabit the GIT were obtained from cultures maintained at the Medical Laboratory Department of UNTH Ituku-Ozala, Enugu State, Nigeria, and used for this study. The selected bacteria include *E. coli*, *S. typhi*, *K. Pneumoniae* and *S. aureus*. White albino rats (Wister strains) weighing between 155 and 300 g were used for the study. Gentamicin sulphate dissolved in distilled water was administered at the following dose levels: 500, 14.28, 10.71, 7.14 and 3.57 mg/kg to the corresponding group of two rats per group. The number of rats was deliberately made small, to enable utilisation of the entire droppings produced by the group in a particular time interval. All the rat droppings were collected at 0-, 1-, 2-, 4- and 6-h interval. A 10% mixture of the dropping was made in distilled water and centrifuged after agitation for about 10 min. Centrifugation was done at 4,000 rpm for 10 min. A 20 µl of the decant was introduced into a 5 mm hole bored in a solidified Mueller–Hinton agar seeded with the respective enterobacterium and incubated for 24 h at 37°C. Zones of inhibition were measured planimetrically.

Assessment of duration of activity of orally administered gentamicin sulphate

The selected enterobacteria were used for this study. White albino rats (Wister strain) weighing 150–250 g were randomly selected for the study. Two rats were given gentamicin sulphate orally at 7.1 mg/kg (500 mg/70 kg). The droppings were collected at 0-, 4-, 6-, 8-, 10-, 12-, 14-, 16- and 18-h interval. A 10% mixture of the droppings was constituted with water and agitated mechanically for 10 min. The mixtures were later centrifuged for 10 min at 4,000 rpm. A 20 µl of the decant was introduced into a 5 mm hole bored in a solidified Mueller–Hinton agar seeded with respective enterobacterium and incubated for 24 h at 37°C. Zones of inhibition were measured as before.

Table 4. Inhibition zone diameter (IZDs) (mm) produced by serum drug level of orally co-administered gentamicin sulphate with permeation enhancers against Staphylococcus aureus.

			IZD	(mm)		
Drug/P. enhancera	**((mg/kg)/%)**	**0 h**	**0.5 h**	**1 h**	**2 h**	**3 h**
Drug	400 500	0.0±0.0 0.0±0.0	0.0±0.0 0.0±0.0	0.0±0.0 8.0±0.0	0.0±0.0 8.7±0.6	0.0±0.0 8.0±0.0
Drug/PEG 4000	400/30 500/30	0.0±0.0 0.0±0.0	0.0±0.0 6.7±0.6	0.0±0.0 7.6±0.6	0.0±0.0 6.7±0.6	0.0±0.0 6.7±0.6
Drug/Tween® 80	400/15 500/15	0.0±0.0 0.0±0.0	0.0±0.0 6.7±0.6	0.0±0.0 6.7±0.6	0.0±0.0 6.7±0.6	0.0±0.0 7.0±1.0
Drug/Kolliphor® 188	400/1.0 500/1.0	0.0±0.0 0.0±0.0	0.0±0.0 8.7±0.6	0.0±0.0 8.7±0.6	0.0±0.0 9.0±0.0	0.0±0.0 9.0±0.0
Drug/Glycerine	400/1.5 500/1.5	0.0±0.0 0.0±0.0	0.0±0.0 7.7±0.6	0.0±0.0 8.3±0.6	0.0±0.0 8.0±0.0	0.0±0.0 7.0±1.0
Drug/SLS	400/2 500/2	0.0±0.0 0.0±0.0	0.0±0.0 0.0±0.0	0.0±0.0 9.0±0.0	0.0±0.0 1 0.0±0.0	0.0±0.0 9.3±0.6
Drug/Citric acid	400/2 500/2	0.0±0.0 0.0±0.0	0.0±0.0 9.0±0.0	0.0±0.0 8.7±0.6	0.0±0.0 8.3±0.6	0.0±0.0 8.3±0.6
Drug/Oleic acid	400/2 500/2	0.0±0.0 0.0±0.0	0.0±0.0 7.7±0.6	0.0±0.0 8.0±0.0	0.0±0.0 7.3±0.6	0.0±0.0 6.3±0.6
[a]P. enhancer =	Permeation	enhancer,	SLS =	Sodium	lauryl	sulphate

Formulation of gentamicin sulphate capsules

Granules of gentamicin sulphate were formed using the formula shown in Table 4. PEG 4000, polyvinyl pyrrolidone (PVP) and gelatine were assessed as granulating aid. PEG 4000 was weighed out and poured into a beaker and melted on a hot plate. The diluent, (N-modified starch, a hydrophobic starch obtained from modifying maize starch with sodium hypochlorite (Nwakile, 2017), desiccant (Carb-o-sil®) and the disintegrant (Ac-di-sol®) were weighed out and mixed. The excipients were dispersed into the melted PEG 4000 and thoroughly mixed. The beaker was removed from the hot plate, and the gentamicin sulphate powder was introduced. The mixture was thoroughly mixed and allowed to cool. It was then granulated to the 0.8 mm sizes. A similar procedure was employed for the batch containing PVP. However, gelatine (3% w/w) was used to prepare batch C. The moisture absorbed by the gentamicin sulphate serves to moisten the mixture and enables lump formation for batch C. Batch B failed to give good granules, as the PVP failed to hold the particles together. Batch C, however, failed to give dry granules because of the presence of absorbed moisture continued to increase and weaken drying. Batch A, which gave quick drying granules, was packaged into gelatine capsule shells (size 00) to desired weight through manual filling.

Evaluation of gentamicin sulphate capsules

Dissolution of batch A-formulated capsules was done using BP basket method. Normal saline was used as the dissolution medium. About 300 ml of the medium was introduced into a 1,000 ml beaker mounted on a hot plate magnetic stirrer and maintained at a temperature of 37±0.5°C and speed of 100 rpm. The capsule contained in a stainless basket was suspended into the medium using a retort stand. The dissolution was allowed to run for 60 min and 2 ml of the sink collected at a 5-min interval with replacement using fresh medium. The collected sink was used to inoculate the *S. aureus* seeded Mueller–Hinton agar whose IZDs were measured after 24 h.

A calibration curve was established using a solution of gentamicin sulphate with the following concentrations: 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml. The organism used was *S. aureus*. Twenty (20) ml of molten Mueller–Hinton agar was poured into sterile Petri dishes, and standardised concentrations (McFarland 0.5) of overnight culture of the selected organism (*S. aureus)* introduced aseptically on the agar plates. The mixtures were gently rocked and allowed to solidify. Holes of 5 mm diameter were made in the agar plates using a sterile metal cork-borer. A 20 µl of the various concentrations of the drug were put in each hole under aseptic condition and kept at room temperature for 1 h to allow for diffusion. The plates were then incubated at 37°C for 24 h, and the IZDs measured and recorded. The size of the cork borer (5 mm) was deducted from the values recorded for the IZDs to get the actual diameter. This procedure was conducted in triplicate, and the mean IZDs calculated and recorded. Thereafter, the IZDs were plotted against the logarithm of the concentrations. Actual concentrations of drug in the serum were obtained by superimposing the IZD of the serum on

Table 5: Average inhibition zone diameter (IZD) (mm) of gentamicin sulphate after 6 h of oral administration at different concentrations.

			Dose (mg/kg)		
Microorganism	500	14.28	10.71	7.14	3.57
E. coli	12.0±0.8	6.3±0.5	6.0±0.0	4.3±0.5	0.0±0.0
S. typhi	13.3±0.5	6.0±0.0	6.3±0.5	4.0±0.0	0.0±0.0
K. pneumonia	15.0±0.8	8.0±0.8	10.0±0.0	8.3±0.5	0.0±0.0
S. aureus	14.3±0.5	10.0±0.8	7.0±0.8	6.3±0.5	0.0±0.0

the IZD axis and extrapolating to the log concentration. The concentration was then obtained by taking the antilogarithm of the log concentration.

Data collection and statistical analyses

The collected data were analysed statistically using mean, standard deviation and chi-square with $p<0.05$ where appropriate.

RESULTS AND DISCUSSIONS

For a possible GIT local active dosage form, there is need to ensure that the drug is not absorbed appreciably from the GIT into the systemic circulation even when administered with potential permeation enhancers. To show that gentamicin sulphate does not get into the systemic circulation when administered PO, a sensitive bacterium was selected and used as a biomarker for the drug permeation study. Various bacteria species were incubated with 4.0 µg/ml of gentamicin sulphate. This represents the highest serum drug level after parenteral administration (CPACPS, 2002). The most sensitive bacterium being *Staphylococcus aureus*, as shown in Table 2, was therefore selected for the permeation studies.

It can be seen from Table 2 that all the bacteria were sensitive to gentamicin sulphate from 30 min after administration to 2 h after administration. Only *B. subtilis* and *S. aureus* were sensitive at the third hour. The *S. Aureus* had the highest sensitivity for all the period under review with IZD of 20, 14, 15.7 and 4 mm corresponded to the periods of 0.5, 1, 2 and 3 h, respectively.

It has been shown that gentamicin sulphate given to rats orally could appear in the blood of the rats if the administered dose is up to 10,000 mg/kg (EMEA, 2001). Hence, to ascertain the minimum concentration of gentamicin sulphate that can appear in the blood of rat after oral administration, gentamicin sulphate was administered orally to the rats at doses from 40 mg/kg and up to 1,000 mg/kg, as shown in Table 3. The oral administration was started at 40 mg/kg because this dose was preliminarily given parenterally (im) and the serum concentration after 1 h inhibited the test bacterium (*S aureus*). The 1,000 mg/kg corresponds to one-tenth of the orally administered dose elsewhere (EMEA, 2001). The serum collected at specified time interval after oral administration of various doses of the drug, as shown in Table 3, was used to inoculate *S. aureus*-seeded Mueller–Hinton agar plates. From Table 3, it can be seen that doses of 400 mg/kg gentamicin sulphate and below did not get into the blood at concentrations that inhibit bacteria growth *in vitro*. However, from gentamicin sulphate concentrations of 500 mg/kg and above, the drug was absorbed into the blood because the sera have concentrations that inhibited bacteria growth *in vitro* (Table 3).

To rule out the possibility of some permeation enhancers, at their pharmaceutically recommended usage levels aiding the drug at 400 mg/kg (and below) to cross the mucosal barriers, the drug was administered to the rats at the dose level of 400 mg/kg (using 500mg/kg as control) and in the presence of the permeation enhancers (Table 4) (Kibbe, 2000). It is quite evident from Table 4 that gentamicin sulphate concentrations of 400 mg/kg and below given orally could not permeate into the systemic circulation even in the presence of the permeation enhancers. However, at the dose of 500 mg/kg and above, the sera from rats inhibited the bacteria growth *in vitro*. This cannot be attributed to the permeation enhancers because similar values of IZDs were obtained in their absence (Table 4).

However formulating the drug at this dose level of 500 mg/kg, which permeates into the blood system, will correspond to giving a 70 kg body weight adult 35 g/dose as tablet of the drug; an outrageous dose. This of course indicates the suitability of the drug being evaluated as a potential drug for GIT local activity. Such evaluation was subsequently done by determining the dose of the drug. The doses of the drug were determined by administering gentamicin sulphate, as shown in Table 5. The filtrate collected from a 10% suspension of rat's droppings inhibited all the test bacteria at the following doses:14.28 mg/kg (1,000 mg/70 kg), 10.71 mg/kg (750 mg/70 kg) and 7.14 mg/kg (500 mg/70 kg) body weights. There was no activity on any of the bacteria when gentamicin sulphate was given at 3.57mg/kg (250 mg/70 kg) body weight. These doses are very much acceptable and realistic in dosage form design.

The duration of activity of gentamicin sulphate administered orally was thereafter determined by monitoring the administered drug for 18 h, as shown in Table 6. The rats

Table 6. Duration of GIT local activity of orally administered gentamicin sulphate.

Time (h)	*E. coli*	*S. typhi*	*K. pneumonia*	*S. aureus*
0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
4	2.5±0.7	0.0±0.0	0.0±0.0	2.0±0.0
6	3.5±0.7	4.0±0.0	2.0±0.0	6.0±0.0
8[b]	6.0±0.0	4.0±0.0	4.0±0.0	8.0±0.0
10	3.5±0.7	0.0±0.0	0.0±0.0	4.0±0.0
12	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
14	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
16	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
18	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

[b]*All organisms were inhibited at this hour*

were given the drug at 7.14 mg/kg (500 mg/70 kg), the lowest dose that produces activity *in vitro*. The filtrate from a 10% suspension of rat droppings inhibited the growth of all the bacteria up to the 8th hour (Table 6). For all the bacteria tested, inhibition peaked at the 8th hour. This implies that the drug should be administered orally on 8-hourly intervals. This frequency of administration will maintain sufficient drug concentration level in the GIT to limit the proliferation of bacteria. However, doses of up to 14.28 mg/kg (1,000 mg/70 kg) and 10.71 mg/kg (750 mg/70 kg), as obtained in Table 5, could be administered. It is very important to note that gentamicin sulphate given orally does not cause lesion on the GIT mucosa at these acceptable doses of 14.28 mg/kg (1,000 mg/70 kg), 10.71 mg/kg (750 mg/70 kg) and 7.14 mg/kg (500 mg/70 kg). Such mucosal damage could not be possible because higher doses of 10,000 mg/kg body weight have been administered without such damage (EMEA, 2001). Hence, important fundamental biopharmaceutical and formulation issues that will enable ushering gentamicin sulphate into usage as oral tablets and solutions for the treatment of GIT localised infections that ravage the greater population of the world are possibly addressed.

Pure gentamicin sulphate powder is known for its hygroscopic character. The pure powder absorbs moisture from the environment and becomes sticky, making the powder difficult to granulate. This presents serious formulation challenges. To overcome this challenge requires the incorporation of excipients that will retard or discourage the absorption of moisture from the atmosphere (Table 1). To this end, N-modified maize starch possesses such attribute judging from its hydrophobic nature (Nwakile, 2017). This attribute of the N-modified maize starch is expected to help limit the atmospheric moisture-absorbing ability of gentamicin sulphate granules formulated with it. To limit the atmospheric moisture-absorption potential of the gentamicin sulphate powders, some other additive like silicone dioxide (Carb-o-sil®) was also included. Carb-o-sil® is a well-known desiccant approved for pharmaceutical use. Other ingredients included

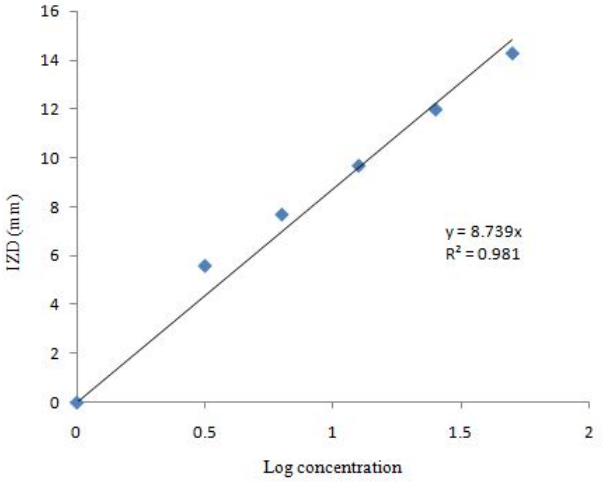

Figure 1. Calibration curve of Gentamicin Sulphate using S. aureus,

in the formulation were Ac-di-sol®. PEG 4000 was employed as the granulating aid because it performs better than PVP and gelatine in preliminary studies. Based on the administrable doses discussed earlier, 250 mg gentamicin sulphate granules were formed and manually packaged into capsules to a total weight of 350 mg of the gentamicin sulphate granules. Dissolution sink was tested for the presence of gentamicin sulphate by incubating the sink with *S. aureus*-seeded molten Mueller–Hinton agar. The inhibition zone diameter was used to deduce the concentration using the calibration curve established for the purpose. The calibration curve was established using S. *aureus* and pure gentamicin sulphate powder, as shown in the Figure 1.

As can be seen from Figure 2, the formulated gentamicin sulphate capsule released enough concentration of the drug that inhibits the test microorganism within 2 min of dissolution (Fig 2).

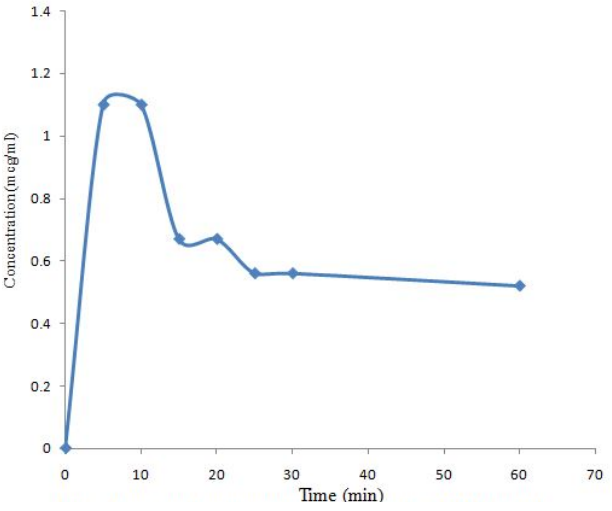

Figure 2. Concentration time curve of gentamicin sulphate capsule in normal saline.

CONCLUSION

Gentamicin sulphate at dose level of 400 mg/kg and below given orally could not get into the systemic circulation even in the presence of commonly available permeation enhancers that include PEG, Tween® 80, kolliphor® 188, glycerine, sodium lauryl sulphate, citric acid and oleic acid. The drug at 14.28 mg/kg (1,000 mg/70 kg), 10.71 mg/kg (750 mg/70kg) and 7.14 mg/kg (500 mg/70 kg) orally administered inhibited some frequently encountered pathogenic enterobacteria. Orally administered gentamicin sulphate was found to maintain effective inhibition concentration in the GIT for 8 h pointing to an 8-hourly administration. The above-stated doses are acceptable in the dosage form design; it is possible to formulate a non-absorbable oral gentamicin sulphate dosage form for local activity in the GIT using existing conventional solid dosage formulating equipment. Such oral dosage(s) will be very important in controlling, in a very simple way, the ever debilitating GIT pathogens without recourse to drastic changes in conventional formulation pattern.

To that effect, gentamicin sulphate 250 mg granules was formulated. The gentamicin sulphate granules, which were quick drying, were packaged into capsules, which released enough concentration of the drug that inhibits the test organism within 2 min of dissolution. Oral dosage forms of gentamicin sulphate will expand the usage of gentamicin sulphate, thereby exploiting the drug's wide spectrum of activity.

FUNDING

This research was not funded by any grant agency or organisation.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENT

Kind supply of gentamicin sulphate powder by Juhel Nigeria Limited is acknowledged.

References

[1] Pignatello, R.; Simerska, P.; Leonardi, A.; Abdelrahim, A.S; Petronio, G.P.; Fuochi, V.; Furneri, P.M.; Ruozi B.; Toth I.Synthesis, characterization and in vitro evaluation of amphiphilic ion pairs of erythromycin and kanamycin antibiotics with liposaccharides. *Eur J Med Chem*., 2016,120,329-337.

[2] Baasov, T.; Atia-Glikin, D.; Kandasamy, J.;Belakhov, V. Technion Research and Development Foundation Ltd, 2015. *Aminoglycosides and uses thereof in treating genetic disorders*. U.S. Patent 9,175,029.

[3] Ito, Y.; Kusawake, T.; Ishida, M.;Tawa, R.; Shibata, N.; Takada, K. Oral solid gentamicin preparation using emulsifier and adsorbent. *J Controlled Release*, 2005,105(1-2),23-31.

[4] Imamura, S.; Adams, J.C. Distribution of gentamicin in the guinea pig inner ear after local or systemic application. *JARO*, 2003, 04, 176-195.

[5] Labiris, N.R.C.; Holbrook, A.M.; Chrystyn, H.;Macleod, S.M.; Newhouse, M.T. Dry powder versus intravenous and nebulized gentamicin in cystic Fibrosis and bronchiectasis; a pilot study. *Am J Respir Crit Care Med* 1999, 160(5), 1711-1716.

[6] Ipsen, T.; Jørgensen, P.S.; Damholt,V.; Tørholm, C. Gentamicin-collagen sponge for local applications:10 cases of chronic osteomyelitis followed for 1 year. *Acta OrthopScand.*,1991, 62(6), 592-594.

[7] Nnamani, P.O.; Kenechukwu, F.C.; Dibua,E.U.; Ogbonna, C.C.; Momoh, M.A.; 1, Olisemeka, A.U.; Obumneme, A.C.; Attama, A.A. Formulation, characterization and *ex-vivo* permeation studies on gentamicin-loaded transdermal patches based on PURASORB® polymers *Sci Res Essays*. 2013, 8(22), 973-982

[8] Pick, J.; Olson, L.L.; Ellis, W.Y.;Lim, P. Development and validation of a method to extract and quantitate paromomycin and gentamicin from an aquaphilic cream formulation. *J Pharm Biomed Anal.*,1997; 16, 131-137.

[9] Ravis, W.R.; Llanos-Cuentas, A.;Sosa,N.Kreishman-Deitrick, M.; Kopydlowski, K.M.; Nielsen, C.; Smith, K.S.; Smith, P.L.; Ransom, J.H.; LinY-J.; Grogl M.Pharmacokinetics and absorption of paromomycin and gentamicin from topical creams used to treat cutaneous leishmaniasis. *Antimicrob. Agents Chemother. 2013,* 57(10), 4809-4815.

[10] Aquino, R.P.; Prota, L.; Auriemma, G.;Santoro, A.; Mencherini, T.; Colombo, G.; Russo, P.Dry powder inhalers of gentamicin and leucine: formulation parameters, aerosol performance and *in vitro* toxicity on CuFi1 cells. *Int J Pharm*., 2012, 426, 100-107.

[11] Goldman, J.M.,Bayston, S.M.;O'Connor,S.,Meigh, R.E.Inhaled micronised gentamicin powder: a new delivery system. *Thorax*., 1990; 45:939-940.

[12] Lim,S.T.; Forbes,B.; Berry,D.J.; Martin, G.P.; Brown, M.B. In vivo evaluation of novel hyaluronan/chitosan microparticulate delivery systems for the nasal delivery of gentamicin in rabbits. *Int J Pharm*. 2002, 231:73-82.

[13] Fix, J.A.; Porter, P.A.; Leppert, P.S.Involvement of active sodium transport in the rectal absorption of gentamicin sulfate in the presence and absence of absorption-promoting adjuvants. *J Pharm Sci*., 1983, 72(6), 698-700.

[14] Krasko, M.Y.; Golenser, J.; Nyska, A.; Nyska, M., Brin, Y.S.; Domb, A.J. Gentamicin extended release from an injectable polymeric implant. *J Control Release*. 2007; 117:90-96.

[15] Soriano, I.; Evora, C. Formulation of calcium phosphates /poly (*d*,*l*-lactide) blends containing gentamicin for bone implantation. *J Controlled Release*, 2000, 68,121-134.

[16] Rama Prasad, Y.V.; Eaimtrakarn, S.; Ishida, M.;Kusawake, Y.; Tawa, R.; Yoshikawa, Y.; Shibata, N.; Takada, K.Evaluation of oral formulations of gentamicin containing labrasol in beagle dogs. *Inter J Pharmaceut*., 2003, 268,13-21.

[17] Gephart, SM., McGrath, JM., Effken, JA. and Halpern, MD. (2012). Necrotizing Enterocolitis Risk. Adv Neonatal Care, 12(2): 77–89. doi: 10.1097/ANC.0b013e31824cee94

[18] Shah D and Sinn JK. (2012). Antibiotic regimens for the empirical treatment of newborn infants with necrotising enterocolitis. Cochrane Database Syst Rev. 15; 8:CD007448. doi: 10.1002/14651858.CD007448.pub2.

[19] Verma, K., Gupta, A.B. and Singh, A., (2014). Optimization of Chlorination Process and Analysis of. *Water Sci*, *4*(3), 231-239.

[20] Anilkumar,P.; Badarinath,A.V.; Naveen, N.;Nirosha, M. A rationalized description on study of intestinal barrier, drug permeability and permeation enhancers. *J Global Trends Pharma Sci*.,2011, 2(4), 431-449.

[21] Shaikh, M.S.; Derle, N.D.; Bhamber,R. Permeability enhancement techniques for poorly permeable drugs: a review.*JAPS*2012,02(06), 34-39.

[22] Umeyor, C.; Attama, A.; Uronnachi, E.; Kenechukwu, F.; N;wakile, C.; Nzekwe, I.; Okoye, E.;Esimone, C.Formulation design and in vitro physicochemical characterization of surface modified self-nanoemulsifying formulations (SNEFs) of gentamicin. *Inter J Pharmaceut* 2016; 497:161-98.

[23] Walker, M.M.; Talley, N.J. Review article: bacteria and pathogenesis of disease in the upper gastrointestinal tract – beyond the era of *Helicobacter pylori*. *Aliment Pharm Ther*.,2014; 39(8), 767-779.

[24] Nwakile, C.D. Production, Characterization and Application of Multiple Modified Maize Starches in the Formulation of Oral Solid Dosage Forms of Gentamicin Sulphate. A thesis submitted to the Department of Pharmaceutics in partial fulfilment for the award of doctor of philosophy (Ph.D) of the University of Nigeria, Nsukka, Enugu State, Nigeria. 2017. Available at: http://dspace.unn.edu.ng/bitstream/handle/123456789/5494/Nwakile%2C%20Calistus%20Dozie.pdf?sequence=1&isAllowed=y, Accessed February 20, 2020.

[25] Canadian Pharmaceutical Association Compendium of Pharmaceuticals and Specialities, the Canadian drug reference for health professionals. Webcom limited, Toronto, 2002.

[26] EMEA. Gentamicin: Summary report (3) - Committee for Veterinary Medicinal Products EMEA/MRL/803/01-final November 2001. Available at: http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-Report/2009/11/WC500014350.pdf.. Accessed March 2, 2020.

[27] Kibbe AH. Hand book of Pharmaceutical excipients, 3rd ed. Washington: American Pharmaceutical Association and Pharmaceutical Press; 2000.

sciendo

Eur. Pharm. J. 2021, 68(2), 16-26.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2021-0015

EUROPEAN PHARMACEUTICAL JOURNAL

Amelioration of the Abnormalities Associated with Metabolic Syndrome by L-Norvaline in Hyperlipidemic Diabetic Rats

Original research article/Review

Dobhal S.[1], Baliyan S.[1], Singh MF.[1]✉, Bisht S.[2], Setya S.[3]

[1]School of Pharmaceutical Sciences, Sardar Bhagwan Singh University, Balawala, Dehradun, PIN-248001, Uttarakhand, India
[2]Babu Banarasi Das University, Lucknow, PIN- 226028, UP, India
[3]SGT College of Pharmacy, SGT University, Gurugram, Haryana, India

Received 3 April, 2021, accepted 1 October, 2021

Abstract The present study was designed to assess the treatment effect of arginase inhibitor, L-Norvaline in abnormalities associated with high fat diet (HFD) and fructose-induced metabolic syndrome. The HFD and fructose was fed to the rats for a period of 45 days. Animals having body weight of 350 g and fasting blood sugar level of more than 250 mg/dl were considered as hyperlipidemic diabetic rats (HDR) and selected for the study. The HDR were divided into three groups having six animals each. The HDR received L–Norvaline (10 mg/kg/day, i.p.) and standard drug, gemfibrozil (60 mg/kg/day, p.o.), for a period of 30 days. Various hormonal, biochemical and tissue parameters were evaluated at the end of the study. Both treatments significantly decreased body weight, BMI, fasting blood sugar and insulin level and improved insulin resistance in HDR as compared to the toxicant control group. A significant improvement was observed in the lipid profile, levels of nitrate, leptin, C-reactive protein and adiponectin in HDR. L-Norvaline also caused slight decrease in the malondialdehyde level, though, no prominent effect was observed on the level of superoxide dismutase and reduced glutathione in the pancreas of HDR, as compared to the toxicant control group. L-Norvaline treatment also improved the histo-architecture of pancreatic cells. Results of the present study concludes that L-Norvaline caused significant alleviation of the abnormalities of MetS indicating that it can be used as potential treatment strategy for managing the symptoms of metabolic syndrome.

Keywords *Gemfibrozil – Insulin resistance – L-Norvaline – Metabolic syndrome – Obesity*

INTRODUCTION

Metabolic syndrome (MetS) or insulin resistance syndrome consisting of obesity, hyperglycemia, hyperinsulinemia, dyslipidemia, and hypertension has become a significant public health problem worldwide (Grundy et al., 2005). MetS is an implication of carbohydrate overloaded diet in addition to decreased physical activity as well as genetic predisposition. It is associated with a prolonged low-grade inflammation, decreased insulin sensitivity, abdominal adiposity, atherogenic dyslipidemia, elevated blood pressure and endothelial dysfunction. Although the exact cause of MetS is still not known, but it is considered that a complex interplay among genetic, metabolic and environmental factors are responsible for the development of the disease. The prevalence of MetS ranges approximately 25% in US, 12.8% - 41% in Asian Pacific region and 17.8%-34% in Europe (Azimi Nezhad et al., 2008).

Central obesity has been hypothesized as the leading factor in the etiological cascade for the development of MetS, which results in adipose dysfunction, adipose tissue mass expansion and increased free fatty acid in the circulation, thus, leading to an increased risk of developing insulin resistance, endothelial dysfunction and cardiovascular diseases (Guilherme et al., 2008). In MetS, increased oxidative stress and vascular inflammation leads to the enhanced activity and expression of arginase. This decreases the amount of L-arginine availability as a substrate for eNOS, causing impaired NO production and endothelial dysfunction. Additionally, synthesis of polyamines and proline is induced that results in vascular remodelling and vascular smooth muscle proliferation, leading to cardiovascular complications related to MetS (Masi et al., 2018). Increased oxidative stress and inflammation possibly through activation of protein kinase C (PKC) and RhoA/Rho

* E-mail: mamta_fr2002@yahoo.co.in

kinase, amplifies the expression of arginase, which plays a pivotal role in various complications related to MetS (Rabelo et al., 2015). Therefore, inhibiting the enzyme arginase can produce promising results in alleviating the complications related to MetS. L-Norvaline [5 -(aminoiminomethyl) amino] with molecular formula - $C_5H_{11}NO_2$ is an amino acid, with potent arginase inhibitory activity. L-Norvaline finds great interest as it is a non-selective inhibitor of arginase and therefore is able to suppress the activity of arginase enzyme and raise the endogenous stocks of L-arginine along with the increased production of NO.

The present study thus aims to evaluate the effect of L-Norvaline on MetS associated obesity, hyperlipidemia, type II diabetes as well as metabolic and vascular abnormalities.

MATERIALS AND METHODS

Animals

In the present study, adult male Wistar rats weighing between 225-250 g and 8-10 weeks of age were used. The rats were housed in standard polypropylene cages under controlled room temperature (24±2°C) and humidity (60-70%). Animals were provided with hyperlipidemic diet and water *ad libitum* throughout the study. All the animals were acclimatized to the laboratory conditions for one week before experiment. The guidelines of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Govt. of India was followed for the care of animals. Prior permission was taken from the Institutional Animal Ethical Committee (Reg. Number 273/PO/Re/S/2000/CPCSEA) for conducting animal studies with approval number of CPCSEA/IAEC/SBS/2018/002.

Reagents and Chemicals

L-Norvaline (N 7627) was purchased from Sigma Aldrich (India). Potassium dihydrogen orthophosphate (RM2951), EDTA (RM1279), thiobarbituric acid (RM1594) and reduced glutathione (MB166) were obtained from Himedia Laboratories, Mumbai, India. 5,5′ -Dithiobis (2-nitrobenzoic acid) (GRM1677) and epinephrine (TC469) were purchased from Sigma Chemical Co., St Louis, MO, USA. All other chemicals were of analytical grade. Diagnostic kits of ERBA were used for biochemical estimation.

Induction of Metabolic Syndrome

Obesity and dyslipidemia were induced by feeding the animals with hyperlipidemic diet for a period of 45 days. Diabetes was induced by fructose (20% w/v in distilled water) which was prepared freshly and given as drinking water to all the test groups. After 45 days, body weight and fasting blood sugar (FBS) level of the rats were checked. The animals with body weight more than 350 g and FBS level more than 200 mg/dl were considered as hyperlipidemic diabetic rats (HDR) and selected for the study.

Selection and Preparation of Doses

The doses of L-Norvaline (10 mg/kg, intraperitoneally) and gemfibrozil (60 mg/kg, p.o.) were selected on the basis of previous studies done by De et al. and Panda et al., respectively (De et al., 2016, Panda et al., 2017). Gemfibrozil was administered as a suspension in distilled water using 1% CMC as the suspending agent.

Experimental Design

The Hyperlipidemic diabetic rats were divided into three groups and one group of normal rats was taken. All groups with six animals in each received the following treatment once in a day for a period of 30 days.

Group I (normal control): Normal animals received distilled water (1 ml/kg/day, p.o.).

Group II (toxicant control): Hyperlipidemic diabetic animals received distilled water (1 ml/kg/day, p.o).

Group III: Hyperlipidemic diabetic animals received L-Norvaline (10 mg/kg/day, i. p.).

Group IV: Hyperlipidemic diabetic animals received gemfibrozil (60 mg/kg/day, p.o.) as a reference drug.

Body weight and Body mass index (BMI) was determined weekly from the first day of the study till the end of the study. Fasting blood sugar was recorded at 0, 15th day and 30th day of study. At the end of the treatment period, all the animals were fasted overnight, anaesthetised with ether and blood was collected from the retro-orbital plexus. Serum was separated from the blood and used for the estimation of FBS, lipid profile and nitrate using autoanalyzer (Erba Chem 5X). Insulin, adiponectin, and leptin levels were measured in serum of the animals using ELISA assay kits. After blood withdrawal, animals were sacrificed by euthanasia (phenobarbitone 150 mg/kg, i.p.). Pancreas was excised immediately from all the group of animals and after processing, tissue homogenate was used for the estimation of oxidative stress and antioxidant enzymes.

Assessment of body weight and body mass index (BMI)

Body weight and BMI were estimated at a regular interval of 7 days.

$BMI = Mass\ (kg)/height(m^2)$

$BMI = Mass\ (lbs.)/height(inch^2) \times 703$

Where m and h are the subject's weight and height respectively.

Estimation of fasting blood sugar level

Fasting blood sugar level was estimated by GOD-POD method from the serum, after induction of diabetes and then at 0, 15th and 30th days of the treatment(Trinder, 1969).

Estimation of lipid profile

Total cholesterol, HDL and triglyceride levels were determined in serum by the CHOD-PAP method (Castelli et al., 1977), phosphotungstic acid method (Miller et al., 1977) and GPO-Trinder method (Bucolo & David, 1973) respectively using Erba diagnostic kits (Mumbai, India).
LDL = Total serum cholesterol- VLDL-HDL
VLDL and LDL were calculated as per Friedewald's equation as follow:

VLDL = Total serum triglycerides/5

The atherogenic index (AI) was calculated by using the formula:

$AI = \log_{10} (TG/HDL)$

Nitrate assay

Nitrate level was estimated in serum using Griess Reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% napthylethylenediamine dihydrochloride in a ratio of 1:1) (Guerra et al., 2005).

Insulin Assay and Insulin Resistance

Estimation of insulin was carried out in the serum by the method of MacDonald 1989 using an ELISA kits of Cell Biolabs, Inc., USA(MacDonald & Gapinski, 1989). Insulin resistance was calculated by the method of HOMA-IR (Homeostasis Model Assessment of insulin resistance)(Matthews et al., 1985) using the following formula:

HOMA-IR= (fasting insulin (μlU/mL) × fasting glucose (mmol/L))/22.5

Measurement of Adiponectin

Serum adiponectin level was estimated by the method of sandwich ELISA assay using adiponectin ELISA kits supplied by Cell Biolabs, Inc., USA(Arita et al., 1999).

Leptin Assay

Serum leptin level was estimated by the method of sandwich ELISA assay using leptin ELISA kits supplied by Cell Biolabs, Inc., USA(Kimura et al., 2000).

C - reactive protein (CRP) assay

CRP level was estimated in serum by a standard latex agglutination test kit of Spectrum Diagnostics, Egypt, as per the method of Schalla et al., 1984 (Schalla et al., 1984).

Estimation of oxidative stress and antioxidant enzymes

At the end of treatment period and after collection of blood sample from the treated animals, pancreas was excised immediately from all group of animals, washed with ice cold saline, weighed and homogenized in phosphate buffer (50 mM, pH 7.4) to prepare a 10% (w/v) solution. The tissue homogenate was used for the assay of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and superoxide dismutase (SOD).

Thiobarbituric acid reactive substances (TBARS) assay

TBARS were estimated spectrophotometrically from the tissue homogenate of pancreas by the method of Ohkawa et al., 1979 (Ohkawa et al., 1979). For estimating TBARS, 0.1 ml of tissue homogenate (10%) was mixed with 0.2 ml of sodium dodecyl sulphate (8.1%), 0.5 ml of acetic acid (pH 3.5) and 0.5ml of TBA (0.67%). The reaction mixture was incubated for 1 hr at 95^{0}C in a water bath. After 1 hour the reaction mixture was cooled, treated with n- butanol -pyridine mixture and absorbance was taken at 532 nm in a spectrophotometer. Results were expressed as nmol/MDA/min/mg of tissue.

Superoxide dismutase (SOD) assay

The level of superoxide dismutase was determined spectrophotometrically from the pancreatic supernatant by the method of Sun and Zigman, 1978 (Sun & Zigman, 1978). For SOD assay, 50 mM of sodium bicarbonate (pH 10.2) was added to 0.5 ml of tissue supernatant. Freshly prepared epinephrine solution was added to this reaction mixture at zero time. Inhibition of autooxidation of epinephrine to adenochrome by SOD was measured at different time intervals at 320 nm.

Reduced glutathione assay

Reduced glutathione was assayed from pancreatic homogenate by the method of Mohandas et al., 1984 using 5,5'-dithiobis (2-nitrobenzoic acid) (Mohandas et al., 1984). A mixture of 0.1M potassium phosphate containing 5 mM of EDTA (pH 8.0) and metaphosphoric acid (25%) in a ratio of 3.75:1 was added to the tissue homogenate. The reaction mixture was then centrifuged and the resulting supernatant was separated out. 5,5'-dithiobis (2- nitrobenzoic acid) was added to the supernatant and the intensity of colour developed in the reaction mixture was read spectrophotometrically at 412 nm. Results were expressed as microgram of GSH/mg of protein.

Histopathology

Pancreatic tissue was isolated and stored in 10% buffered formalin. It was then embedded in paraffin and sections of tissue of 5 μm thickness were cut followed by staining with

Table 1. Effect of L-Norvaline on body weight and BMI in hyperlipidemic diabetic rats.

Groups	Body weight (g)			BMI		
	0 day of treatment	15th day of treatment	30th day of treatment	0th day of treatment	15th day of treatment	30th day of treatment
Normal animals (1ml/kg normal saline, p.o.)	230 ± 0.05	235 ± 0.42	233 ± 0.18	1.96 ± 0.06	1.95 ± 0.04	1.94 ± 0.04
Toxicant control HDR received distilled water (1ml/kg, p.o)	360 ± 1.32[a]	356 ± 1.96[a]	356 ± 2.96[a]	3.1 ± 0.03[a]	3.05 ± 3.46[a]	3.10 ± 1.36[a]
HDR received L-Norvaline (10 mg/kg, i.p.)	365 ± 2.70	310 ± 1.62	205 ± 0.28***	3.3 ± 0.06	3.00 ± 1.62	2.34 ± 0.13***
HDR received gemfibrozil 60 mg/kg, p.o)	360 ± 1.32	270 ± 3.84*	200 ± 1.12***	2.9 ± 0.08	2.78 ± 1.19*	2.26 ± 3.72***

*All values are expressed as mean ± SEM; N=6, one way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: *P<0.05 and ***P<0.001 when the results of day 30 of treatment was compared with the results of day 0 for each group.*
[a]P<0.001 when the results of toxicant control group were compared with the normal group of animals.

eosin and hematoxylin. The sections were observed in light microscope for histo-architectural study.

Statistical analysis

Statistical analysis of results was carried out by using Graph pad prism 7.0 software. The results were expressed as mean ± SEM from six animals. Multiple comparison between different groups was carried out by using one-way ANOVA (Analysis of Variance) followed by Dunnett's multiple comparison test. Comparison between two groups was done by student t- test. P values less than 0.05 was considered as indicative of significance.

RESULTS

Effect of L - Norvaline on body weight and BMI of hyperlipidemic diabetic rats

The effect of L-Norvaline on body weight and body mass index (BMI) of HDR is shown in Table 1. Results show that L-Norvaline at a dose of 10 mg/kg administered once in a day for a period of 30 days caused significant (p<0.001) decrease in weight of the hyperlipidemic diabetic rats as compared to the day 1 of the treatment. Gemfibrozil also caused significant (p<0.001) reversal of HFD induced weight gain in HDR.
Administration of L-Norvaline restored the BMI of HDR in a gradual manner. There was significant (p<0.001) improvement in the BMI of HDR rats on day 30 of the treatment as compared to day 1 in all the groups of animals except the toxicant control.

Effect of L- Norvaline on FBS of hyperlipidemic diabetic rats

Results in Fig. 1 indicate that after treatment period of 30 days, there was significant reduction in FBS level in all groups as compared to toxicant group. L-Norvaline treatment caused significant (p<0.001) reduction in FBS in hyperlipidemic diabetic rats as compared to day 1 of the study. In this study, administration of gemfibrozil caused less significant (p<0.05) reduction in FBS level of hyperlipidemic diabetic rats.

Effect of L-Norvaline on lipid profile and atherogenic index of hyperlipidemic diabetic rats

Results summarised in Table 2 indicates the effect of L – Norvaline on lipid profile in HFD and fructose fed hyperlipidemic diabetic rats. Feeding the animals with HFD and fructose for a period of 45 days caused significant (p<0.001) increase in total cholesterol, triglyceride, LDL and VLDL levels and decrease in HDL level. Hyperlipidemic diabetic rats treated with L-Norvaline for a period of 30 days showed significant increase in HDL level and decrease in total cholesterol, triglyceride, LDL and VLDL as compared to the toxicant control group. Administration of gemfibrozil also caused significant improvement (p<0.001) in the lipid profile of HDR.
The effect of L-Norvaline and gemfibrozil on atherogenic index in HDR is summarised in the Fig. 2. Results show that both L-Norvaline and gemfibrozil caused significant decrease (p<0.001) in atherogenic index of hyperlipidemic diabetic rats.

Effect of L-Norvaline on hormonal parameters and insulin resistance of hyperlipidemic diabetic rats

Results summarised in Table 3 indicates the effect of L-Norvaline on the level of insulin, leptin and adiponectin in HDR. Administration of HFD for a period of 45 days caused significant (p<0.001) increase in serum insulin, leptin and adiponectin levels. Treatment of HDR with L-Norvaline moderately restored (p<0.01) the level of insulin and leptin,

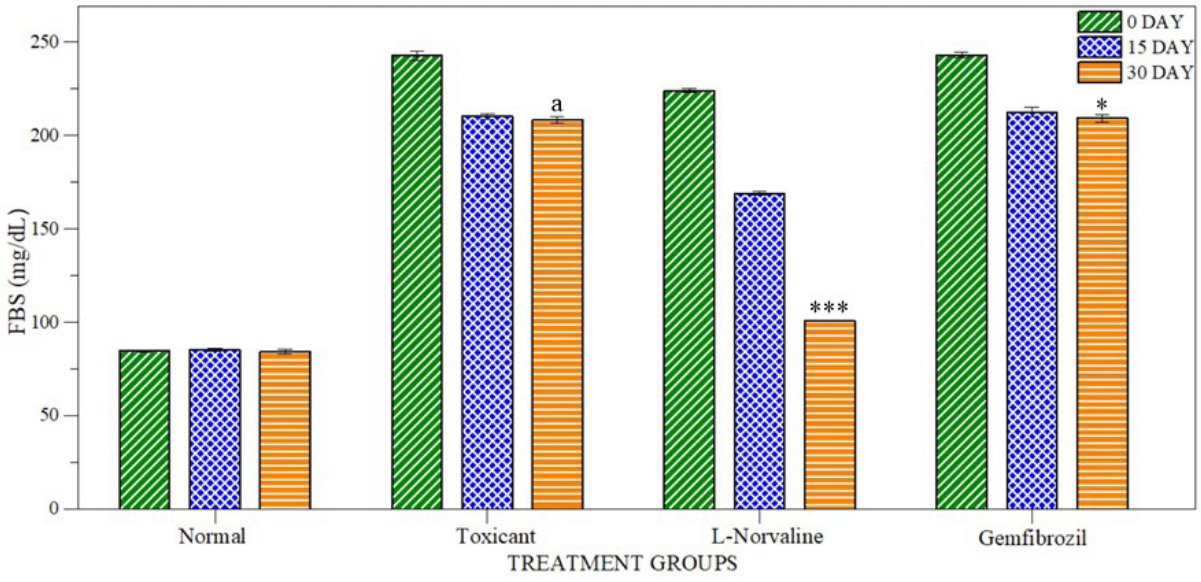

Figure 1. Effect of L-Norvaline on fasting blood sugar level in hyperlipidemic diabetic rats.
*All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: *P<0.05, ***P<0.001 when the results of treatment groups were compared with the toxicant control groups.*
[a]P<0.001 when the results of toxicant control group were compared with the normal group of animals.

Table 2. Effect of L-Norvaline on lipid profile in hyperlipidemic diabetic rats.

Groups	HDL (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal animals (1ml/kg normal saline, p.o.)	54.4 ± 1.06	60.8 ± 2.0	76.6 ± 0.56	90.2 ± 1.43	18.66 ± 1.25
Toxicant control HDR received distilled water (1ml/kg, p.o)	20.2 ± 2.94[a]	130.6 ± 0.64[a]	153.6 ± 1.19[a]	182.6 ± 1.47[a]	43.32 ± 2.98[a]
HDR received L-Norvaline (10 mg/kg, i.p.)	48.8 ± 3.76***	60.6 ± 1.42***	84.4 ± 0.82***	76.2 ± 1.3***	20.42 ± 0.76***
HDR received gemfibrozil 60 mg/kg, p.o)	52.2 ± 2.31***	57.0 ± 0.84***	79.6 ± 0.61***	74.6 ± 1.8***	19.74 ± 2.93***

*All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: ***P<0.001 when the results of treatment groups were compared with the toxicant control groups.*
[a]P<0.001 when the results of toxicant control group were compared with the normal group of animals.

Table 3. Effect of L-Norvaline on hormonal parameters and nitrate level in hyperlipidemic diabetic rats.

Groups	Insulin (mIU/L)	Leptin (ng/ml)	Adiponectin (µg/ml)	Nitrates (µmol/L
Normal animals (1ml/kg normal saline, p.o.)	20.5 ± 0.8	7.8 ± 0.72	9.8 ± 0.72	350.5 ± 0.6
Toxicant control HDR received distilled water (1ml/kg, p.o)	34.7 ± 1.12[a]	12.2 ± 0.25[a]	28.2 ± 3.26[a]	165.23 ± 0.43[a]
HDR received L-Norvaline (10 mg/kg, i.p.)	24.6 ± 0.65**	9.6 ± 0.82**	10.6 ± 0.82***	320.7 ± 0.5***
HDR received gemfibrozil 60 mg/kg, p.o)	31.6 ± 1.5	7.4 ± 0.68***	10.4 ± 1.21***	320.7 ± 0.5***

*All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: **P<0.01 and ***P<0.001 when the results of treatment groups were compared with the toxicant control groups.*
[a]P<0.001 when the results of toxicant control group were compared with the normal group of animals.

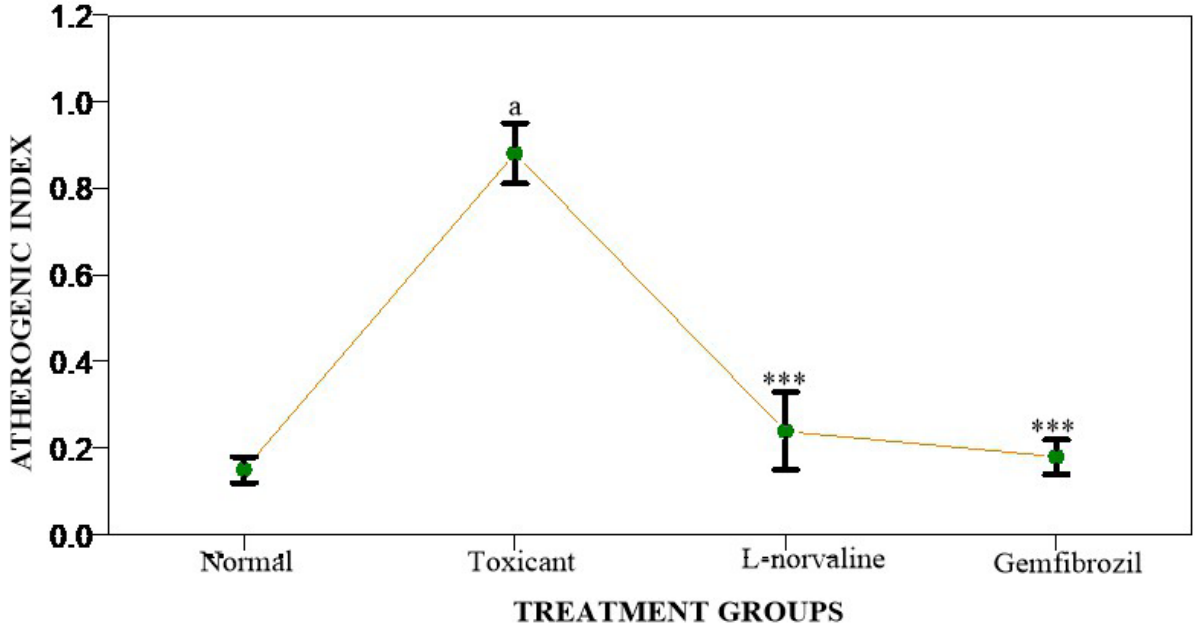

Figure 2. Effect of L-Norvaline on atherogenic index in hyperlipidemic diabetic rats.
All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: $^{***}P<0.001$ when the results of treatment groups were compared with the toxicant control groups. $^{a}P<0.001$ when the results of toxicant control group were compared with the normal group of animals.

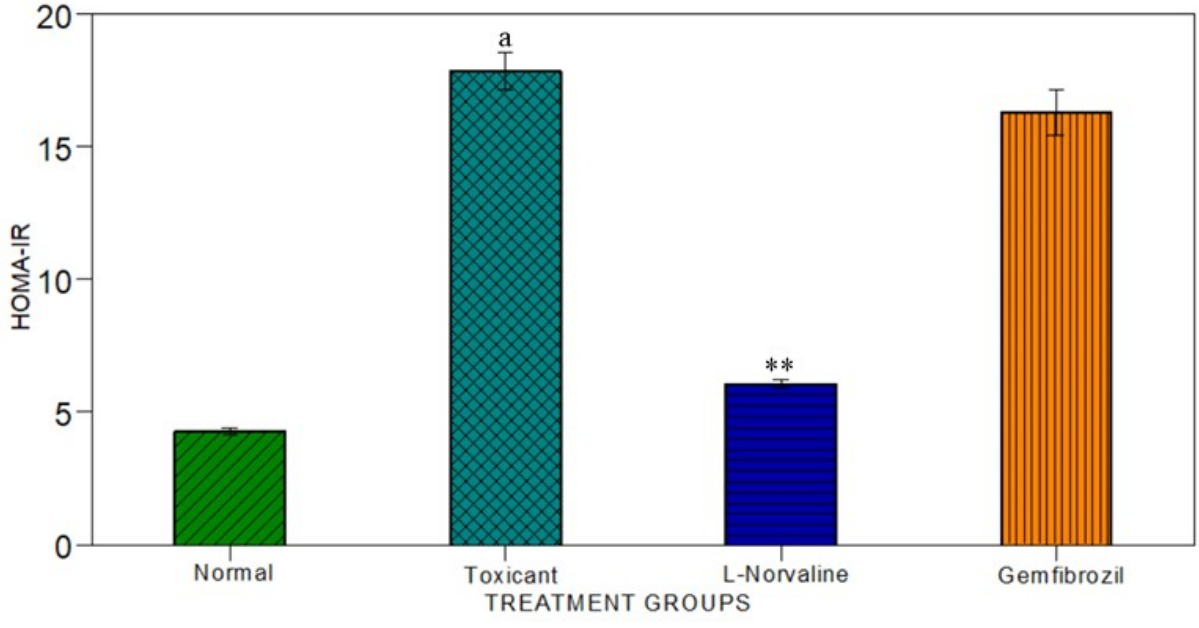

Figure 3. Effect of L-Norvaline on HOMA-IR in hyperlipidemic diabetic rats.
All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: $^{**}P<0.01$ when the results of treatment groups were compared with the toxicant control groups. $^{a}P<0.001$ when the results of toxicant control group were compared with the normal group of animals.

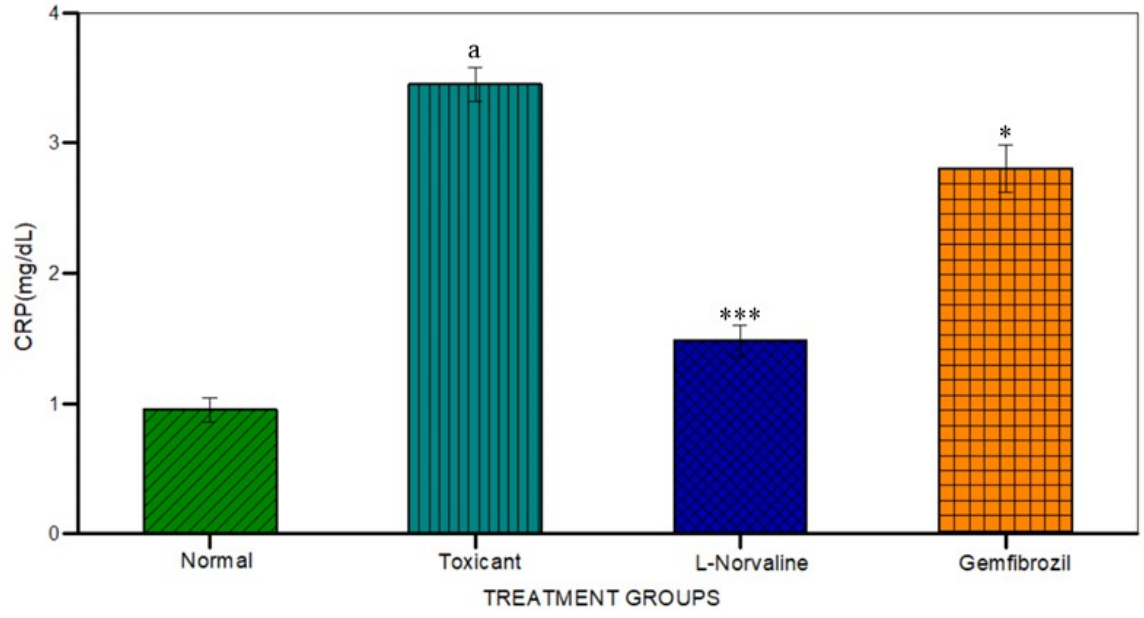

Figure 4. Effect of L-Norvaline on C - reactive protein in hyperlipidemic diabetic rats.
All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: $^{*}P<0.05$ and $^{***}P<0.001$ when the results of treatment groups were compared with the toxicant control groups.
$^{a}P<0.001$ when the results of toxicant control group were compared with the normal group of animals.

though the most significant ($p<0.001$) effect was seen on adiponectin level. Gemfibrozil caused significant ($p<0.001$) decrease in serum leptin and adiponectin levels but only slight decrease in serum insulin levels. Fig. 3 indicates the effect of the treatment on insulin resistance (HOMA-IR). The treatment group has shown significant ($p<0.01$) decrease in HOMA-IR scores of HDR. However, the standard drug gemfibrozil was found to be ineffective.

Effect of L-Norvaline on nitrate and C-reactive protein of hyperlipidemic diabetic rats

Table 3 indicates that HFD and fructose caused significant ($p<0.001$) decrease in serum nitrate level. Both, L-Norvaline and gemfibrozil caused significant ($p<0.001$) increase in serum nitrate level of HDR as compared to the toxicant control.

Fig. 4 shows effect of the treatment on serum CRP level in HDR rats. The CRP level was significantly increased in HFD and fructose fed animals as compared to normal animals. L-Norvaline caused significant ($p<0.001$) reduction in serum CRP levels as compared to the toxicant control group. Gemfibrozil, however, caused less significant ($p<0.05$) reduction in the level of CRP.

Effect of L-Norvaline on oxidative stress and antioxidant enzymes level of hyperlipidemic diabetic rats

The effect of L-Norvaline and gemfibrozil on lipid peroxidation and antioxidant enzymes level is summarised in the Table 4. Results show that the level of malondialdehyde (MDA) was significantly ($p<0.001$) increased in animals after treatment with HFD and fructose for a period of 45 days. The level of SOD and GSH were also significantly ($p<0.001$) reduced after administration of HFD and fructose. L-Norvaline caused mild decrease ($p<0.05$) in the level of MDA with no effect on the level of GSH and SOD. Gemfibrozil treatment significantly decreased ($p<0.01$) the MDA level, significantly ($p<0.01$) increased SOD level and caused slight increase ($p<0.05$) in GSH level in hyperlipidemic diabetic rats.

Histopathological studies

Fig. 5a shows the histopathology of pancreatic tissue of the normal group of animals and reveals an intact pancreatic tissue without signs of necrosis, degeneration of pancreatic islets and vacuolization in the cytoplasm of β cells. The pancreatic tissue of toxicant control group indicates shrinkage and degeneration of β cells mainly in the central region. Vacuolization was also observed in the cytoplasm of islet cells (Fig. 5b). In some previous studies, similar effects were also seen on the histology of pancreas after chronic administration of high fat sugar diet and high fat diet (Zhou et al., 2018, Gulen et al., 2015).

Treatment with L-Norvaline (10 mg/kg) has shown marked increase in the number of intact and normal beta cells as well as decrease in the number of degenerated β cells and vacuolization (Fig. 5c). L-Norvaline treatment exhibited protective effect as pancreatic tissue of rats manifested histo-architecture comparable to the pancreatic tissue of normal

Table 4. Effect of L-Norvaline on oxidative stress and antioxidant enzymes level in hyperlipidemic diabetic rats.

Groups	Lipid peroxidation (nmol MDA/min/mg of tissue)	Superoxide Dismutase (EU/mg of protein)	Reduced Glutathione (microgram/mg of protein)
Normal animals (1ml/kg normal saline, p.o.)	45.5 ± 1.80	25.7 ± 1.42	4.4 ±1.40
Toxicant control HDR received distilled water (1ml/kg, p.o)	79.8 ± 1.25[a]	13.3 ± 1.31[a]	1.3 ±1.35[a]
HDR received L-Norvaline (10 mg/kg, i.p.)	68.0 ± 1.18[*]	15.2 ± 1.06	1.6 ± 0.54
HDR received gemfibrozil 60 mg/kg, p.o)	60.5 ± 1.88[**]	16.4 ± 0.75[**]	2.1 ± 0.22[*]

All values are expressed as mean ± SEM; N=6. One way ANOVA followed by Dunnetts multiple comparison test was applied for statistical analysis. P values: [*]P<0.05 and [**]P<0.01 when the results of treatment groups were compared with the toxicant control groups.
[a]P<0.001 when the results of toxicant control group were compared with the normal group of animals.

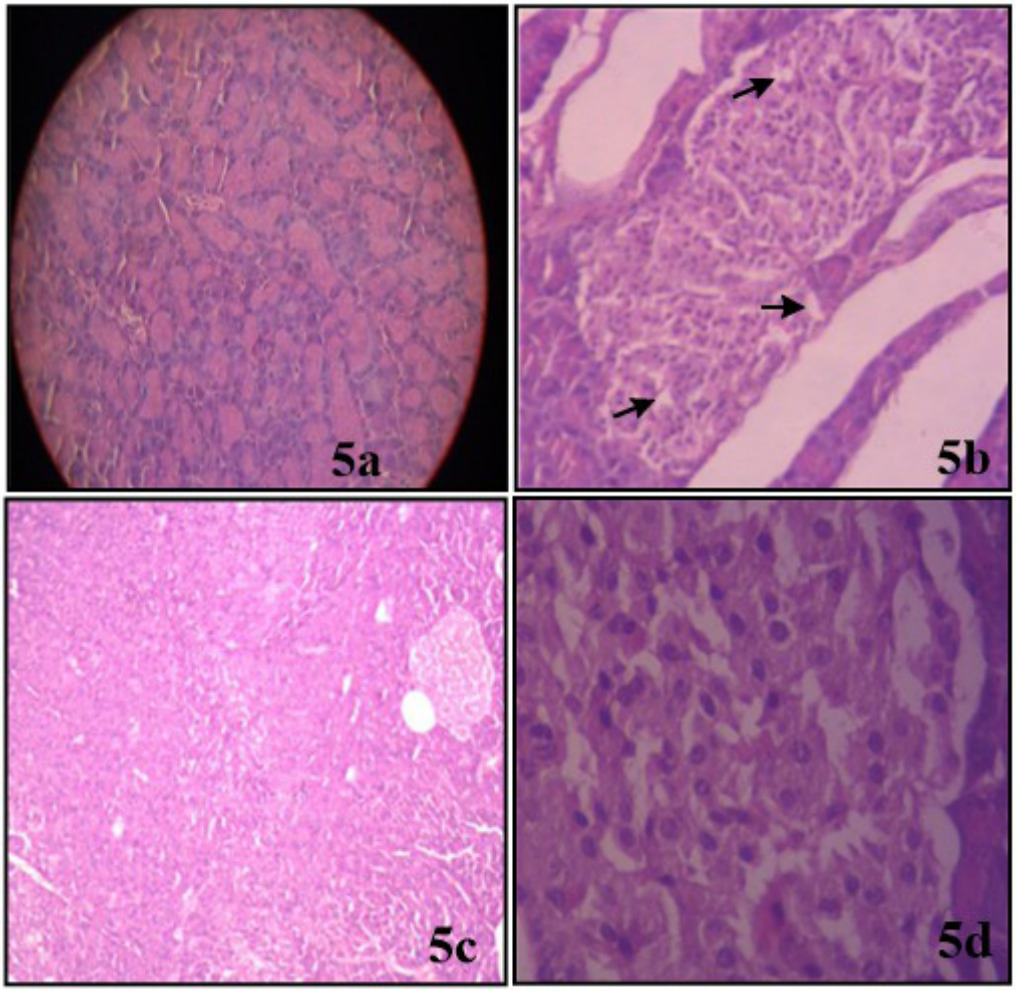

Figure 5. Effect of L-Norvaline on histology of pancreas in hyperlipidemic diabetic rats.
(a) Microscopic section of pancreas of rats from normal control group (Hematoxylin-eosin, magnification X 100 (H&E, X 100)). (b) Microscopic section of pancreas of rats from toxicant control group (H&E, X 100). (c) Microscopic section of pancreas of rats from L-Norvaline (10 mg/kg, i.p.) treated group (H&E, X 100). (d) Microscopic section of pancreas of rats from gemfibrozil (60 mg/kg, p.o.) treated group (H&E, X 100).

animals. However, gemfibrozil treatment has only mild effect on pancreatic histology with less improvement in the structure of and number of β cell (Fig. 5d).

DISCUSSION

Metabolic syndrome is characterized by various abnormal medical conditions such as central obesity, dyslipidemia, hypertension and diabetes mellitus. In the present study, HFD and fructose given to the animals for a period of 45 days caused significant increase in the body weight and BMI of animals. As visceral adiposity is a main trigger for most of the pathways involved in MetS, increased calorie intake through high fat diet acts as a causative factor in the initiation of conditions associated with MetS (Panchal et al., 2011). Fructose intake is associated with hypertriglyceridemia, lipogenesis and formation of reactive oxygen species (ROS) with subsequent increase in oxidative stress. Increased production of ROS is associated with endothelial injury and increased expression of nuclear factor kappa B cells (NF-kB) on the endothelium and vascular smooth muscle cells that contributes to the development of dyslipidemia, type II diabetes mellitus and cardiovascular disease.

HDR treated with L-Norvaline have shown significant decrease in the body weight and BMI, the predictors of obesity-related cardiovascular complications. It may be assumed that L-Norvaline mediated upregulation of peroxisome proliferator activated receptor-α (PPAR-α) and downregulation of PPAR-c2, Stearoyl-CoA desaturase-1 (SCD-1), adipose differentiation related protein (ADRP) mRNA and gene expression are responsible for its anti-obesity effect (Moon et al., 2014).

In the present study, HFD along with fructose caused significant increase in fasting blood sugar, serum insulin level and insulin resistance. HFD augments the adipose tissue mass and triggers increased secretion of adipokines into the bloodstream giving rise to obesity related insulin resistance and inflammation. Fructose plays an important role in the development of insulin resistance. It is mainly taken up by hepatocytes and the excess fructose then undergoes hepatic de novo lipogenesis resulting in FFA surge in hepatic circulation which by phosphorylation of IRS1 gives rise to an insulin resistant state. L-Norvaline caused decrease in fasting blood sugar and insulin level and improved insulin sensitivity. Improvement in insulin sensitivity may be due to L-Norvaline mediated increase in the bioavailability of NO and subsequent vasodilation leading to increased blood perfusion to skeletal muscles. This amplifies glucose and insulin supply to skeletal muscles and boost glucose disposal for the production of ATP (Ouellet et al., 2017). Previous studies done on L-Norvaline indicates that the NO produced by L-Norvaline phosphorylates the AMPK, which in turn activates the GLUT4 receptors, thus facilitating entry of glucose into the cell and its oxidation (Hu et al., 2017).

In the present study, HFD and fructose administration is associated with the lipid accumulation in various tissues, altered lipid metabolism, increased TG, LDL and VLDL levels and dyslipidemic state in animals. HFD upregulates expression of mRNA of various lipogenic proteins like lipoprotein lipases in the liver. These lipoproteins mediate uptake of lipids from circulation into the liver and the skeletal muscles which gets accumulated in these peripheral tissues. This leads to a state of insulin resistance resulting in inhibition of the anti-lipolytic effect of insulin, thereby increasing non-esterified fatty acids levels and lipid oxidation. Fructose given in diet, is taken up by the hepatocytes, where it increases de novo lipogenesis and the level of triglyceride in hepatocytes and LDL and VLDL in plasma resulting a dyslipidemic state (Pereira et al., 2017).

L-Norvaline treatment decreased the level of TG, cholesterol, LDL, VLDL and also improved HDL level in hyperlipidemic diabetic rats. Study by Hong et al., reported that down regulation of FAS involved in de novo lipogenesis, HMG-CoA reductase and SREBP-2 involved in synthesis of cholesterol, SREBP-1 that transcribes genes for fatty acid synthesis might be responsible for the protective effect of arginase inhibitors in dyslipidemic state (Hong et al., 2018).

In our study, feeding HFD and fructose has attributed to hyperleptinemia, the endocrine cause of weight gain and development of adiposity in HFD and fructose induced MetS model. The possible mechanism behind the decrease in plasma leptin concentration after L-Norvaline administration might be due to an increase in the level of NO by the arginase inhibitor, that activates guanylate cyclase resulting in the elevation of cGMP and inhibition of leptin release (Fain et al., 2003).

Adiponectin is a hormone like peptide inversely related to BMI and body fat. Generally, increase in adiponectin level occurs in the early stage till complete development of MetS, followed by a decrease in the level of adiponectin (Moreno-Fernández et al., 2018). In the present study, administration of HFD and Fructose caused initial increase in adiponectin level, followed by a fall in serum adiponectin level in HDR, which is in accordance with the previous study. L-Norvaline administration for a period of 30 days significantly increased adiponectin level in HDR. It can be assumed that L-Norvaline by increasing NO bioavailability, triggers p38 MAPK pathway and activates PPAR and its binding to PPRE, leading to transcriptional activation of adiponectin gene and subsequent rise in serum adiponectin level.

CRP is an inflammatory biomarker released into the plasma by hepatocytes in response to pro- inflammatory cytokines. In the present study, HFD and fructose administration caused significant increase in the level of CRP in hyperlipidemic diabetic rats indicating a chronic low-grade inflammatory state. Results from previous studies also reveals that increased level of CRP in plasma binds to lipoproteins (LDL and VLDL) and upregulates the expression of adhesion molecules ultimately causing development of atherosclerosis and MetS (Pereira et al., 2017). L-Norvaline treatment significantly decreased CRP level in HDR. Though much information is not available on how L-Norvaline decreases serum CRP levels, but it can be

hypothesized that increased NO bioavailability as a result of arginase inhibition by L-Norvaline results in AMPK activation and inhibition of IL-6 mediated CRP release (McCarty, 2004). In HDR, excess energy provided by HFD results an increase in oxidative activity leading to mitochondrial dysfunction, that leads to increase in the level of ROS and weakening of anti-oxidant mechanisms of the body. Fructose metabolism in hepatocytes occur by utilizing ATP, thereby increasing the levels of ADP and AMP. Increased AMP level causes activation of AMP deaminase enzyme and initiates the hypoxanthine pathway producing uric acid, that increases the oxidative stress in the adipose tissues by activation of NADPH oxidase. In the present study, L-Norvaline caused mild reduction in the level of lipid peroxidation in HDR with no effect on the level of SOD and GSH. However, studies in the past indicated that L-Norvaline by increasing the expression of SOD, GSH and CAT exhibits antioxidant activity (Liang et al., 2018).

CONCLUSION

The present study recapitulates the effect of L-Norvaline on the development of obesity, diabetes, hyperlipidemia and metabolic syndrome. Results of the study concludes that L-Norvaline, an arginase inhibitor shows promising improvement in the body weight, BMI, lipid profile, fasting blood sugar level, insulin, insulin resistance, leptin and adiponectin level. It also caused improvement in CRP and nitrate level as well as caused mild decrease in the oxidative stress. Results confirm that L-Norvaline can be developed as a potential treatment strategy for MetS and its associated conditions. However further preclinical and clinical studies are sought to explore the actual mechanism of action so that L-Norvaline can be used as a promising treatment for metabolic syndrome associated complications.

ACKNOWLEDGEMENT

The authors are immensely thankful to the management SBS University, Balawala, Dehradun for providing all the facilities. We are also thankful to the management AIIMS Rishikesh, Uttarakhand for helping in estimation of various biochemical and hormonal parameters.

DECLARATION OF INTEREST

None

FUNDING STATUS

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

References

[1] Arita Y, Kihara S, Ouchi N, et al. Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity. Biochem Biophys Res Commun. 1999; 257: 79–83.

[2] Azimi Nezhad M, Ghayour-Mobarhan M, Parizadeh SMR, et al. Metabolic syndrome: Its prevalence and relationship to socio-economic parameters in an Iranian population. Nutr Metab Cardiovasc Dis. 2008;18: e11–2.

[3] Bucolo G, David H. Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem. 1973; 19:476–82.

[4] Castelli WP, Doyle JT, Gordon T, et al. HDL cholesterol and other lipids in coronary heart disease. The cooperative lipoprotein phenotyping study. Circulation. 1977; 55:767–72.

[5] De A, Singh MF, Singh V, Ram V, Bisht S. Treatment effect of l-Norvaline on the sexual performance of male rats with streptozotocin induced diabetes. Eur J Pharmacol. 2016; 771:247–54.

[6] Fain JN, Kanu A, Bahouth SW, Cowan GSM, Hiler ML. Inhibition of leptin release by atrial natriuretic peptide (ANP) in human adipocytes. Biochem Pharmacol. 2003; 65(11):1883–8.

[7] Grundy SM, Cleeman JI, Daniels SR, et al. Diagnosis and Management of the Metabolic Syndrome: An American Heart Association/National Heart, Lung and Blood Institute scientific statement. Circulation. 2005;112: 2735–52.

[8] Guerra J, De Jesus A, Santiago-Borrero P, Roman-Franco A, Rodríguez E, Crespo MJ. Plasma nitric oxide levels used as an indicator of doxorubicin-induced cardiotoxicity in rats. Hematol J. 2005; 5: 584–8.

[9] Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol. 2008; 9:367–77.

[10] Gulen MI, Bagla AG, Yavuz O, Hismiogullar AA. Histopathological changes in rat pancreas and skeletal muscle associated with high fat diet induced insulin resistance. Biotech Histochem. 2015; 90(7): 495–505.

[11] Hong MY, Beidler J, Hooshmand S, Figueroa A, Kern M. Watermelon and l-arginine consumption improve serum lipid profile and reduce inflammation and oxidative stress by altering gene expression in rats fed an atherogenic diet. Nutr Res. 2018;58:46–54.

[12] Hu S, Han M, Rezaei A, Li D, Wu G, Ma X. L-Arginine Modulates Glucose and Lipid Metabolism in Obesity and Diabetes. Curr. Protein Pept. Sci. 2017; 18:599–608.

[13] Kimura E, Matsumoto K, Samori T, Kato S, Kawahara T. One-step enzyme-linked immunosorbent assay (ELISA) for measurement of serum free leptin. Clin Chim Acta. 2000; 296: 45–57.

[14] Liang M, Wang Z, Li H, et al. L-Arginine induces antioxidant response to prevent oxidative stress via stimulation of glutathione synthesis and activation of Nrf2 pathway. Food Chem Toxicol. 2018;115:315–28.

[15] MacDonald MJ, Gapinski JP. A rapid ELISA for measuring insulin in a large number of research samples. Metabolism. 1989; 38: 450–2.

[16] Masi S, Colucci R, Duranti E, et al. Aging modulates the influence of arginase on endothelial dysfunction in obesity. Arterioscler Thromb Vasc Biol. 2018;38: 2474–83.

[17] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia. 1985; 28:412–9.

[18] McCarty MF. AMPK activation may suppress hepatic production of C-reactive protein by stimulating nitric oxide synthase. Med. Hypotheses. 2004 ;63:328–33.

[19] Miller NE, Thelle DS, Forde OH, Mjos OD. The Tromsø heart-study. High-density lipoprotein and coronary heart-disease: a prospective case-control study. Lancet. 1977;1: 965–8.

[20] Mohandas J, Marshall JJ, Duggin GG, Horvath JS, Tiller DJ. Low activities of glutathione-related enzymes as factors in the genesis of urinary bladder cancer. Cancer Res. 1984; 44:5086–91.

[21] Moon J, Do HJ, Cho Y, Shin MJ. Arginase inhibition ameliorates hepatic metabolic abnormalities in obese mice. PLoS One. 2014; 9:1–10.

[22] Moreno-Fernández S, Garcés-Rimón M, Vera G, Astier J, Landrier JF, Miguel M. High fat/high glucose diet induces metabolic syndrome in an experimental rat model. Nutrients. 2018;10:1–15.

[23] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. 1979; 95: 351–8.

[24] Ouellet V, Weisnagel SJ, Joanisse DR, et al. Beneficial Impact of Cod Protein, l-Arginine, and Other Amino Acids on Insulin Sensitivity. In: Patel VB, Preedy VR, Rajendram R, editors. L-Arginine in Clinical Nutrition. Cham: Springer International Publishing; 2017. p. 433–47.

[25] Panchal SK, Poudyal H, Iyer A, et al. High-carbohydrate high-fat diet-induced metabolic syndrome and cardiovascular remodeling in rats. J Cardiovasc Pharmacol. 2011;57:51–64.

[26] Panda V, Mistry K, Sudhamani S, Nandave M, Ojha SK. Amelioration of Abnormalities Associated with the Metabolic Syndrome by *Spinacia oleracea* (Spinach) Consumption and Aerobic Exercise in Rats. Oxid Med Cell Longev. 2017; 1: 1-15.

[27] Pereira RM, Botezelli JD, da Cruz Rodrigues KC, et al. Fructose consumption in the development of obesity and the effects of different protocols of physical exercise on the hepatic metabolism. Nutrients. 2017; 9:1–21.

[28] Rabelo LA, Ferreira FO, Nunes-Souza V, Fonseca LJS Da, Goulart MOF. Arginase as a Critical Prooxidant Mediator in the Binomial Endothelial Dysfunction-Atherosclerosis. Oxid Med Cell Longev. 2015; 2:1–12.

[29] Schalla WO, Arko RJ, Thompson SE. Evaluation of a C-reactive protein latex agglutination detection test with sera from patients with sexually transmitted diseases. J Clin Microbiol. 1984; 20: 1171–3.

[30] Sun M, Zigman S. An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. Anal Biochem. 1978; 90:81–9.

[31] Trinder P. Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol. 1969; 22:158–61.

[32] Zhou J, Zeng Q, Cai D, Zeng X, Chen Y, Gan H et al. Evaluation of type 2 diabetic mellitus animal models via interactions between insulin and mitogen-activated protein kinase signalling pathways induced by a high fat and sugar diet and streptozotocin. Mol Med Rep. 2018; 17: 5132-5142.

sciendo

Eur. Pharm. J. 2022, 68(2), 27-40.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2021-0017

EUROPEAN PHARMACEUTICAL JOURNAL

Effects of Demographic Characteristics and Consumer Behavior in the selection of Retail Pharmacies and Over-the-Counter Medicine

Original Paper

Kevrekidis D.P.[1]✉, Mináriková D.[1], Markos A.[2]

[1]Comenius University in Bratislava Faculty of Pharmacy: Univerzita Komenskeho v Bratislave Farmaceuticka Fakulta, Bratislava, SLOVAKIA
[2]Democritus University of Thrace School of Education: Demokriteio Panepistemio Thrakes Schole Epistemon Agoges, Alexandroupolis, GREECE

Received 14 April, 2018, accepted 1 June, 2018

Abstract

***Aim*:** The study aimed to provide new insights into consumer behaviour by identifying the key demographic factors that influence the choice of pharmacy and over-the-counter (OTC) medicine.
***Materials and Methods*:** A cross-sectional study was conducted in an urban area (Thessaloniki, Greece), surveying a convenience sample of 314 consumers with a structured questionnaire. Data analysis was conducted using the chi-square test, one-way analyses of variance (ANOVAs) and Spearman's rho correlation coefficient.
***Results*:** Respondents with a lower educational level and retired consumers tended to make their purchases in a single pharmacy ($p < 0.001$). Older participants were more likely to consider the pharmacy staff and additional services to be important factors ($p < 0.01$). Students were the only group to prefer a formal relationship with the pharmacy staff ($p < 0.001$). Participants with a lower educational level tended to know exactly what they would buy ($p < 0.05$), whereas women made more unscheduled purchases of OTCs ($p < 0.05$). Respondents with a higher income assigned more importance to the product's country of origin ($p < 0.05$) and manufacturing company ($p < 0.01$) and less importance to the pharmacist's opinion than those of a lower income ($p < 0.05$).
***Conclusions*:** The educational level, occupation and age of consumers have a marked effect in their selection of pharmacy, and along with gender and personal income, in their choice of OTC medicine. Our findings yield implications for the management of community pharmacies.

Keywords *Self-medication – OTC – community pharmacy – demographic factors – marketing strategy*

INTRODUCTION

Community pharmacies are providers of emergency medication, expert advice and specialist healthcare services. Due to their vital contribution to the community, retail pharmacies have traditionally been protected with heavy regulation in many countries (Schmidt & Pioch, 2004). However, over the past few decades, liberalisation measures have been introduced in several EU member states, leading to a more competitive environment in the community pharmacy sector (Castaldo et al., 2016; Schmidt & Pioch, 2004). Such measures include the reclassification of prescription medications as over the counter (OTC; European Commission, 2014; Martins et al., 2002), the availability of OTC medicine outside of pharmacies and the deregulation of the ownership and establishment rules for community pharmacies (Vogler, 2014). This competitive market environment requires pharmacies to adjust to the new circumstances, so as to maintain their patronage, attract new clients and develop customer loyalty.

Furthermore, with the pressure set on healthcare systems in the EU and elsewhere by limited resources and an aging population (European Commission, 2014; Krishnan & Schaefer, 2000) that necessitate a more efficient distribution of health resources (European Commission, 2014), the practice of self-care will continue to grow in importance (Krishnan & Schaefer, 2000). This inevitable demographic change will be influencing the need for pharmaceutical products and the landscape of their consumption (Ogura & Jakovljevic, 2014). Self-medication is a modern and increasingly important mode of self-care (Figueiras et al., 2000; Jakovljevic & Souliotis, 2016). It is used in order to relieve, ameliorate, cure or prevent

* E-mail: dphkevrekidis@gmail.com

the symptoms of various illnesses (Wertheimer & Serradell, 2008) and is conducted, for the most part, by the use of non-prescription or OTC medicine (Dean, 1986). The use of OTC medications is steadily rising (Lessenger & Feinberg, 2008), with half of all medicines sold in the EU having a non-prescription status (European Commission, 2014).

In order for self-care and self-medication to be successful, professional guidance and impartial advice are needed (Krishnan & Schaefer, 2000). The purchase of OTC medicines is consistently performed in community pharmacies (Haramiova et al., 2017). Although as an initial step, individuals will take actions based on their own knowledge and beliefs or seek advice and support from their family and friends, pharmacists still have significant opportunities to provide and support a vast array of health-related questions. That is based on the fact that pharmacists offer a great degree of accessibility to patients (Wertheimer & Serradell, 2008). Thanks to this, the wide distribution of pharmacies across Europe (European Commission, 2014) and the fact that no prior appointment is required (Wertheimer & Serradell, 2008), pharmacists are the first and, sometimes, only health professionals who will assist patients in self-care (European Commission, 2014).

Current information concerning pharmacy customer behaviour is needed in order for community pharmacists to focus their marketing strategy and provide patient-centred service adjusted to the self-medication needs of their consumers. Contemporary data on customer behaviour regarding the factors influencing the choice of pharmacy and the purchase of OTC medicines would be particularly useful as a consumer's purchasing behaviour is mediated by cultural, social and personal factors (Kotler & Keller, 2012). Therefore, information concerning pharmacy and OTC medicine selection in conjunction with demographic information of the consumers is particularly required.

Various studies conducted in different countries have examined the factors influencing customer choice of community pharmacy. Functional attributes relating to the customers' convenience, mainly the pharmacy's location, but also attributes of the pharmacy staff have generally been reported as important factors influencing the selection of pharmacy (Arneson et al., 2011; El Hajj et al., 2011; Gavilan et al., 2014; Kouwenberg & Jaiboon, 2019; Merks et al., 2014; Minarikova et al., 2016a, 2016b; Patterson et al., 2019; Villako & Raal, 2007; Wirth et al., 2010). In addition, the influence of demographic factors such as age, gender, income, occupation and education on pharmacy customer behaviour has been reported in pharmacy literature (Kouwenberg & Jaiboon, 2019; Patterson et al., 2019; Rabbanee et al., 2015; Shiyanbola et al., 2016; Villako & Raal, 2007). However, country-specific differences, as well as differences between older and more recent studies, have also been reported with regard to factors that influence the patient's choice of community pharmacy. They mainly concerned the ranking of factor importance (Merks et al., 2014; Patterson et al., 2019).

Several studies have also examined the factors influencing OTC purchases, the offered range of which is widening in community pharmacies in several countries (Castaldo et al., 2016). Both medical factors, including previous experience with the OTC medicines and the pharmacist's recommendations, and business factors, such as familiarity with the name or brand and the OTC medicine's price, have been reported as important factors influencing the consumers' selection of OTC medicines (Chan & Tran, 2016; Habash & Al-Dmour, 2020; Hanna & Hughes, 2011; Lodorfos et al., 2006; Paddison & Olsen, 2008; Srivastava & Wagh, 2020; Temechewu, 2020; Wazaify et al., 2005). However, in these studies, the need for contemporary research, due to the dynamic and evolving nature of the OTC market, has been highlighted (Paddison & Olsen, 2008). The international literature is limited when it comes to examining the influence of demographic factors on the OTC medicine's selection; the little available information indicates that demographic characteristics also have an impact on assigning value to OTC products (Paddison & Olsen, 2008).

Within the context of Greece, the literature on the aforementioned topics is much more limited (Kevrekidis et al., 2018), and therefore, it is important to provide such domestic information, which could also be utilised in other comparable countries in terms of healthcare systems, levels of pharmaceutical care, financial situations, liberalisation schemes and so on. In Greece, the value of total drug sales was reduced by 28.2% between 2009 and 2012, as the market's dynamic was halted by the financial crisis (Athanasiadis et al., 2013). As a result of the signing of the Memorandum of Understanding between Greece and its creditors, there were changes in the healthcare sector targeting cost containment measures legislated within very tight timelines, aiming to reduce both the cost and volume of prescribed medicines (Jakovljevic & Souliotis, 2016). This included an expansion of the OTC list and the maintenance of a cap on OTC prices based on the average of the three lowest prices in EU member states, while allowing prices to move downwards (IMF, 2014).

The main goal of the present study is to provide new insights into consumer behaviour as it relates to the purchase of OTC products and the selection of pharmacies, in order to contribute to the development of an effective marketing strategy by community pharmacies. In particular, we have investigated the potential influence of demographic factors of the consumers, particularly gender, age, educational level, occupation and personal income, on their selection of community pharmacy and OTC medicines. The present study is an extension of a previous study (Kevrekidis et al., 2018) dealing with customer segmentation in relation to community pharmacy and OTC product criteria; three distinct clusters of pharmacy customers were detected and classified as 'convenience customers' (49%), 'loyal customers' (35%) and 'convenience and price-sensitive customers' (16%). Both studies were conducted in the metropolitan area of Thessaloniki, Greece. This second paper attempts to

answer the following research questions: (1 and 2) How do the demographic characteristics of the consumers relate to their preferences concerning the choice of pharmacy and the purchase of OTC medicine, and which are the key factors affecting their choice? (3) What is the relationship between the factors influencing the choice of pharmacy and the purchase of OTC medicine?

THEORETICAL FRAMEWORK

According to the American Marketing Association, consumer behaviour is defined as 'the dynamic interaction of affect and cognition, behavior, and environment by which human beings conduct the exchange aspects of their lives' (Bennett, 1995). It is the decision-making process and physical activity that occurs during the acquisition, evaluation, use and disposal of goods and services, and it precedes any kind of purchase (Khan, 2006). Consumer behaviour is a vital and necessary topic as its influence affects our daily lives and purchase decisions.

A consumer's typical buying process is described by a five-stage model: (a) Problem recognition: The consumer recognises a need to buy a product. (b) Information search: The consumer attempts to gain knowledge on the product. (c) Evaluation of alternatives: The products which can fulfil the needs are evaluated in terms of plus and minus points. (d) Purchase decision: After consideration of a number of factors, the actual purchase is made from the store. (e) Post-purchase behaviour: How the consumer feels after the use of the product (Khan, 2006).

Problem recognition is the perception of a gap between the existing and the desired consumer position (Khan, 2006). The circumstances that trigger particular needs may be identified by gathering information from a number of consumers (Kotler & Keller, 2012). An information search is the process by which the consumer surveys his or her environment for appropriate data in order to make a reasonable decision (Solomon et al., 2006). An aroused consumer who recognises a problem will be inclined to search for more information. This arousal may be distinguished into two levels of engagement: (a) heightened attention (at the milder search state), in which the consumer simply becomes more receptive to information concerning the product, and (b) active information search level, in which the consumer actively surveys the Internet, talks with friends and visits stores to learn more about the product. A survey regarding painkiller purchasing in the UK has shown that externalised information searching started when the symptoms appeared or when a certain brand was unavailable (Paddison & Olsen, 2008). The information sources may include (a) personal sources, (b) commercial sources, (c) public sources and (d) experiential sources (Kotler & Keller, 2012). The consumer may also consult unbiased third parties or other consumer reports published in various European countries (Beales et al., 1981). The existing knowledge of a consumer on a product may be because on a previous occasion, they had already searched for relevant information or experienced some of the alternatives (directed learning) or in a passive manner, through exposure to advertising, packaging and sales promotion activities (incidental learning) (Solomon et al., 2006).

Through this stage in the process, the consumer is informed about the various competing brands. From a total set of brands available to the consumer, they will only become aware of some of them (the awareness set). Some of these will meet the consumer's initial buying criteria (the consideration set). And as more information is gathered, only a few strong contenders will remain, the choice set from which the final choice is made (Narayana & Markin, 1975). The company must strategise in order to enter the consumer's awareness, consideration and choice set. Moreover, they must identify other brands in the choice set, so as to plan competitive appeals. The company must also identify the sources of information that the consumer uses and evaluate their relative importance, so that it can prepare a range of effective communications for the target market (Kotler & Keller, 2012). After the information searching, the consumer must make a final value judgement. This is mostly done on a conscious and rational basis (Kotler & Keller, 2012). The choice may be influenced by integrating information from sources such as prior experience with the product, information that was present at the time of the purchase and brand beliefs formed through advertising (Smith, 1993). While evaluating alternatives, the consumer develops a set of brand beliefs about where each brand stands on each attribute. Ultimately, consumers develop various attitudes towards different brand alternatives through an attribute evaluation procedure. A brand which knows how buyers evaluate alternatives and form preferences can influence their consumer behaviour (Kotler & Keller, 2012).

Once the relevant options from a category have been assembled and evaluated, the consumer must choose among them (Putsis & Narasimhan, 1994). The criteria on which products differ from one another carry more weight in the decision process than those where they are similar. Attributes used to differentiate among choices are called determinant attributes. Consumers often form assumptions about brands, products or outlets. These market beliefs become short cuts that guide the consumer's decisions, regardless of their accuracy (Duncan, 1990). A consumer who is engaged in extended problem-solving may carefully evaluate several brands, while someone who makes a habitual decision may not consider any alternatives to their normal brand. Furthermore, more extended processing may occur in situations where negative emotions are aroused by conflicts among the available choices, especially where difficult trade-offs are involved (Solomon et al., 2006).

A consumer's buying behaviour is influenced by personal, cultural and social factors (Kotler & Keller, 2012). Personal characteristics that influence a buyer's decision include age and state in the life cycle, occupation and economic

circumstances, personality and self-concept, lifestyle and values. Many of them have a direct impact on consumer behaviour; so, it is important for marketers to follow them closely. Consumption is also shaped by the family life cycle and the number, age and gender of people in the household at any point in time (Kotler & Keller, 2012). Consumption patterns are also influenced by occupation. Both brand and product choice are greatly affected by economic circumstances such as spendable income (level, stability and time pattern), savings and assets (as well as the percentage of those that is liquid), debts, borrowing power and attitudes towards spending and saving (Kotler & Keller, 2012). Consumers are also influenced by their core values, the belief systems that underlie their attitudes and behaviours. They go much deeper than behaviour or attitude and determine people's choices and desires over the long term at a basic level.

Culture is a fundamental determinant of a person's wants and behaviour, and cultural factors have the broadest and deepest influence towards a consumer's buying behaviour (Kotler & Keller, 2012). Groups with a direct influence are called membership groups (Khan, 2006; Kotler & Keller, 2012). Some of them are primary groups with whom the consumer interacts fairly continuously and informally, like family, friends, neighbours and co-workers (Kotler & Keller, 2012). Some are secondary groups, such as religious, professional groups that tend to be more formal and require less interaction (Kotler & Keller, 2012). They have lower frequency of contact and are not so closely knit (Khan, 2006). The family is a closely knit social group and an earning, consuming and decision-making unit, being the most immediate and pervasive influence on decision-making. Family members are the most influential primary reference group and have effects on all purchase decisions (Khan, 2006; Moore et al., 2002). They must fulfil not only their individual needs, but also their shared needs by drawing on a common and shared, relatively fixed supply of sources (Khan, 2006). Because the bonds in a family are more powerful than they are in other groups, a reciprocal influence acts in all family decisions. Every member of the family has his or her own motives, beliefs and predisposition and influences and is affected by other family members in the decision process. Interactions take place in a family that develop tastes, preferences, shopping styles, how much money to spend, where to buy from, what to use at which occasions and so on (Khan, 2006).

Background factors like age, sex, social class and others exist. There are also socialising agents from whom young people learn, such as the media, family members (through instrumental training, modelling and mediation), peers and teachers. Moreover, advertising and promotional activities have a strong influence on consumer socialisation. These influence the learning mechanism and result in a socialised consumer (Khan, 2006).

METHODS

Study Design and Data Collection

A cross-sectional study was conducted between February and March of 2016 in a convenience sample of consumers recruited in the metropolitan area of Thessaloniki. A total of 314 consumers were surveyed (approximately 0.1% of the target population) via anonymous, face-to-face interviews using a structured written questionnaire. The interviews were typically conducted during weekdays from 9 am to 3 pm and from 6 pm to 9 pm in public spaces (shopping malls, public transportation stations, etc.) and written consent was obtained from all participants. The sample was stratified by age and employment status, per the results of the 2011 Population and Housing Census for this administrative region (ELSTAT, 2014). The differences in the distribution of age and employment status between the study sample and the target population were minor; there was a notable difference in regard to gender, but this is consistent with the fact that women are more likely to go shopping and tend to make more frequent OTC purchases. Ten questionnaires were excluded due to invalid or incomplete answers and four due to uniform questionnaire scores. The final sample comprised 300 participants: 185 (61.7%) female and 115 (38.3%) male respondents.

Survey Instrument

The main instrument used for data collection was a structured questionnaire with close-ended, multiple-choice questions. The instrument was developed by the first author following an in-depth review of the literature concerning consumer behaviour as it pertains to the selection of community pharmacies (Arneson et al., 1989; Boström, 2011; Merks et al., 2014; Villako & Raal, 2007; Wirth et al., 2010) and the purchase of OTC medicines (Boström, 2011; Gyaneshwari, 2015; Lodorfos et al., 2006; Paddison & Olsen, 2008; Wazaify et al., 2005). The content and face validity of the questionnaire were assessed by an independent panel of three specialists in pharmacy management and organisation. Three questions were revised for ease of comprehension and clarity and further assessed by a convenience sample of nine adults recruited at a shopping mall in Thessaloniki. The pilot phase responses were not included in the study results. The final version of the instrument comprised of three sections (available upon request from the first author).

The first section collected the demographic information of the participants (gender, age, educational level, employment status, personal income). The second section contained questions regarding the consumers' choice of pharmacy. The participants were asked to answer multiple-choice, close-ended questions regarding their tendency to make purchases in a single pharmacy and their desired relationship

Table 1. Spearman's correlations among factors influencing (a) the choice of pharmacy and (b) the purchase of OTC medicine.

(a) Pharmacy criteria	1	2	3	4	5	6	7	8
The pharmacy's location (1)	-							
The pharmacy staff (2)	0.29**	-						
The product range (3)	0.26**	0.25**	-					
The additional services (4)	0.15**	0.25**	0.40**	-				
The membership programme (5)	0.04ns	0.21**	0.22**	0.46**	-			
Anonymity/confidentiality (6)	0.11ns	0.35**	0.30**	0.29**	0.22**	-		
The store's atmosphere (7)	0.22**	0.40**	0.39**	0.35**	0.21**	0.41**	-	
The opening hours (8)	0.30**	0.19**	0.21**	0.21**	0.18**	0.22**	0.27**	-
(b) OTC product criteria	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
The drug's country of origin (1)	-							
The manufacturing company (2)	0.77**	-						
Packaging (3)	0.36**	0.38**	-					
Experience of a previous use (4)	0.26**	0.28**	0.13*	-				
The product's advertisement (5)	0.01ns	0.06ns	0.29**	0.11ns	-			
The pharmacist's opinion (6)	0.05ns	0.06ns	0.02ns	0.18**	0.13*	-		
Family's/friends' opinion (7)	0.01ns	0.06ns	0.17**	0.10ns	0.38**	0.21**	-	
The product's price (8)	0.07ns	0.09ns	0.19**	0.05ns	0.00ns	0.30**	0.15**	-

**p < 0.05, **p < 0.01, ns: not significant at 0.05 level.*
OTC: over the counter

to the pharmacy staff and to rate, on a five-level Likert-type scale, their agreement with statements regarding the degree that certain pharmacy factors (location, staff, product range, additional services, membership programmes, anonymity/confidentiality, atmosphere, opening hours) affect their selection of pharmacy. The third section concerned the selection of OTC medicine. It contained multiple-choice questions assessing the confidence, spontaneity and specificity of the consumer's selection. Finally, the participants were asked to indicate the degree to which each of eight factors (the product's country of origin, manufacturing company, packaging, experience of previous use, advertisement, pharmacist's opinion, family or friends' opinion, price) affected their purchase of OTC medicines.

Data Analysis

The relationships between the demographic characteristics of the participants and their choice of pharmacy, as well as their purchase preferences of OTC medicine (research questions 1 and 2) were assessed using the chi-square test followed by Cramer's *V*, one-way analysis of variance (ANOVA) with Tukey's post hoc test and Spearman's rho correlation coefficient, where appropriate. Effect sizes for ANOVA were calculated with eta squared (η^2), where 0.01 represents a small effect size, 0.06 a medium effect size and 0.14 a large effect size. Spearman's correlation coefficients were computed to identify the inter-correlations among factors influencing the choice of pharmacy and OTC products (research question 3; Table 1). All tests were two tailed, and the significance level was set at 5%. Statistical analyses were performed using the IBM Statistical Package for Social Science statistics software, version 21.

RESULTS

Inter-correlations Among Factors Influencing the Choice of Pharmacy and Purchase of Otc Medicine

The relationships among the factors influencing the choice of pharmacy and the factors influencing the purchase of OTC medicine (research question 3) were examined. Table 1 presents the Spearman's inter-correlations among the factors influencing the choice of pharmacy. All values were positive and ranged from very low to moderate. The highest correlations were observed between the following pairs of factors: membership programme and additional services that are provided, rho (300) = 0.46, $p < 0.01$; the store's atmosphere and anonymity/confidentiality, rho (300) = 0.41, $p < 0.01$; the store's atmosphere and pharmacy staff, rho (300) = 0.40, $p < 0.01$; the store's atmosphere and the product range, rho (300) = 0.39, $p < 0.01$; and the additional services and the product range, rho (300) = 0.40, $p < 0.01$.

The correlations among factors influencing the purchase of OTC medications are shown in Table 1. The strongest correlation was found between the product's country of

origin and the manufacturing company, rho (300) = 0.77, $p < 0.01$. The packaging was found to moderately correlate with both the product's country of origin, rho (300) = 0.36, $p < 0.01$, and the manufacturing company, rho (300) = 0.38, $p < 0.01$. Moreover, family's/friends' opinion was also found to moderately correlate with the product's advertisement, rho (300) = 0.38, $p < 0.01$.

Effect of the Demographic Characteristics of the Participants on the Choice Of Pharmacy

While examining the differences in the choice of pharmacy and the factors influencing this choice in relation to the demographic characteristics of the participants (research question 1), statistically significant relationships emerged for their educational level, occupation and age and are reported in detail below.

The relationship between the participants' educational level and answers to the question, 'I tend to make my purchases in a single pharmacy', was found to be statistically significant (χ^2 (10) = 24.90, $p = 0.006$, Cramer's $V = 0.29$) (Table 2). Those who have completed primary school or lower secondary (gymnasium) reported that they always favour making their purchases in a single pharmacy (73.3% and 46.7%, respectively). On the contrary, respondents with higher completed levels of education tend to do so as well, but only most of the time (45.5%–56%).

The relationship between occupation and answers to the question, 'I tend to make my purchases in a single pharmacy', was found to be statistically significant (χ^2 (12) = 68.41, $p < 0.001$, Cramer's $V = 0.338$) (Table 2). A significant percentage of students (40%), unemployed people (44.1%) and part-time workers (40%) tend to make their purchases in various pharmacies. This is in contrast to the frequency of retired people who always made their purchases in a single pharmacy (61.9%).

A significant relationship was also observed between the participants' occupation and their reported relationship with the pharmacy staff (χ^2 (12) = 47.08, $p < 0.001$, Cramer's V = 0.281). Students strongly preferred their relationship to be formal (80.0%), while others mostly wanted it to be familiar (Table 2).

The importance of the pharmacy staff was found to be significantly different across educational levels (F (5, 296) = 2.76, $p = 0.019$, $\eta^2 = 0.02$). Tukey's post hoc test revealed that the pharmacy staff is significantly more important to consumers who have completed gymnasium (lower secondary) (4.55) and attained a doctorate (4.50) than to those who have undergraduate-level (3.95) education (Table 3).

Results also revealed statistically significant differences in the importance of the pharmacy staff across occupations (F (6, 298) = 7.41, $p < 0.001$, $\eta^2 = 0.05$). Post hoc tests revealed that retired consumers (4.63) and homemakers (4.33) consider the pharmacy staff as a significantly more important factor than students (3.60), unemployed (3.63), part-time workers (3.90), full-time workers (4.18) and self-employed respondents (3.83) (Table 3).

Spearman's rho correlations were calculated between age and the eight factors affecting the choice of pharmacy. Low to moderate positive correlations were observed between age and the pharmacy staff, $rho(299) = 0.32$, $p < 0.01$, and between age and additional services that are provided, $rho(298) = 0.19$, $p < 0.01$. Therefore, older participants tend to rate these factors as significantly more important than younger participants.

Effect of the Demographic Characteristics of the Participants on the Purchase of Otc Medicine

This section summarises the purchase preferences of OTC medicines and the factors influencing this purchasing behaviour in relation to the demographic characteristics of the participants (research question 2).

A significant relationship was observed between the participants' educational level and purchase patterns of OTC medicine (χ^2 (10) = 22.90, $p = 0.011$, Cramer's $V = 0.197$). A vast majority of primary school graduates (80%) reported that they know exactly what they need when they purchase OTC medicine, in comparison with respondents of other educational levels who are evenly split between those who know 'exactly' and those who know 'approximately' what they need (Table 2).

A significant relationship was found between gender and unscheduled purchases of OTC medicine (χ^2 (2) = 6.32, $p = 0.042$, Cramer's $V = 0.146$). As shown in Table 2, most female respondents tend to sometimes make unscheduled purchases of OTCs (52.5%), whereas most male respondents tend to never do so (55.3%).

There is a marginal statistically significant relationship between occupation and answers to the question, 'When I am in a pharmacy, I tend to make unscheduled purchases of OTC medicine' (χ^2 (12) = 21.25, $p = 0.047$, Cramer's $V = 0.189$). More specifically, the majority of homemakers (59.5%) and part-time workers (60.0%) reported that they sometimes make unscheduled purchases of OTC medicine, whereas the majority of retired people (56.6%) claimed that they never tend to do so (Table 2).

The importance attributed to the product's advertisement, as a factor that potentially influences the purchase of OTC medicine (Table 3), was statistically significantly different across occupations (F (6, 299) = 2.15, $p = 0.048$, $\eta^2 = 0.01$). Tukey's post hoc test revealed that this factor is considered significantly more important by full-time workers (3.28) than self-employed participants (2.33).

In addition, the results of ANOVA indicated significant differences in the importance given to the pharmacist's opinion across different occupation levels (F (6, 298) = 2.93, $p = 0.009$, $\eta^2 = 0.02$). Tukey's post hoc tests revealed that homemakers (4.52) and retired respondents (4.47) consider this factor as significantly more important than students

Table 2. (a) Tendency to make purchases in a single pharmacy across educational level and occupation; (b) relationship with the pharmacy staff across occupation; (c) purchase patterns of over-the-counter medicine across educational level; (d) unscheduled purchases of over-the-counter medicine across gender and occupation.

(a) 'I tend to make my purchases in a single pharmacy'				
Educational level	Yes, always	Yes, most of the time	No, I make my purchases in various pharmacies	Total
Primary school	11 (73.3%)	3 (20.0%)	1 (6.7%)	15 (100.0%)
Gymnasium (lower secondary)	21 (46.7%)	19 (42.2%)	5 (11.1%)	45 (100.0%)
Lyceum (upper secondary)	32 (29.1%)	50 (45.5%)	28 (25.5%)	110 (100.0%)
University	24 (26.7%)	41 (45.6%)	25 (27.8%)	90 (100.0%)
Post-graduate	3 (12.0%)	10 (56.0%)	8 (32.0%)	25 (100.0%)
Doctorate	3 (25.0%)	6 (50.0%)	3 (25.0%)	12 (100.0%)
Total	94 (31.6%)	133 (44.8%)	70 (23.6%)	297 (100.0%)
Occupation	Yes, always	Yes, most of the time	No, I make my purchases in various pharmacies	Total
Student	1 (6.7%)	8 (53.3%)	6 (40.0%)	15 (100.0%)
Unemployed	5 (14.7%)	14 (41.2%)	15 (44.1%)	34 (100.0%)
Homemaker	14 (34.1%)	18 (43.9%)	9 (22.0%)	41 (100.0%)
Part-time worker	4 (20.0%)	8 (40.0%)	8 (40.0%)	20 (100.0%)
Full-time worker	16 (18.4%)	46 (52.9%)	25 (28.7%)	87 (100.0%)
Self-employed	3 (16.7%)	10 (55.6%)	5 (27.8%)	18 (100.0%)
Retired	52 (61.9%)	30 (35.7%)	2 (2.4%)	84 (100.0%)
Total	95 (31.8%)	134 (44.8%)	70 (23.4%)	299 (100.0%)
(b) 'Regarding my relationship with the pharmacy staff'				
Occupation	I want it to be familiar	I want it to be formal	I don't want the staff to recognise me	Total
Student	3 (20.0%)	12 (80.0%)	0 (0.0%)	15 (100.0%)
Unemployed	17 (50.0%)	14 (41.2%)	3 (8.8%)	34 (100.0%)
Homemaker	28 (68.3%)	11 (26.8%)	2 (4.9%)	41 (100.0%)
Part-time worker	13 (65.0%)	7 (35.0%)	0 (0.0%)	20 (100.0%)
Full-time worker	57 (65.5%)	25 (28.7%)	5 (5.7%)	87 (100.0%)
Self-employed	13 (72.2%)	5 (27.8%)	0 (0.0%)	18 (100.0%)
Retired	75 (89.3%)	7 (8.3%)	2 (2.4%)	84 (100.0%)
Total	206 (68.9%)	81 (27.1%)	12 (4.0%)	299 (100.0%)
(c) 'When I purchase an over-the-counter drug, I usually'				
Educational level	I know exactly what I need	I know approximately what I need	I don't know what I need	Total
Primary school	12 (80.0%)	0 (0.0%)	3 (20.0%)	15 (100.0%)
Gymnasium (lower secondary)	23 (51.1%)	21 (46.7%)	1 (2.2%)	45 (100.0%)
Lyceum (upper secondary)	57 (51.4%)	49 (44.1%)	5 (4.5%)	111 (100.0%)
University	42 (48.3%)	43 (49.4%)	2 (2.3%)	87 (100.0%)
Post-graduate	14 (56.0%)	11 (44.0%)	0 (0.0%)	25 (100.0%)
Doctorate	7 (58.3%)	5 (41.7%)	0 (0.0%)	12 (100.0%)
Total	155 (52.5%)	129 (43.7%)	11 (3.7%)	295 (100.0%)

Continued Table 2. (a) Tendency to make purchases in a single pharmacy across educational level and occupation; (b) relationship with the pharmacy staff across occupation; (c) purchase patterns of over-the-counter medicine across educational level; (d) unscheduled purchases of over-the-counter medicine across gender and occupation.

(d) 'When I am in a pharmacy, I tend to make unscheduled purchases of over-the-counter drugs'				
Gender	Yes, often	Yes, sometimes	No, never	Total
Female	8 (4.4%)	96 (52.5%)	79 (43.2%)	183 (100.0%)
Male	8 (7.0%)	43 (37.7%)	63 (55.3%)	114 (100.0%)
Total	16 (5.4%)	139 (46.8%)	142 (47.8%)	297 (100.0%)
Occupation	Yes, often	Yes, sometimes	No, never	Total
Student	0 (0.0%)	7 (46.7%)	8 (53.3%)	15 (100.0%)
Unemployed	4 (12.1%)	14 (42.4%)	15 (45.5%)	33 (100.0%)
Homemaker	5 (11.9%)	25 (59.5%)	12 (28.6%)	42 (100.0%)
Part-time worker	1 (5.0%)	12 (60.0%)	7 (35.0%)	20 (100.0%)
Full-time worker	0 (0.0%)	42 (48.8%)	44 (51.2%)	86 (100.0%)
Self-employed	1 (5.6%)	8 (44.4%)	9 (50.0%)	18 (100.0%)
Retired	5 (6.0%)	31 (37.3%)	47 (56.6%)	83 (100.0%)
Total	16 (5.4%)	139 (46.8%)	142 (47.8%)	297 (100.0%)

(3.93), self-employed (4.00) and unemployed respondents (4.05) (Table 3).

The importance of the product's country of origin was found to be significantly different across levels of monthly personal income (F (5, 297) = 2.78, p = 0.018, η^2 = 0.02). Tukey's post hoc tests showed that the product's country of origin is significantly more important to respondents with an income of 801–1200 € (3.93) than to those with an income below 400 € (3.39) (Table 4).

Furthermore, significant difference was found in the importance attributed to the product's manufacturing company across income levels (F (5, 296) = 4.03, p = 0.001, η^2 = 0.04). Tukey's post hoc tests indicated that respondents with a higher income (more than 801 €) tend to consider the medicine's manufacturing company as more important than those with a lower income (less than 800 €) (Table 4).

Finally, the results of ANOVA suggested the existence of significant differences in the importance attributed to the pharmacist's opinion across income levels (F (5, 298) = 2.41, p = 0.036, η^2 = 0.02). Tukey's post hoc tests revealed that respondents with an income between 400 and 1200 € consider this factor as more important than those with an income between 1201 and 1600 € (Table 4).

Moreover, a low negative correlation was found between age and the product's advertisement (rho (300) = -0.14, $p < 0.05$) and a positive correlation between age and the pharmacist's opinion (rho (299) = 0.22, $p < 0.01$).

DISCUSSION

Effect of the Demographic Characteristics of the Participants on the Choice of Pharmacy

The results presented indicate that in the urban area of Thessaloniki, Greece, pharmacy customer behaviour regarding the choice of pharmacy is influenced by demographic factors, particularly age, occupation and education. In agreement with our findings, in the pharmacy literature, pharmacy customer behaviour has been reported to be greatly influenced by demographic factors (Castaldo et al., 2016; Kouwenberg & Jaiboon, 2019; Rabbanee et al., 2015; Shiyanbola et al., 2016).

A recent study suggests that a large portion of the urban residents of Thessaloniki have established customer loyalty to a specific community pharmacy (77%), as well as a preference for a familiar relationship with the pharmacy staff (69%) (Kevrekidis et al., 2018). The results of the present study suggest that, among these consumers, retired consumers and consumers of low educational level particularly display a significantly higher level of customer loyalty to a particular pharmacy.

These findings are compatible with past literature indicating high pharmacy patronage among elderly customers (Rabbanee et al., 2015). This, in turn, may suggest that the relationship between the pharmacist and elderly customers is trusting. The customer–pharmacist interpersonal relationship has been reported to be critical in the retail pharmacy sector (Antunes et al., 2015; Athavale, et al., 2015; Castaldo et al., 2016; Minarikova et al., 2016b), especially with regard to elderly customers (Shiyanbola et al., 2016).

Table 3. Importance of the (a) pharmacy staff in the choice of pharmacy across educational level and occupation, importance of the (b) product's advertisement and the (c) pharmacist's opinion in the choice of over-the-counter medicine across occupation.

Variable	Educational level	*n*	Mean	SD
(a) Pharmacy staff	Primary school	15	4.33	1.39
	Gymnasium (lower secondary)	45	4.55	0.69
	Lyceum (upper secondary)	110	4.20	0.92
	University	90	3.95	1.06
	Post-graduate	25	4.16	0.80
	Doctorate	12	4.50	0.52
	Total	297	4.19	0.96
	Occupation			
(a) Pharmacy staff	Student	15	3.60	1.12
	Unemployed	33	3.63	1.24
	Homemaker	42	4.33	0.75
	Part-time worker	20	3.90	0.91
	Full-time worker	87	4.18	0.99
	Self-employed	18	3.83	0.78
	Retired	84	4.63	0.67
	Total	299	4.20	0.96
	Occupation			
(b) Product's advertisement	Student	15	2.93	1.22
	Unemployed	34	2.82	1.35
	Homemaker	42	2.92	1.31
	Part-time worker	20	3.05	1.23
	Full-time worker	87	3.28	1.04
	Self-employed	18	2.33	1.13
	Retired	84	2.84	1.19
	Total	300	2.97	1.20
(c) Pharmacist's opinion	Student	15	3.93	0.59
	Unemployed	34	4.05	0.98
	Homemaker	42	4.52	0.77
	Part-time worker	20	4.20	0.69
	Full-time worker	86	4.31	0.77
	Self-employed	18	4.00	0.76
	Retired	84	4.47	0.71
	Total	299	4.31	0.78

SD: standard deviation

The observation that students strongly preferred for their relationship with the pharmacy staff to be formal, as opposed to the remaining respondents, could be attributed to a lack of interest for a familiar relationship due to their age difference with the staff or the fact that these younger consumers tend to be healthier. By not having to manage as many chronic conditions, the members of this demographic segment are less likely to constitute regular pharmacy customers or to have a strong motivation to establish a good rapport with the pharmacist.

The pharmacy's location, opening hours, pharmacy staff and anonymity/confidentiality have previously been reported to be among the most important factors that influence the choice of pharmacy of residents of the metropolitan area of Thessaloniki, whereas the availability of a membership programme, the provision of additional services, the

Table 4. Importance of the (a) product's country of origin, (b) manufacturing company and (c) the pharmacist's opinion in the choice of over-the-counter medicine across income level.

Variables	Income	*n*	Mean	SD
(a) Product's country of origin	Under 400 €	86	3.39	1.22
	400–800 €	96	3.48	1.20
	801–1200 €	77	3.93	1.13
	1201–1600 €	24	3.91	0.88
	1601–2000 €	9	3.55	1.13
	Over 2000 €	6	4.33	0.81
	Total	298	3.63	1.17
(b) Product's manufacturing company	Under 400 €	86	3.29	1.28
	400–800 €	95	3.24	1.26
	801–1200 €	77	3.87	1.03
	1201–1600 €	24	3.87	0.89
	1601–2000 €	9	3.88	1.16
	Over 2000 €	6	4.16	0.98
	Total	297	3.50	1.20
(c) Pharmacist's opinion	Under 400 €	86	4.18	0.86
	400–800 €	96	4.44	0.73
	801–1200 €	78	4.42	0.71
	1201–1600 €	24	4.04	0.75
	1601–2000 €	9	4.22	0.83
	Over 2000 €	6	3.83	0.98
	Total	299	4.31	0.78

SD: standard deviation

product range and the store's atmosphere are among the least important (Kevrekidis et al., 2018). The results of the present study indicate that the importance of the pharmacy staff's quality for the choice of pharmacy depends on the demographic characteristics of consumers in Thessaloniki; in particular, this criterion appeared to be the most important for elderly consumers, consumers of low and high educational level, as well as retired consumers and homemakers.

The above findings, which seem to be incongruous with regard to the educational level, could possibly be interpreted as consumers of different demographic characteristics emphasising different aspects of the quality of the pharmacy staff. This explanation is supported by the findings of earlier studies, which illustrated that consumers of different demographic categories favour different aspects of the pharmacy staff quality (Shiyanbola et al., 2016; Villako & Raal, 2007). For instance, older adults identify a quality pharmacy as one in which the pharmacist provides patient-centred care (Shiyanbola et al., 2016). Quick service has been found to be valued by younger customers, with that appraisal decreasing with age (Villako & Raal; 2007).

The observation that the perceived importance of additional services that are offered by the pharmacist appears to increase with the consumers' age could be related to the fact that the elderly might have a growing demand for pharmaceutical services due to age-related health challenges (Francis et al., 2005; Pelicano-Romano et al., 2015).

The perceived importance of the pharmacy's atmosphere was found to intercorrelate with that of the provision of anonymity and confidentiality, the pharmacy staff and the available range of products. The pharmacy staff (El Hajj et al., 2011; Merks et al., 2014; Villako & Raal, 2007; Wirth et al., 2010) and the provision of a good range of products and services (El Hajj et al., 2011; Villako & Raal, 2007) have been independently shown to be among the primary factors of pharmacy choice, and both functional experience and hedonic experience factors have been found to positively correlate with consumer satisfaction and with loyalty and purchases (Gavilan et al., 2014). Castaldo et al. (2016) have also found that equivalent retail-level levers, such as the store's environment, the product assortment and communication, indirectly influence trust in pharmacies. Therefore, these may simply be factors that are consistently valued by consumers who seek pharmacies that can inspire their loyalty. Alternatively, the pharmacy's layout and atmosphere could be set up in such a way as to make finding sought products or products of interest more convenient (Emmett et al., 2006), achieve privacy (Hattingh et al., 2015) and encourage communication (De Vera et al., 2018).

An inter-correlation between the provision of additional services and a membership programme was also found. Both features could reward health-conscious regular customers, the former in terms of continuous lifestyle management and the latter in terms of incentivising repeat purchases, possibly of self-medication products, both of which can be broadly tied to self-care.

Effect of the Demographic Characteristics of the Participants on the Purchase Of Otc Medicine

Kevrekidis et al. (2018) investigated the consumers' preferences concerning the selection of OTC medicines in the metropolitan area of Thessaloniki and noted the reported tendency of about half of the respondents to know exactly what they need when purchasing OTC medicines and of most of them to 'never' or only 'sometimes' make unscheduled OTC purchases, as well as frequently favouring a specific product from a certain OTC medicine category. The results of the present study, in accordance with preceding information (Paddison & Olsen, 2008), indicate that the demographic characteristics of these consumers influence their perception of OTC products.

In particular, the results presented indicate that the respondents who had only completed primary school and

retired participants were more decisive consumers than those of higher educational levels, with their vast majority claiming that they know exactly what product they need during their shopping. When taken at face value, this decisiveness appears to be counterintuitive and incompatible with the fact that educated people are more knowledgeable and competent in making OTC purchase choices as well as less likely to trust their physicians (Figueiras et al, 2000), as well as with literature that indicates that a higher level of education is a predictor of direct product requests (Collins et al., 2020). Nevertheless, as these patients with a higher education are more likely to self-medicate when suffering from an acute disorder (Figueiras et al., 2000), they may also simply be more willing to experiment with their purchases while engaging in self-care behaviours and being more open to a wider variety of OTC products, including ones with which they have no prior experience.

While participants tend not to make completely compulsive OTC purchases (Kevrekidis et al., 2018), women were more likely to do so, as they reported a higher likelihood of making unscheduled purchases of OTC medicines. This is in line with the fact that women are more self-care oriented (Papakosta et al., 2014), and that they use more types of medicines (Zadoroznyj & Svarstad, 1990). Additionally, self-medication is more prevalent among them (Figueiras et al, 2000; Ya-Ning, 2006). While there is little consensus in the literature about why it is so, there may be biomedical, social (Zadoroznyj & Svarstad, 1990) and cultural elements (Figueiras et al., 2000) that factor into this tendency.

According to Kevrekidis et al. (2018), the most influential factors in the purchase of OTC medicines for the residents of the urban area of Thessaloniki are experience of previous use and the pharmacist's opinion, followed by product price, the product's country of origin and manufacturer; on the contrary, the packaging, the product's advertisement and the opinion of friends or family were reported as the least important factors in an OTC purchase. The results of the present study indicate that respondents with higher incomes tend to place more importance on a product's country of origin and manufacturing company than those in lower brackets. It would appear that by judging such non-medical product features, they are looking for a more premium and reputable product, or at the very least take more care in finding a 'proper' one. The country of origin has been reported to drive purchase decisions (Kauppinen-Räisänen et al., 2012; Sanyal & Datta, 2011; Temechewu, 2020). A domestic company would especially act as a risk reduction cue (Pecotich & Ward, 2007). A study conducted in Finland showed that between two equally priced choices, consumers would choose the domestic product (Honkanen, 2013). Moreover, well-known brands associated with high perceived quality can also act as risk reduction cues in OTC drugs (Aufegger et al., 2021).

In addition, the product's country of origin, manufacturing company and packaging appeared to be interrelated attributes of OTC product choice. A drug's package identifies the country and company by displaying their information and can also serve as a means for the aforementioned search for a reputable product, with the consumers essentially 'judging a book by its cover'.

The pharmacist's opinion, which is a medical criterion, carried more weight with low-to-middle income brackets, instead. One aspect of this might be the role that pharmacists play by recommending cheaper products to price-sensitive consumers (Ricks & Mardanov, 2012). Nevertheless, homemakers and retirees appeared to value the pharmacist's opinion more than students, self-employed and unemployed consumers. Considering the latter, the association between occupation and the importance of the pharmacist's opinion could not be exclusively linked to price-sensitive consumers. Homemakers and retirees are largely made up of older consumers, and therefore, the previously described relationship of trust between pharmacists and older consumers, who have a reported preference towards patient-centred care (Shiyanbola et al, 2016), could play a role in rendering homemakers and retired consumers more receptive towards the pharmacist's opinion. Self-employed workers were also more likely to discount the importance of the product's advertisement, another external source that could impact the information search stage of the buyer decision process.

The product's advertisement moderately correlated with the opinion of family and friends. This could be the case of laymen, without previous experience with a certain product, seeking information from such sources, which may not come from caregivers, but are nonetheless easily accessible. The desired impact of a direct-to-consumer advertisement of an OTC is dependent on whether the ad was informative (Spake & Joseph, 2007). These advertisements could function by supporting the word of mouth that has already been received by family and friends. For interviewees of Paddison and Olsen (2008), the consumer's interpersonal communication with family and friends was vital due to its credibility and empathy. In fact, some preferred certain painkillers due to a sense of family tradition. For them, the effect of advertisements was that of reinforcement rather than conversion to a different brand.

Loyalty appears to develop through time and interaction with the pharmacy staff themselves, as consumers tended to appreciate them and trust the pharmacist's opinion more, the older they get. With age comes increased morbidity, an increasing tendency to visit the pharmacy as well as increased importance of additional services; consumers undergo point-of-care testing (such as glucose, cholesterol and blood pressure measurements). A previous study has found that younger community pharmacy customers (<40 y.o.) were more likely to exhibit information-seeking behaviour prior to visits to the pharmacy (Burghle et al., 2020). This cohort may rely less on the pharmacy staff's input into self-care decisions or the choice of OTC products, and therefore value it less than older customers. There was a low negative correlation between respondent age and the product's advertisement.

The younger the people were, the more they relied on advertisements. A survey by Paddison and Olsen (2008) found that younger consumers are more likely to recount adverts.

MANAGERIAL IMPLICATIONS

As noted earlier, the most important factors for pharmacy and OTC medicine selection for the Thessaloniki sample as a whole are the pharmacy's location, opening hours, staff and experience of previous use, the pharmacist's opinion and product price, respectively (Kevrekidis et al., 2018). In the context of a pharmacy looking to develop a marketing strategy that focuses on attracting and maintaining customers on the basis of their demographic characteristics, the manager is facing a conundrum on whether to focus on developing the loyalty and maintaining the patronage of demographic groups who already make their purchases in a single pharmacy, such as older, retired consumers with a lower educational level or to, in competition with other pharmacies, court groups which are comparatively more likely to make purchases in various pharmacies, such as students, unemployed, part-time employed consumers and those who have attained higher educational status. It could be argued that the former should act as a foundation of loyal consumers and that the latter should be more actively pursued so as to expand the establishment's patronage. In addition, groups that are less certain in their purchases and relatively more likely to make unscheduled sales of OTC products, such as women, homemakers, part-time workers and those who have graduated from secondary education or higher, could be the object of managerial focus, in order to increase the volume of OTC product sales.

Emphasis should be placed on the pharmacy staff's quality and ability to offer an interaction that focuses on the patient's needs and helps build a rapport of familiarity in order to facilitate the patronage of the elderly. The pharmacist's advice for OTC medicines should be integrated into this interaction, and additional services should be made available. Efforts made to attract younger consumers and students could focus on the OTC medicine's advertisement and quicker and more formal service.

A pharmacy marketing plan directed towards women and other groups, such as homemakers and part-time workers who are more likely to make unscheduled OTC purchases, should consider expanding the product range of OTC medicines, while to engage consumers of both low and post-graduate educational level, the pharmacy staff's quality should be prioritised.

Retired consumers and homemakers could be drawn by focusing on the pharmacy staff and the pharmacist's advice for OTC medicine selection. OTC medicine advertisements could be utilised to attract full-time workers.

Finally, the pharmacy marketing plan to attract and develop loyalty among consumers of lower personal income should consider the integration of pharmacist advice for OTC medicines, and for those with higher income, to have available products of known brands, possibly non-generic, and domestic, or of other countries of origin that are considered to be more reputable among consumers.

CONCLUSIONS

In conclusion, the results presented indicate that demographic characteristics can potentially influence the consumer behaviour of community pharmacy customers. In particular, age, occupation and the educational level of consumers appear to have a marked effect on their preferences regarding the selection of pharmacy, and along with gender and personal income, on their preferences regarding the selection of OTC medicines.

The present study provides insight into the consumer behaviour of community pharmacy customers. Our findings could be utilised in order for community pharmacies within a competitive market environment to develop an effective marketing strategy for engaging new clients, maintaining their patronage and developing customer loyalty. Future research could investigate consumer behaviour in the general population of Greece and in other countries comparable in terms of healthcare systems and economic frameworks, by helping to generalise the results and extract safer conclusions. Further investigation could, in the context of developing efficient and effective community pharmacy marketing strategies, take the rate of self-medication usage and self-care orientation as well as their relationship to demographic characteristics into account. It would also be of interest to further explore what kind of qualities or behaviours from the pharmacy staff facilitate better care, self-medication outcomes and customer satisfaction and whether these differ among demographic groups.

ACKNOWLEDGEMENT

Thanks are due to the two anonymous referees for their helpful comments.

References

1] Antunes LP, Gomes JJ, Cavaco AM. How pharmacist-patient communication determines pharmacy loyalty? Modeling relevant factors. Res Social Adm Pharm. 2015;11: 560-570.

[2] Arneson DL, Jacobs EW, Scott DM, Murray WJ. Patronage motives of community pharmacy patrons. J Pharm Mark Manage. 2011;4:3-22.

[3] Athanasiadis T, Maniatis G, Ntemousis F, Health Economics Unit, Foundation for Economic & Industrial Research (IOBE). The pharmaceutical market in Greece. Annual Report 2012. Athens, Greece: IOBE; 2013.

[4] Athavale AS, Banahan BF, Bentley JP, West-Strum DS. Antecedents and consequences of pharmacy loyalty behavior. Int J Pharm Healthc Mark. 2015;9:36-55.

[5] Aufegger L, Yanar C, Darzi A, Bicknell C. The risk-value trade-off: price and brand information impact consumers' intentions to purchase OTC drugs. J Pharm Policy Pract. 2021;14.

[6] Beales H, Jagis MB, Salop SC, Staelin R. Consumer search and public policy. J Consum Res. 1981;8:11-22.

[7] Bennett PD. AMA Dictionary of Marketing Terms. Lincolnwood, IL: NTC Business Books; 1995.

[8] Boström K. Consumer behaviour of pharmacy customers-choice of farmacy and over-the-counter medicines [dissertation]. Helsinki, Finland: Arcada University of Applied Sciences; 2011.

[9] Burghle A, Abrahamsen B, Lundby C, Rossing C, Hansen RN, Nørgaard LS, Pottegård A. Customers' information seeking behavior prior to community pharmacy visits: A community pharmacy survey. Res Social Adm Pharm. 2020;16:1442-1446.

[10] Castaldo S, Grosso M, Mallarini E, Rindone M. The missing path to gain customers loyalty in pharmacy retail: The role of the store in developing satisfaction and trust. Res Social Adm Pharm. 2016;12:699-712.

[11] Chan V, Tran H. Purchasing over-the-counter medicines from Australian pharmacy: What do the pharmacy customers value and expect? Pharm Pract (Granada). 2016;14:782.

[12] Collins JC, Schneider CR, El-Den S, Moles, RJ. Self-care–seeking behaviors in the community pharmacy: A cross-sectional exit survey of Australian consumers. J Am Pharm Assoc. 2020;60:827-834.

[13] De Vera MA, Campbell NKJ, Chhina H, Galo JS, Marra C. Practical strategies and perceptions from community pharmacists following their experiences with conducting pharmacy practice research: a qualitative content analysis. Int J Pharm Pract. 2018;26:302-309.

[14] Dean K. Lay care in illness. Soc Sci Med. 1986;22:275-284.

[15] Duncan CP. Consumer Market Beliefs: A Review of the Literature and an Agenda for Future Research. Adv Consum Res. 1990;17:729-735.

[16] El Hajj MS, Salem S, Mansoor H. Public's attitudes towards community pharmacy in Qatar: A pilot study. Patient Prefer Adherence. 2011;5:405-422.

[17] Emmett D, Paul DP, Chandra A, Barrett H. Pharmacy Layout: What Are Consumers' Perceptions?. J Hosp Mark Public Relations. 2006;17: 67-77.

[18] European Commission. Report of the working group on promoting good governance of non-prescription drugs in Europe. Ref Ares (https://ec.europa.eu/docsroom/documents/7623?locale=en). Published November 19, 2014. Accessed March 25, 2021.

[19] Figueiras A, Caamaño F, Gestal-Otero JJ. Sociodemographic factors related to self-medication in Spain. Eur J Epidemiol. 2001;6:19-26.

[20] Francis SA, Barnett N, Denham M. Switching of prescription drugs to over-the-counter status: Is it a good thing for the elderly? Drugs Aging, 2005;22:361-370.

[21] Gavilan D, Avello M, Abril C. Shopper marketing: A new challenge for Spanish community pharmacies. Res Social Adm Pharm. 2014;10:125-136.

[22] Gyaneshwari K. A study on consumer behavior towards OTC products. Int J Manag Soc Sci Res Rev. 2015;1:134-137.

[23] Habash R, Al-Dmour H. Factors influencing the intention to buy over-the-counter medicines: empirical study. Int J Pharm Healthc Mark.2020;14.

[24] Hanna L-A, Hughes CM. Public's views on making decisions about over-the-counter medication and their attitudes towards evidence of effectiveness: A cross-sectional questionnaire study. Patient Educ Couns. 2011;83:345-351.

[25] Haramiova Z, Kobliskova Z, Soltysova J. Purchase of prescription and OTC medicines in Slovakia: factors influencing patients' expectations and satisfaction. Braz J Pharm Sci. 2017;53:e16035

[26] Hattingh HL, Emmerton L, Ng Cheong Tin P, Green C. Utilization of community pharmacy space to enhance privacy: a qualitative study. Health Expect. 2015;19:1098-1110.

[27] Hellenic Statistical Authority. 2011 Population and Housing Census. Economic characteristics of the resident population of Greece. Press Release; Piraeus, Greece, 2014.

[28] Honkanen E. Understanding Brand Relationships with OTC. Pharmaceutical Products and Corporate Brand Experienced by the Consumers: Case Orion Oyj [dissertation]. Espoo, Finland: Aalto University, School of Business; 2013.

[29] International Monetary Fund (IMF). Greece: Letter of Intent, Memorandum of Economic and Financial Policies, and Technical Memorandum of Understanding; 2014.

[30] Jakovljevic MB, Souliotis K. Pharmaceutical expenditure changes in Serbia and Greece during the global economic recession. SEEJPH, 2016. Published April 19, 2016. Accessed March 25, 2021.

[31] Kauppinen-Räisänen H, Owusu R, Bamfo B. Brand salience of OTC pharmaceuticals through package appearance. Int J Pharm Healthc Mark, 2012;6:230-249.

[32] Kevrekidis DP, Mináriková D, Markos A, Malovecka I, Minarik P. Community pharmacy customer segmentation based on factors influencing their choice of pharmacy and over-the-counter drugs. Saudi Pharm J. 2018;26:33–43.

[33] Khan M. Consumer Behaviour and Advertising Management. New Dehli, India: New Age International; 2006.

[34] Kotler P, Keller KL. Marketing Management, Fourteenth Edition. Upper Saddle River, NJ: Prentice Hall; 2012.

[35] Kouwenberg R, Jaiboon R. Factors influencing consumers on selecting a particular modern phamacy in Bangkok [dissertation]. Bangkok, Thailand: College of Management Mahidol University; 2020.
[36] Krishnan HS, Schaefer M. Evaluation of the impact of pharmacist's advice giving on the outcomes of self-medication in patients suffering from dyspepsia. Pharm World Sci. 2000;22:102-108.
[37] Lessenger JE, Feinberg SD. Abuse of prescription and over-the-counter medications. J Am Board Fam Med. 2008;21:45-54.
[38] Lodorfos GN, Mulvana KL, Temperley J. Consumer behaviour: Experience, price, trust and subjective norms in the OTC pharmaceutical market. Innov Market. 2006;2:41-59.
[39] Martins AP, da Costa Miranda A, Mendes Z, Soares MA. Self-medication in a Portuguese urban population: a prevalence study. Pharmacoepidemiol Drug Saf. 2002;11:409-414.
[40] Merks P, Kaźmierczak J, Olszewka AE, Koltowska-Häggstrom M. Comparison of factors influencing patient choice of community pharmacy in Poland and in the UK, and identification of components of pharmaceutical care. Patient Prefer Adher. 2014;14:715-726.
[41] Minarikova D, Malovecka I, Foltan V. Patient choice of pharmacy and satisfaction with pharmaceutical care – Slovak regional comparison. Farmacia. 2016;64:473–480.
[42] Minarikova D, Malovecka I, Lehocka L, Snopkova M, Foltan V. The Assessment of Patient Satisfaction and Attendance of Community Pharmacies in Slovakia. Eur Pharm J. 2016;63:23-29.
[43] Moore ES, Wilkie WL, Lutz RJ. Passing the Torch: Intergenerational Influences as a Source of Brand Equity. J Mark. 2002;66:17-37.
[44] Narayana CL, Markin RJ. Consumer Behavior and Product Performance: An Alternative Conceptualization. J Mark. 1975;39:1-6.
[45] Ogura S, Jakovljevic M. Health Financing Constrained by Population Aging-An Opportunity to Learn from Japanese Experience. SJECR. 2014;15:175-181.
[46] Paddison A, Olsen K. Painkiller purchasing in the UK. An exploratory study of information search and product evaluation. Int J Pharm Healthc Mark. 2008;2:284-306.
[47] Papakosta M, Zarvas D, Niakas D. Investigating factors of self-care orientation and self-medication use in a Greek rural area. Rural Remote Health. 2014;14: 23-49.
[48] Patterson JA, Holdford DA, Spencer E, Harpeb SE. Patient preferences for objective quality metrics during community pharmacy selection: A discrete choice experiment. Res Social Adm Pharm. 2019;15:641–649.
[49] Pecotich A, Ward S. Global branding, country of origin and expertise: an experimental evaluation, Int Market Rev. 2007;24:271-296.
[50] Pelicano-Romano J, Neves MR, Amado A, Cavaco AM. Do community pharmacists actively engage elderly patients in the dialogue? Results from pharmaceutical care consultations. Health Expect. 2015;18:1721-1734.
[51] Putsis WPJ, Narasimhan S. Buying or just browsing? The duration of purchase deliberation. J Mark Res. 1994;31:393-402.
[52] Rabbanee FK, Burford O, Ramaseshan B. Does employee performance affect customer loyalty in pharmacy services? J Serv Theor Pract. 2015;25:725-743.
[53] Ricks J, Mardanov I. The effect of pharmacists on drug purchasing behavior of price-sensitive consumers, J Med Mark. 2012;12:177-187.
[54] Sanyal S, Datta S. The effect of country of origin on brand equity: and empirical study on generic drugs. J Prod Brand Manag. 2011;20:130-140.
[55] Schmidt RA, Pioch EA. Community pharmacies under pressure: issues of deregulation and competition. Int J Retail Distrib Manag. 2004;32:354-357
[56] Shiyanbola, OO, Mott DA, Croes KD. The structural and process aspects of pharmacy quality: older adults perceptions. Int J Clin Pharm. 2016;38:96-106.
[57] Smith RE. Integrating information from advertising and trial: Processes and effects on consumer response to product information. J Mark Res. 1993;30:204-219.
[58] Solomon M, Bamossy G, Askegaard S, Hogg MK. Consumer Behaviour A European Perspective Third Edition. London, UK: Prentice Hall; 2006.
[59] Spake DF, Joseph M. Consumer opinion and effectiveness of direct-to-consumer advertising. J Consum Mark. 2007;24:283-293.
[60] Srivastava RK, Wagh S. Factors impacting consumer purchase behaviour for pharmaceutical products. Int J Healthc Manag. 2020;13:113-121.
[61] Temechewu MW. Factors Affecting Consumers' Purchase Decision of Over-The-Counter (OTC) Medicines: Empirical Evidences from Community Pharmacies in Ethiopia. Journal of Medicine, Physiology and Biophysics. 2020;65:8-25.
[62] Villako P, Raal A. A survey of Estonian consumer expectations from the pharmacy service and a comparison with the opinions of pharmacists. Pharm World Sci. 2007;29:546-550.
[63] Vogler S. Liberalization in the Pharmacy Sector. Paper presented at: Global Forum on Competition; March 18, 2014; Paris, France.
[64] Wazaify M, Shields E, Hughes CM, McElnay JC. Societal perspectives on over-the-counter (OTC) medicines. Fam Pract. 2005;22:170-176.
[65] Wertheimer AI, Serradell J. A discussion paper on self-care and its implications for pharmacists. Pharm World Sci. 2008;30:309-315.
[66] Wirth F, Tabone F, Azzopardi LM, Gauci M, Zarb-Adami M, Serracino-Inglott A. Consumer perception of the community pharmacist and community pharmacy services in Malta. J Pharm Health Serv Res. 2010;1:189-194.
[67] Ya-Ning L. Consumers' expectations of over-the-counter (OTC) medicines: Location of sale [dissertation]. Saskatoon, Canada: College of Pharmacy and Nutrition, University of Saskatchewan; 2006.
[68] Zadoroznyj M, Svarstad BL. Gender, Employment and Medication Use. Soc Sci Med. 1990;31:971-978.

sciendo

Eur. Pharm. J. 2021, 68(2), 41-48.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2021-0018

EUROPEAN PHARMACEUTICAL JOURNAL

Cultural Adaptation and Validation of the EORTC QLQ-BR45 to Assess Health-Related Quality of Life of Breast Cancer Patients

Original Paper

Ehab Barakat H.[1]✉, Hussein R.R.S.[2,3], Abdullah Elberry A.[4], Ahmed Zaki M., Elsherbiny Ramadan M.[5]

[1]*Clinical Pharmacy Department, Faculty of Pharmacy, Ahram Canadian University, Giza, Egypt*
[2]*Clinical Pharmacy Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt*
[3]*Clinical Pharmacy Department, Faculty of Pharmacy, Modern University for Technology and Information, Cairo, Egypt*
[4]*Clinical Pharmacology Department, Faculty of Medicine, Beni-Suef University, Beni-Suef, Egypt*
[5]*Medical Oncology Department, Faculty of Medicine, Beni-Suef University, Beni-Suef, Egypt*

Received 20 April, 2021, accepted 13 December, 2021

Abstract

Background: The European Organization for Research and Treatment of Cancer (EORTC) QLQ-BR23 is considered a premier module for breast cancer patients that is utilised synchronously with the core questionnaire. However, new and scalable treatments on breast cancer patients' quality of life (QoL) need a more accurate and comprehensive tool to be assessed. Therefore, the EORTC introduced the newly updated module EORTC QLQ-BR45. Hence, the current study aims to perform cultural adaptation, pilot testing and assessment of the psychometric properties of the Egyptian Arabic translation of the EORTC QLQ-BR45 module on Egyptian breast cancer patients.

Patients and Methods: First, a review of the existing Arabic translation and the modified preliminary translation was sent to a professional proofreader. Then, comprehensibility of the Egyptian Arabic translation was pilot tested on a sample of 13 breast cancer patients. Afterwards, 74 patients with proven locally advanced breast cancer receiving neoadjuvant chemotherapy at Beni-Suef University Hospital, Beni-Suef, Egypt were interviewed. A second interview was conducted post-surgery for patients receiving target therapy, endocrine therapy or radiotherapy. The psychometric properties of the EORTC QLQ-BR45 were assessed in terms of reliability, convergent and divergent validity.

Results: Adequate internal consistency reliability (Cronbach's α coefficients >0.7) was demonstrated for the questionnaire, except for body image scale (α = 0.51) and systemic therapy side effects scale (α = 0.63). Multi-trait scaling analysis exhibited acceptable convergent and divergent validity, and scaling success was observed for all questionnaire items.

Conclusion: The Egyptian Arabic version of the EORTC QLQ-BR45 module is valid and adequately reliable. These results support using the EORTC QLQ-BR45 in future breast cancer clinical trials.

Keywords *Breast cancer – Quality of life – Module development – EORTC QLQ-BR45 – Validation – Reliability*

INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women and the most prevalent cause of cancer mortality in females [1, 2]. Since the past decades, health-related quality of life (HRQoL) has been considered a main clinical outcome of cancer research reflecting patient-reported outcomes (PROs) [3]. The vital importance of PROs is the expression of patient satisfaction and endurance to the disease or treatment impacts on the patients' daily life. The HRQoL is a multidimensional structure that covers several major aspects such as disease-related and treatment-related symptoms, physical, psychological and social functioning [4]. Therefore, one of the main objectives in the treatment of breast cancer patients is to maintain their quality of life (QoL).

The European Organization for Research and Treatment of Cancer (EORTC) established an integrated framework for assessing the QoL among cancer patients. The EORTC

* *E-mail: hadeer.ehab7785@gmail.com*

QLQ-C30 version 1.0 module was introduced in 1993 as a core questionnaire used to assess the QoL among any type of cancer patients. Then, this core questionnaire was updated to version 2.0 and version 3.0 in 1997 and 2000, respectively. Version 3.0 is currently the standard version of QLQ-C30 [5]. Besides, EORTC QLQ-BR23 was developed in 1996 as a premier module to be used in conjunction with the core questionnaire for breast cancer patients [6]. Over the course of the last decade, newer therapeutic options were introduced for breast cancer. Whereas tamoxifen treatment was the gold standard in postmenopausal women for hormonal breast cancer therapy, aromatase inhibitors have become the first option in patients, although they suffer from new toxicities including arthralgia, bone loss and cognitive impairment [7, 8]. In addition, chemotherapeutic treatments have expanded to include taxanes and anthracyclines that have become standard treatments for breast cancer patients. Moreover, targeted agents represent a novel class of anticancer treatments for breast cancer patients. These anticancer therapies have a substantial effect on the QoL of patients with breast cancer due to their toxicity profile [9, 10]. Furthermore, new surgical techniques can exhibit new consequences for the QoL of the patients [11]. Consequently, due to the impact of newer treatment alternatives, it became apparent that the initial 23-item QLQ-BR23 might be ineffective for solving a variety of crucial QoL problems and possible side effects. Therefore, in 2020, the EORTC quality of life group (QLG) updated this module into the EORTC QLQ-BR45. This module can assess more accurately and comprehensively the impact of new and scalable treatments on breast cancer patients' QoL [12].

Extensive literature research is thus needed to assess the reliability and validity of the EORTC QLQ-BR45. Accordingly, the main objectives of the current study are to:

1) adapt and pilot test the available Arabic translation of the EORTC QLQ-BR45 module into an Egyptian Arabic version, which is currently available as the first native Arabic version and
2) evaluate the internal consistency reliability and validity of the Egyptian Arabic translation of the EORTC QLQ-BR45 module for determining the QoL of Egyptian breast cancer patients.

PATIENTS AND METHODS

Trial design and participants

Between June 2019 and November 2020, subjects were recruited from Beni-Suef University Hospital, Beni-Suef, Egypt. Subjects were eligible if they were female patients of age between 18 and 65 years, spoke Egyptian Arabic, had proven locally advanced breast cancer and would receive first-line neoadjuvant chemotherapy regimen (four cycles of doxorubicin/cyclophosphamide, followed by 12 cycles of weekly paclitaxel). Patients were excluded if they had a previous history of breast cancer treatment or suffered any psychiatric disorders, physical disabilities or severe medical condition.

In the current study, sample size was calculated using Raosoft sample size calculator [13], with 0.95 confidence level and 0.1 margin error. Hence, 80 non-diabetic breast cancer patients were enrolled in the study. Six patients were excluded from the study because of protocol deviation, early withdrawal or loss to follow-up. Thus, 74 patients were interviewed on day 1 of receiving the first cycle of neoadjuvant chemotherapy treatment. After the last chemotherapy cycle, all patients underwent breast surgery, either mastectomy or breast conservative surgery. Eventually, a second interview was conducted 3–4 weeks post-surgery for 19 patients receiving targeted therapy, 34 patients receiving endocrine treatment and six patients receiving radiotherapy.

The translated Arabic EORTC QLQ-BR45 version was permitted for use in the current study by the EORTC-QLG. The EORTC-QLG provided us with the existing Arabic version for review and adaptation to the Egyptian culture. Then, the reviewed version was proofread by a professional translator who was arranged by the EORTC-QLG. Afterwards, the results of the proofreading were sent to us for review. Thus, the new version was pilot tested on 13 patients. The results of pilot testing were reviewed by the EORTC-QLG, and an approval for the use of the EORTC QLQ-BR45 was provided to the current study by the EORTC.

The study was approved by the Institutional Review Board (IRB) of the Faculty of Pharmacy, Beni-Suef University (REC-H-PhBSU-20012). The study was conducted in accordance with the Declaration of Helsinki, Good Clinical Practice norms and local and national regulatory requirements. The respondent's right to confidentiality was always respected, and any legal requirements on data protection were adhered to. Approval of the written informed consent was acquired by all the patients before enrolment in the study. The consent form to the patients included a statement explaining the aim of the study, the potential length of time for participation and a description of all the procedures that would be completed during enrolment in the clinical trial. In addition, it included their right to cease their participation in the clinical trial at any time and the contact information of the researchers.

In the current study, most of our patients had received less education and could not read the consent form. Therefore, an oral presentation of the consent document, stating the requisite aspects of informed consent, was given, wherein it was thoroughly explained to the patients or the patient's legally authorised representative and a witness to the oral presentation. The witness can be a family member or someone independent of the research team. The consent document must be signed by both the patient (or the patient's representative) and the witness. The IRB approved a written summary of what should be said in the oral presentation to the patient or the representative to ensure all the required aspects are presented.

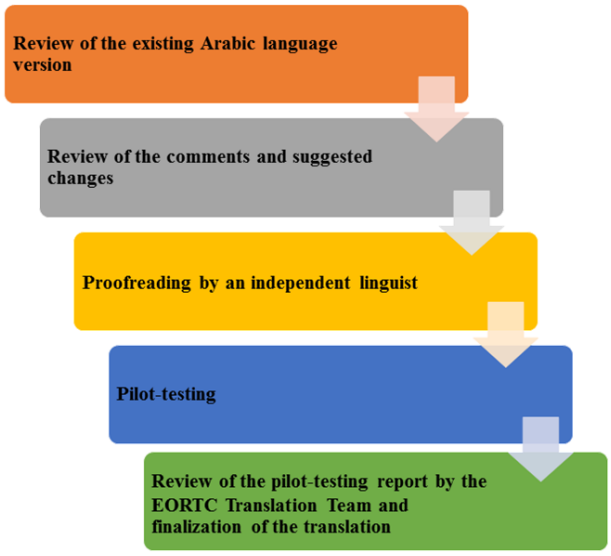

Figure 1. Flowchart of the developmental sequences of the Egyptian Arabic version of the QLQ-BR45 module.

Instrument, procedure and scoring

The breast cancer module, EORTC QLQ-BR45 questionnaire, is a supplementary questionnaire module to be used in conjunction with QLQ-C30. The EORTC QLQ-BR45 is composed of 45 items related to breast cancer or its treatment [6]. The numbering of the items in the module starts from item 31 till item 75. The module consists of three multi-item functional scales (body image, sexual functioning and breast satisfaction), two single-item functional scales (sexual enjoyment and future perspective), three multi-item symptom scales (systemic side effects, breast symptoms and arm symptoms), one single-item symptom scale (upset by hair loss) and three multi-item target therapy scales (endocrine therapy symptoms, skin mucosis symptoms and endocrine sexual symptoms). The time frame of the QLQ-BR45 module is 'during the past week' in item numbers 31–43, 47–69 and 74–75. The time frame is 'during the past 4 weeks' in item numbers 44–46 and 70–73. All the items of the questionnaire are scored on a four-point Likert scale as follows: 'not at all' (1), 'a little' (2), 'quite a bit' (3), 'very much' (4).

An appointment was arranged with all eligible patients who visited the breast cancer oncology clinic, agreed to participate in the trial and signed the informed consent to perform a face-to-face interview on day 1 of the first dose of neoadjuvant chemotherapy. Then, the patients were followed up to determine the day of the second interview after the first dose of their indicated adjuvant treatment. Finally, the second face-to face interview was conducted. The interviews were conducted in a private room by a female trained clinical pharmacist using the structured interview questions. Then, scoring of the EORTC QLQ-BR45 was conducted following the EORTC scoring manual [14]. Afterwards, linear transformation to a 0–100 scale was done for all the responses to Likert scale items. Higher scores for functional scales indicate a better QoL. On the other side, higher scores for symptom scales and single items indicate higher severity of the symptoms and worse QoL.

Cultural adaptation and pilot testing

Cross-cultural adaptation and pilot testing of the EORTC QLQ-BR45 were performed according to the EORTC guidelines and the international guidelines for cross-cultural adaptation of HRQOL as shown in Figure 1 [15, 16]. Some terminologies in several questionnaire items were substituted to adapt to the local culture in Egypt. Then, pilot testing was conducted on 13 breast cancer patients to check its comprehensibility in Arabic (Egypt) and debriefing. The content was assessed for items that were difficult to understand or answer and confusing or offensive items, and the questionnaire was amended accordingly. Finally, the release of an elaborated version of the EORTC QLQ-BR45 in Egyptian Arabic was done. The approval of the EORTC team was obtained at each step.

Statistical analysis

Socio-demographic data of the patients was analysed with frequencies and percentages. Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, version 26.0) was used to perform the statistical analysis. The psychometric properties of the EORTC QLQ-BR45 were evaluated in terms of reliability, convergent and divergent validity tests. Cronbach's α was used to evaluate the internal consistency reliability, whereas Pearson's correlation was used to evaluate the convergent and divergent validity.

Reliability Test

Cronbach's α coefficient was used to evaluate the questionnaire's internal consistency reliability, with the acceptable value being ≥0.70 [17, 18].

Validity

Evaluation of the convergent and divergent validity was performed using multi-trait scaling analysis. Convergent validity was anticipated in case the correlation of each item and its own scale was ≥0.40, while divergent validity was anticipated in case the correlation of each item with its own scale exceeded its correlation with other scales. Scaling success was predicted in case the correlation of each item with its own scale exceeded that with other scales [17, 19].

RESULTS

Socio-demographic characteristics of the patients

Table 1 summarises the socio-demographic characteristics and the tumour characteristics of the patients at baseline.

Table 1. Socio-demographic data and tumour characteristics of the patients in the study at baseline (n = 74).

Variable	Frequency	Percentage
Age, years		
<35	8	10.81%
35–45	26	35.13%
46–55	21	28.38%
56–65	19	25.68%
Marital status		
Married	58	78.37%
Single	5	6.77%
Widow	11	14.86%
Body mass index		
Normal weight	17	22.97%
Overweight	26	35.17%
Obese	31	41.89%
Menopausal state		
Pre-menopause	38	51.35%
Post-menopause	36	48.65%
Family history of breast cancer		
Yes	15	20.27%
No	59	79.73%
Comorbidity		
Yes	16	21.62%
No	58	78.38%
Educational level		
Low level	47	63.51%
Intermediate level	18	24.32%
High level	9	12.16%
ECOG performance status		
0	19	25.68%
1	47	63.51%
2	8	10.81%
3	0	0.00%
4	0	0.00%
Histological type		
IDC	67	90.54%
ILC/mixed IDC/ILC	7	9.46%
Clinical stage		
IIB	10	13.52%
IIIA	31	41.89%
IIIB	33	44.59%
Hormone receptor status		
ER and/or PR positive	63	85.14%
ER and PR negative	11	14.86%
HER2 status		
Positive	30	40.54%
Negative	44	59.46%

ECOG: Eastern Cooperative Oncology Group, ER: estrogen receptor, PR: progesterone receptor HER2: human epidermal growth factor receptor-2, IDC: invasive ductal carcinoma, ILC: invasive lobular carcinoma.

The mean age of the patients was 48 ± 10.83 years, and the mean body mass index (BMI) was 30.32 ± 5.62. Also, 78.37% of the patients were married and 63.51% had received low education level. The Eastern Cooperative Oncology Group (ECOG) performance status of the patients ranged from 0 to 2.

Cultural adaptation and pilot testing

In the process of adaptation of the questionnaire, several terminologies were substituted to adapt to the local culture in Egypt. The reviewed version was proofread by a professional translator. The new version was pilot tested on 13 patients. Accordingly, the patients' comments were summarised and sent to the EORTC translation team. Most of the patients refused to answer questions related to their sexual behaviour, with numbers 44, 45, 46, 72 and 73. Patients had neither understanding problems nor linguistic issues; however, they felt unwilling to answer these questions. In addition, there was an inquiry by the interviewer about question numbers 74 and 75 and if the QLQ-BR45 module should be conducted only after breast surgery. The EORTC commented that there are no specific guidelines for a specific time for the BR45 module. Furthermore, the patients who did not undergo surgical operation could answer 'not at all' to the surgery items or could skip them, and then these scales would not be scored.

Reliability test

Table 2 shows that the internal consistency reliability of most scales demonstrated an acceptable level (Cronbach's α coefficients above 0.70), except for body image (α = 0.54) and systemic therapy side effects (α = 0.63).

Validity test

As shown in Table 3, the magnitude of the correlation coefficient of almost all items–scale was >0.40, showing convergent validity of the items of each scale, except for systemic therapy side effects and endocrine therapy symptoms. Furthermore, the correlation coefficient of each item with its own scale exceeded its correlation with the other scales. Thus, scaling success criteria were met and the item–scales correlation revealed divergent validity.

DISCUSSION

The development of QoL questionnaires is mainly conducted in English-speaking countries. A main objective of HRQoL studies is to measure the applicability of the QoL questionnaires to other languages and traditions. Another objective is to demonstrate the degree of implementation of acceptable intercultural validity. The current study contributes to the cross-cultural adaptation and validation of the EORTC QLQ-BR45 questionnaire in the Egyptian breast cancer population.

Table 2. Reliability of EORTC QLQ-BR45 Arabic version in female breast cancer patients in Egypt (n = 74).

Scale	Cronbach's α value[a]
Body image	0.54
Sexual functioning	0.85
Breast satisfaction	0.95
Systemic therapy side effects	0.63
Arm symptoms	0.77
Breast symptoms	0.74
Endocrine therapy symptoms	0.76
Skin mucosis symptoms	0.75
Endocrine sexual symptoms	0.88
Future perspective Sexual enjoyment Upset by hair loss	Single item Single item Single item

[a]For single items, reliability test is not applicable
EORTC: European Organization for Research and Treatment of Cancer

The EORTC procedures and the international guidelines for cross-cultural adaptation of HRQOL were rigorously followed to culturally adapt and validate the EORTC QLQ-BR45 instruments into local Egyptian Arabic [15, 16]. The available Arabic version provided by the EORTC-QLG was not for a native Arabic country. This could be justified because this version was developed in a non–Arabic-speaking country in the Middle East. Therefore, certain expressions in the available Arabic version of the EORTC QLQ-BR45 necessitate modifications to be suitable for the Egyptian culture. Furthermore, the questionnaire items may require adaptation when used in different languages and cultural settings in order to achieve equivalence between the original and the new versions. Therefore, if questionnaires are to be utilised across cultures, they must not only be adequately translated linguistically, but also culturally altered to maintain the instrument's content validity on a conceptual level [16]. Hence, it is necessary to adapt the Arabic version among different countries to ensure the validity of the instrument.

Pilot testing was supplemented for Egyptian breast cancer patients to identify problematic wording and sentence structures for the target population. The mean time required for interviewing the patients was 25 min. Then, the psychometric properties of the adapted questionnaire were evaluated. Target therapy scale was used for patients receiving targeted therapy (n = 19) or endocrine therapy (n = 34), and breast satisfaction scale was used for patients post-surgery (n = 74).

In the current study, the internal consistency reliability of the majority of scales in the EORTC QLQ-BR45 was found to be satisfactory. This conclusion was drawn from Cronbach's α values exceeding 0.70 for all scales, except body image and systemic therapy side effects. The results of the current study

Table 3. Multi-trait scaling analysis for convergent and divergent validity of EORTC-QLQ-BR45 Arabic version in female breast cancer patients in Egypt (n = 74).

Scale	Subscale	Item–own scale correlation[a]	Item–other scale correlationa	Scaling success
Functional scales				
	Body image	0.487–0.798	0.032–0.638	4/4
	Sexual functioning	0.902–0.924	0.001–0.027	2/2
	Breast satisfaction	0.977–0.978	-0.041–0.03	2/2
Symptoms scales				
	Systemic therapy side effects[b]	0.308–0.743	0.002–0.507	7/7
	Arm symptoms	0.765–0.872	0.022–0.480	3/3
	Breast symptoms	0.649–0.827	0.016–0.308	4/4
Target therapy scales				
	Endocrine therapy symptoms[b]	0.397–0.879	0.003–0.773	10/10
	Skin mucosis symptoms	0.425–0.796	0.055–0.541	6/6
	Endocrine sexual symptoms	0.940–0.959	0.004–0.635	4/4

[a]Pearson's correlation coefficient
[b]Systemic therapy side effects and Endocrine therapy symptoms are below 0.4
EORTC: European Organization for Research and Treatment of Cancer

are in agreement with the Amharic version of EORTC QLQ-BR23 that showed internal consistency reliability value of the body image scale α = 0.51, which was below the required Cronbach's α values [15, 20]. Also, the Iranian version of the EORTC QLQ-BR23 showed minimum internal consistency for the systemic therapy side effects with Cronbach's α = 0.63 [21]. In the current study, other scales showed adequate internal consistency, and their Cronbach's α values ranged from 0.74 to 0.95.

On the other hand, it was observed that in the Mexican-Spanish version of the EORTC QLQ-BR23 questionnaire, all multi-item scales presented an internal reliability of Cronbach's α >0.7, except for the breast symptoms scale (α = 0.65) [22]. In addition, the validation of the Turkish version of the QLQ-BR23 showed an internal reliability of Cronbach's α >0.7, except for the breast and arm symptoms scale, which presented with Cronbach's α values of 0.65 and 0.61, respectively [23]. In addition, the Taiwan-Chinese version of BR23 showed an internal reliability of Cronbach's α >0.7, except for the arm symptoms α = 0.59 [24]. Moreover, the validation of the BR23 for women in the United Arab Emirates showed an internal reliability of Cronbach's α >0.7, except for the breast symptoms scale (α = 0.51) [25].

Consequently, construct validity was assessed by the evaluation of convergent and divergent validity of items. The multi-trait scaling analysis showed a sufficient convergent validity of the instruments, indicating good correlations of most items with their respective subscale ($r \geq 0.4$) [17]. However, systemic therapy side effects and endocrine therapy symptoms did not show adequate convergent validity ($r < 0.4$). Furthermore, there were no scaling errors observed, since the correlation of each item with its own scale exceeded its correlation with other scales demonstrating divergent validity.

In the current study, the sexual functioning and endocrine sexual functioning scales exhibited strong psychometric properties that yielded adequate convergent and divergent validity. However, the results should be cautiously considered as the sexuality items were calculated from a small number of patients (11 patients for the sexual functioning scale and 13 patients for the endocrine sexual functioning scale) because most of the patients refused to answer these questions and felt that sexuality items were upsetting and intrusive. A trial on Iranian patients found that both the patients and the therapist were reluctant to talk about sexual health, although it was deteriorated tremendously in breast cancer patients compared to healthy women [26]. This trial proposed the requirement of valid clinical guidelines to provide suitable evidence-based care for sexual health in breast cancer cohorts.

In agreement with the current study, the construct validity of the Moroccan Arabic QLQ-BR23 determined by evaluating the items' convergent and discriminant validity showed that the sexual functioning scores had strong psychometric characteristics and satisfactory multi-trait scaling findings [19]. Furthermore, the multi-trait scaling analysis of validation of the EORTC QLQ-BR45 among the Ethiopian breast cancer patients showed a strong convergent and divergent validity in most items. However, item number 38 on the endocrine therapy scale had a low correlation ($r < 0.4$) with its own scale. Additionally, the most obvious scaling failure was observed for systemic therapy side effect [27]. Furthermore, the Amharic version of the EORTC QLQ-BR23 showed that almost

all the items had strong correlations with their corresponding scale ($r \geq 0.6$), indicating a strong convergent validity of the questionnaire, except for hair loss [20].

The aforementioned results of the current study for multi-trait scaling could justify that the hypothesised scale structure of the EORTC QLQ-BR45 is relevant including both item translation and response choices. In addition, the scores of the scales derived from the Egyptian Arabic version can be involved in cross-cultural comparisons.

Even though the EORTC QLQ-BR45 is a more comprehensive module for breast cancer patients, the following points were observed in the questionnaire that need more clarification or amendment in the module's final version. The hypothesised target therapy scale, which considers evaluation of the QoL of breast cancer patients receiving target therapies, includes some side effects that could be experienced by patients receiving some classes of chemotherapeutic agents, such as tingling, numbness, mucositis and vaginal dryness. Hence, the EORTC-QLG should illustrate the items included in the target therapy scale that could be used for interviewing patients receiving chemotherapeutic agents. Also, according to 'the international update of the EORTC questionnaire for assessing the QoL in breast cancer patients using EORTC QLQ-BR45', more clarification is required for the cases in which the new target scale could be used as one scale or three separate scales [12].

One of the limitations of the current study is that most of the patients had a low education level. This leads to the difficulty of self-administration of the questionnaires, and all the participants were interviewed. Interviewing the patients leads to the refusal of most patients to answer sexual-related questions. On the other side, interviewing the patients provided some points of strength, which included that the interviewer conducted direct verbal and non-verbal communication with the patients. Therefore, all the questions were interpreted carefully to the patients and the missing responses were almost negligible. In addition, this data collection approach enables spontaneous reporting of in-depth assessments of patients' experiences with their diagnosis and/or treatment. Hence, this approach provides evidence that the collected PROs followed a robust validation process [28].

CONCLUSION

The Egyptian Arabic translation of the EORTC QLQ-BR45 is an adequately reliable and valid tool to measure the QoL in Egyptian breast cancer patients. Hence, clinical trials concerned with evaluating the HRQoL of breast cancer patients could effectively use this tool.

DISCLOSURE

The authors declare that they have no conflicts of interest.

FUNDING

None to declare.

ACKNOWLEDGEMENT

None to declare.

AUTHOR CONTRIBUTIONS

All authors have agreed on the submitted version of the manuscript and meet the **International Committee of Medical Journal Editors** (ICMJE) recommendations for authorship.

References

[1] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A: **Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries**. *CA: a cancer journal for clinicians* 2018, **68**(6):394-424.

[2] Mokhatri-Hesari P, Montazeri A: **Health-related quality of life in breast cancer patients: review of reviews from 2008 to 2018**. *Health Qual Life Outcomes* 2020, **18**(1):338.

[3] Bonnetain F, Borg C, Adams RR, Ajani JA, Benson A, Bleiberg H, Chibaudel B, Diaz-Rubio E, Douillard JY, Fuchs CS *et al*: **How health-related quality of life assessment should be used in advanced colorectal cancer clinical trials**. *Annals of oncology : official journal of the European Society for Medical Oncology* 2017, **28**(9):2077-2085.

[4] Velikova G, Coens C, Efficace F, Greimel E, Groenvold M, Johnson C, Singer S, van de Poll-Franse L, Young T, Bottomley A: **Health-Related Quality of Life in EORTC clinical trials — 30 years of progress from methodological developments to making a real impact on oncology practice**. *European Journal of Cancer Supplements* 2012, **10**(1):141-149.

[5] **EORTC. EORTC QLQ-C30 (Version 3). EORTC Study Group on Quality of Life. 1995** [https://qol.eortc.org/]

[6] **EORTC. EORTC QLQ-BR23. (version 1). EORTC Study Group on Quality of Life. 1994** [https://qol.eortc.org/]

[7] Perez EA: **Safety profiles of tamoxifen and the aromatase inhibitors in adjuvant therapy of hormone-responsive early breast cancer**. *Annals of Oncology* 2007, **18**:viii26-viii35.

[8] van Nes JGH, Fontein DBY, Hille ETM, Voskuil DW, van Leeuwen FE, de Haes JCJM, Putter H, Seynaeve C, Nortier JWR, van de Velde CJH: **Quality of life in relation to tamoxifen or exemestane treatment in postmenopausal breast cancer patients: a Tamoxifen Exemestane Adjuvant Multinational (TEAM) Trial side study**. *Breast Cancer Research and Treatment* 2012, **134**(1):267-276.

[9] Gianni L, Dafni U, Gelber RD, Azambuja E, Muehlbauer S, Goldhirsch A, Untch M, Smith I, Baselga J, Jackisch C *et al*:

Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomised controlled trial. *The Lancet Oncology* 2011, **12**(3):236-244.

[10] Harbeck N, Iyer S, Turner N, Cristofanilli M, Ro J, André F, Loi S, Verma S, Iwata H, Bhattacharyya H *et al*: **Quality of life with palbociclib plus fulvestrant in previously treated hormone receptor-positive, HER2-negative metastatic breast cancer: patient-reported outcomes from the PALOMA-3 trial**. *Annals of Oncology* 2016, **27**(6):1047-1054.

[11] Winters ZE, Afzal M, Rutherford C, Holzner B, Rumpold G, da Costa Vieira RA, Hartup S, Flitcroft K, Bjelic-Radisic V, Oberguggenberger A *et al*: **International validation of the European Organisation for Research and Treatment of Cancer QLQ-BRECON23 quality-of-life questionnaire for women undergoing breast reconstruction**. *British Journal of Surgery* 2018, **105**(3):209-222.

[12] Bjelic-Radisic V, Cardoso F, Cameron D, Brain E, Kuljanic K, da Costa RA, Conroy T, Inwald EC, Serpentini S, Pinto M *et al*: **An international update of the EORTC questionnaire for assessing quality of life in breast cancer patients: EORTC QLQ-BR45**. *Annals of oncology : official journal of the European Society for Medical Oncology* 2020, **31**(2):283-288.

[13] **Raosoft sample size calculator** [http://www.raosoft.com/samplesize.html]

[14] **EORTC. EORTC Scoring Manual (Third Edition). 2001** [https://www.eortc.org/]

[15] **Eortc Quality Of Life Group Translation Procedure, Fourth edition** [https://www.eortc.org/app/uploads/sites/2/2018/02/translation_manual_2017.pdf]

[16] Beaton DE, Bombardier C, Guillemin F, Ferraz MB: **Guidelines for the process of cross-cultural adaptation of self-report measures**. *Spine* 2000, **25**(24):3186-3191.

[17] Sprangers MA, Groenvold M, Arraras JI, Franklin J, te Velde A, Muller M, Franzini L, Williams A, de Haes HC, Hopwood P *et al*: **The European Organization for Research and Treatment of Cancer breast cancer-specific quality-of-life questionnaire module: first results from a three-country field study**. *J Clin Oncol* 1996, **14**(10):2756-2768.

[18] Cronbach LJJp: **Coefficient alpha and the internal structure of tests**. 1951, **16**(3):297-334.

[19] El Fakir S, Abda N, Bendahhou K, Zidouh A, Bennani M, Errihani H, Benider A, Bekkali R, Nejjari C: **The European Organization for Research and Treatment of Cancer quality of life questionnaire-BR23 Breast Cancer-Specific Quality of Life Questionnaire: psychometric properties in a Moroccan sample of breast cancer patients**. *BMC research notes* 2014, **7**:53.

[20] Gadisa DA, Gebremariam ET, Ali GY: **Reliability and validity of Amharic version of EORTC QLQ-C30 and QLQ-BR23 modules for assessing health-related quality of life among breast cancer patients in Ethiopia**. *Health Qual Life Outcomes* 2019, **17**(1):182.

[21] Montazeri A, Harirchi I, Vahdani M, Khaleghi F, Jarvandi S, Ebrahimi M, Haji-Mahmoodi M: **The EORTC breast cancer-specific quality of life questionnaire (EORTC QLQ-BR23): translation and validation study of the Iranian version**. *Quality of life research : an international journal of quality of life aspects of treatment, care and rehabilitation* 2000, **9**(2):177-184.

[22] Cerezo O, Oñate-Ocaña LF, Arrieta-Joffe P, González-Lara F, García-Pasquel MJ, Bargalló-Rocha E, Vilar-Compte D: **Validation of the Mexican-Spanish version of the EORTC QLQ-C30 and BR23 questionnaires to assess health-related quality of life in Mexican women with breast cancer**. *European journal of cancer care* 2012, **21**(5):684-691.

[23] Demirci S, Eser E, Ozsaran Z, Tankisi D, Aras AB, Ozaydemir G, Anacak Y: **Validation of the Turkish versions of EORTC QLQ-C30 and BR23 modules in breast cancer patients**. *Asian Pac J Cancer Prev* 2011, **12**(5):1283-1287.

[24] Chie WC, Chang KJ, Huang CS, Kuo WH: **Quality of life of breast cancer patients in Taiwan: validation of the Taiwan Chinese version of the EORTC QLQ-C30 and EORTC QLQ-BR23**. *Psychooncology* 2003, **12**(7):729-735.

[25] Awad MA, Denic S, El Taji H: **Validation of the European Organization for Research and Treatment of Cancer Quality of Life Questionnaires for Arabic-speaking Populations**. *Annals of the New York Academy of Sciences* 2008, **1138**:146-154.

[26] Roshandel S, Lamyian M, Azin SA, Haghighat S, Mohammadi E: **Development and validation of a guideline on sexual and reproductive health of breast cancer survivors in Iran: a mixed methods study protocol**. *Health Research Policy and Systems* 2021, **19**(1):87.

[27] Mikiyas Amare G, Panpan W, Eva Johanna K, Edom S, Changying C, Adamu A: *Health Qual Life Outcomes* 2021.

[28] Brédart A, Marrel A, Abetz-Webb L, Lasch K, Acquadro C: **Interviewing to develop Patient-Reported Outcome (PRO) measures for clinical research: eliciting patients' experience**. *Health Qual Life Outcomes* 2014, **12**:15-15.

sciendo

Eur. Pharm. J. 2021, 68(2), 49-58.
ISSN 1338-6786 (online) and ISSN 2453-6725 (print version),
DOI: 10.2478/afpuc-2021-0019

EUROPEAN PHARMACEUTICAL JOURNAL

Development and Validation of Bioanalytical Method for Determination of Nebivolol and Valsartan in Human Plasma by Using RP-HPLC

Original Paper

Kachave R. N.[1]✉, Mundhe A. G.[2]

[1]Amrutvahini College of Pharmacy, Sangamner
Sangamner, Maharashtra INDIA
[2]Amrutvahini College of Pharmacy

Received 14 April, 2018, accepted 1 June, 2018

Abstract

Aim: Nebivolol and valsartan are used in the treatment of hypertension. So, this study was conducted for the purpose of determining bioavailability/bioequivalence of nebivolol and valsartan in human plasma.

Materials and Methods: The chromatographic separation was performed on Symmetry C18 (150 × 4.6 mm, 5 µm) column using 0.01 N potassium dihydrogen phosphate (pH 3.0):acetonitrile (60:40) as the mobile phase at a flow rate of 1.0 mL/min and a detector wavelength of 280 nm. The retention times of nebivolol and valsartan in plasma were found to be 3.1 and 4.3 min, respectively.

Results: The method was validated statistically and by recovery studies. The linearity concentration was acceptable in the range of 0.5–10 ng/mL for nebivolol and 400–8000 ng/mL for valsartan. The lower limits of quantification were 0.5 ng/mL for nebivolol and 400 ng/mL for valsartan, which reached the levels of both drugs possibly found in human plasma. Per cent recoveries were obtained as 97.78% and 98.11% for nebivolol and valsartan, respectively.

Conclusion: The proposed method is simple, rapid, accurate, precise and gives us knowledge about the pharmacokinetics and therapeutic drug monitoring in clinical laboratories.

Keywords *Nebivolol – Valsartan – Atorvastatin – RP-HPLC – Method development – ICH Guidelines (ICH M10) – Validation*

INTRODUCTION

Nebivolol (NL) is chemically described as 1-(6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-yl)-2-{[2-(6-fluoro-3, 4-dihydro-2H-1-benzopyran-2-yl)-2-ydroxyethyl]amino} ethan-1-ol (Figure 1a). Its molecular formula is $C_{22}H_{25}F_2NO_4$ and its molecular weight is 405.435 g/mol. Valsartan (VL) is chemically described as (2S)-3-methyl-2-[N-({4-[2-(2H-1, 2, 3, 4-tetrazol-5-yl) phenyl] phenyl}methyl) pentanamido] butanoic acid (Figure 1b). Its empirical formula is $C_{24}H_{29}N_5O_3$ and its molecular weight is 435.52 g/mol [1-5].

VL belongs to the angiotensin II receptor blocker (ARB) family of drugs, which also includes telmisartan, candesartan, losartan, olmesartan and irbesartan. ARBs selectively bind to angiotensin receptor-1 (AT1) and prevent the protein angiotensin II from binding and exerting its hypertensive effects, which include vasoconstriction, stimulation and synthesis of aldosterone and ADH, cardiac stimulation and renal reabsorption of sodium, among others. Overall, the physiological effects of VL lead to reduced blood pressure, lower aldosterone levels, reduced cardiac activity and increased excretion of sodium. NL is a racemic mixture of two enantiomers, wherein one is a β-adrenergic antagonist and the other acts as a cardiac stimulant without β-adrenergic activity. Treatment with NL leads to a greater decrease in systolic and diastolic blood pressure than atenolol, propranolol or pindolol. NL and other β-blockers are generally not first-line therapies as many patients are first treated with thiazide diuretics. They are indicated for hypertension to lower the blood pressure and reduce the risk of fatal and non-fatal cardiovascular events, primarily strokes and myocardial infarction. NL is a competitive and selective β1-receptor antagonist, has little or no effect on β2 receptors at doses <10 mg, lacks intrinsic sympathomimetic and membrane-stabilising activity at therapeutically relevant concentrations and reduces systemic vascular resistance. VL blocks the binding of angiotensin II to type 1 angiotensin receptors, causing a reduction in blood pressure, and blocks vasoconstrictor and aldosterone-secreting effects of angiotensin II. Byvalson (VL and NL tablet)

* E-mail: ramanlalkachave26@gmail.com

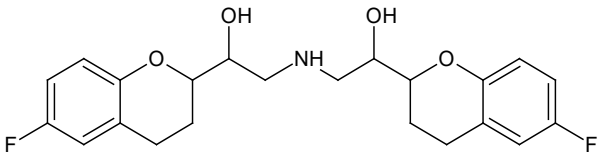

Figure 1a. Structure of Nebivolol.

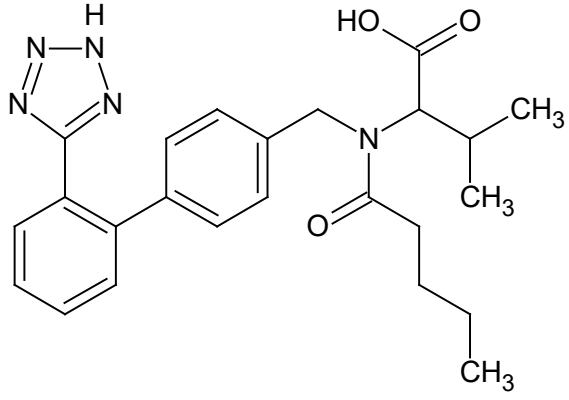

Figure 1b. Structure of Valsartan.

80 and 5 mg is a fixed-dose combination for the treatment of hypertension [1-8].

The literature survey presents different methods available for the detection of NL and VL separately and also in a combined form. Ultra-performance liquid chromatography (UPLC) method for detection of both drugs [9], an isocratic (high-performance liquid chromatography [HPLC]) method [10], an ion-pair HPLC method [11], RP-HPLC method for the estimation of both drugs [12], ultraviolet (UV) simultaneous estimation of NL and VL [13] – these all are the methods available for the detection of NL and VL.

This study aims to develop a rapid analytical technique for the estimation of NL and VL in human plasma. The proposed method is a rapid, precise, selective and sensitive RP-HPLC method that has short and simple extraction procedures that consume small amounts of solvent and biological fluid for extraction and time. This method is used in the bioavailability/bioequivalence (BA/BE) study for analysis of NL and VL in biological samples like blood, plasma and urine.

MATERIALS AND METHODS

NL and VL active pharmaceutical ingredients (APIs) were received as a gift sample from Spectrum Pharma Research solutions. HPLC-grade acetonitrile and methanol were procured from Rankem, Avantor Performance Material India Limited. Plasma used for the study was received from Deccan pathological lab, Hyderabad. Analytical grade phosphate buffer, potassium dihydrogen phosphate and orthophosphoric acid were also procured from Rankem, Avantor Performance Material India Limited.

Chromatographic conditions

Chromatographic separation was performed at 30°C of column temperature with the mobile phase consisting of 0.01 N potassium dihydrogen phosphate pH 3.0:acetonitrile in the ratio of 60:40. Separation of NL and VL was achieved on Symmetry C18 (150 × 4.6 mm, 5 µm) at a flow rate of 1.0 mL/min; the injection volume was 50 µL and detector wavelength was 280 nm.

Preparation of standard stock solution

Diluents

Based upon the solubility of the drugs, diluent was selected; 0.01 N potassium dihydrogen phosphate and acetonitrile were taken in the ratio of 50:50.

Preparation of NL stock solution (5000 ng/mL)

Take 5 mg of NL in a 100-mL volumetric flask and add diluent to give a volume of 50,000 ng/mL. Take 1 mL of the prepared solution and dilute to 10 mL with diluent to produce 5000 ng/mL.

Preparation of NL spiking solutions

From the above-mentioned NL stock solution, 0.01, 0.02, 0.03, 0.08, 0.10, 0.12, 0.16 and 0.20 mL was pipetted and transferred to eight individual 10-mL volumetric flasks and the volume was made up to the mark with diluent to produce 5, 10, 15, 40, 50, 60, 80 and 100 ng/mL. Calibration standards and quality control (QC) samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 0.5, 1, 1.5, 4, 5, 6, 8 and 10 ng/mL.

Preparation of VL stock solution (400 µg/mL)

Take 40 mg of VL in a 100-mL volumetric flask and add diluent to give a volume of 400,000 ng/mL.

Preparation of VL spiking solutions

From the above-mentioned VL stock solution, 0.1, 0.2, 0.3, 0.8, 1.0, 1.2, 1.6 and 2.0 mL was pipetted and transferred to eight individual 10-mL volumetric flasks, and the volume was made up to the mark with diluent to produce 4.0, 8.0, 12, 32, 40, 48, 64 and 80 µg/mL. Calibration standards and QC samples were prepared by spiking blank plasma with working stock dilutions of analytes to produce 400, 800, 1200, 3200, 4000, 4800, 6400 and 8000 ng/mL.

Preparation of atorvastatin (internal standard [IS]) spiking solutions

Fifty milligrams of atorvastatin was weighed, dissolved and diluted to 100 mL by diluent (500 µg/mL). From this stock solution, 2 mL was pipetted, diluted to 100 mL with diluent and used as a spiking solution (10 µg/mL).

EXTRACTION PROCEDURE

Protein precipitation method

Preparation of calibration standard and QC samples

Take 750 µL of plasma and add 500 µL of IS, 250 µL of NL and 250 µL of VL from the spiking solutions into a centrifuging tube; add 1 mL of acetonitrile. Mix by cyclomixer for 15 sec. Then vertex for 2 min and finally centrifuge for 5 min at 3200 rpm. After centrifugation, collect the supernatant, filter it and directly inject 50 µL into HPLC.

Preparation of patient's samples

Take 1250 µL of the patient's sample and add 500 µL of IS into a centrifuging tube; add 1 mL of acetonitrile. Mix by cyclomixer for 15 sec. Then vertex for 2 min and finally centrifuge for 5 min at 3200 rpm. After centrifugation, collect the supernatant, filter it and directly inject 50 µL into HPLC.

Method validation

Method validation for an analytical method is a process used to verify/confirm whether the newly developed method is suitable for its intended purpose as per the ICH guidelines [14-19]

Parameters involved in bioanalytical validation are as follows:

1. System suitability test
2. Selectivity
3. Matrix factor evaluation
4. Precision and accuracy
5. Linearity
6. Recovery
7. Stability

1. System suitability test

System suitability test was performed before each batch sample analysis to ensure the reproducibility of the chromatographic system. This test was performed by running six injections of the diluted drug and IS in the linear region of the calibration curve and measuring the percentage of relative standard deviation (% RSD).

2. Selectivity/specificity

To establish the selectivity of the method, possible interferences at the retention time (RT) of NL, VL and IS due to endogenous plasma components were checked during validation. Selectivity was performed by testing six lots of K_2EDTA blank plasma, and the analyte detection of extracted plasma has good selectivity of both drugs and IS.

3. Matrix factor evaluation

The combined effect of all components of the sample other than the analyte on the measurement of quantity. Three blank specimens from each of not less than six batches of the matrix under screening were extracted. For the matrix effect, LQC and HQC spiking dilutions were spiked, and IS dilutions were also spiked in the previously extracted blank specimens.

4. Precision and accuracy

The intra-day and inter-day accuracy and precision were assessed by analysing six replicates at four different QC levels like LLOQ, LQC, MQC and HQC. Accuracy and precision method performance was evaluated by six replicate analyses for NL at four concentration levels, that is, 0.5 ng/mL (LLOQ), 1.5 ng/mL (LQC), 5 ng/mL (MQC) and 8 ng/mL (HQC), and for VL at 400 ng/mL (LLOQ), 1200 ng/mL (LQC), 4000 ng/mL (MQC) and 6400 ng/mL (HQC). The intra-day and inter-day accuracies of plasma samples were assessed and excellent mean % accuracies were calculated. The precision of the method was expressed in terms of % RSD, and accuracy was expressed as a percentage of theoretical concentration (observed concentration × 100/theoretical concentration).

5. Linearity

The calibration curve in a bioanalytical method is a linear relationship between concentration (independent variable) and response (dependent variable) using a least-squares method. This relationship is built to predict the unknown concentrations of the analyte in a complicated matrix. The linearity sample should consist of a blank sample, a zero sample and six to eight non-zero samples covering the expected range, including LLOQ.

6. Recovery

Recovery was determined by comparing the peak areas obtained from prepared plasma samples with those extracted from blank plasma spiked with the same amount of NL and VL standards at three QC concentration levels.

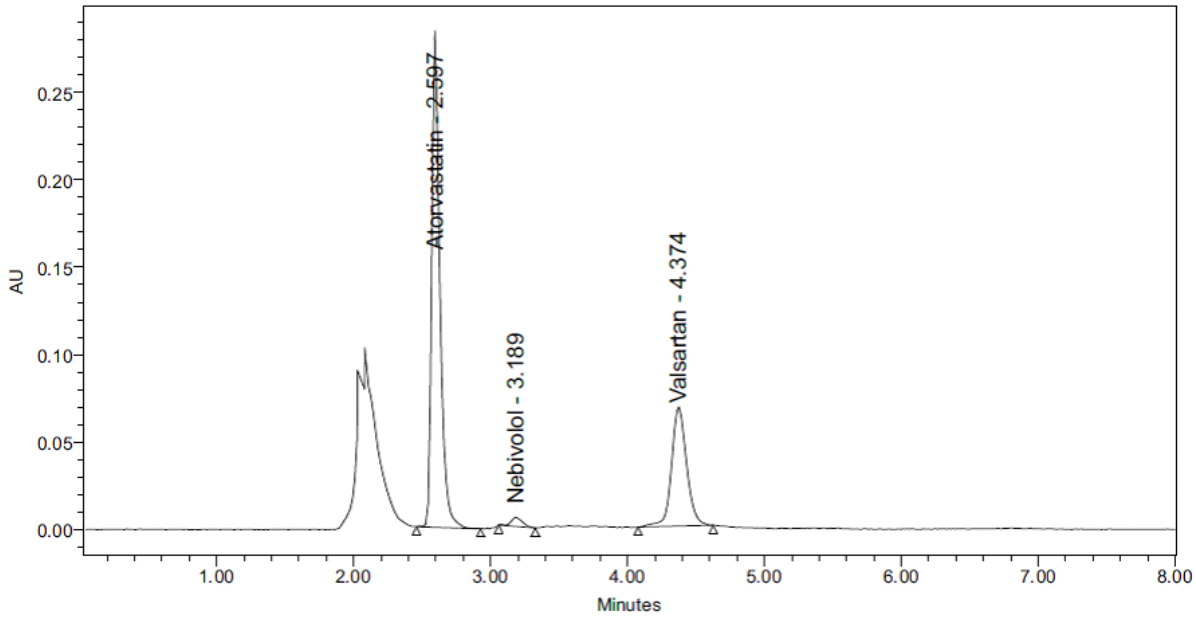

Figure 2. Chromatogram of Optimized Chromatographic Method.

7. Stabilities

Long-term stock solution stability for NL: In bench-top stability, six replicates of LQC and HQC samples (1.5 and 8 ng/mL) were analysed after 9 h of storage at room temperature in the laboratory bench. The % mean stability was calculated. **Long-term stock solution stability for VL:** In bench-top stability, six replicates of LQC and HQC samples (1200 and 6400 ng/mL) were analysed after 9 h of storage at room temperature in the laboratory bench. The % mean stability was calculated.

Matrix samples' stability at -20°C ± 5°C and -70°C ± 5°C after for 37 days of storage

Long-term stock solution stabilities for NL and VL were determined at LQC and HQC concentration levels after a storage period of 37 days at -20°C and -70°C in the deep freezer. The % means stabilities of NL and VL were calculated.

RESULTS

Optimisation of chromatographic method

Chromatographic separation of NL and VL by RP-HPLC method was performed by various mobile phase trials. The ideal separation of NL and VL was obtained by using a mobile phase consisting of 0.01 N potassium dihydrogen phosphate (pH 3.0):acetonitrile in the ratio of 60:40. A representative chromatogram of the optimised chromatographic method is shown in Figure 2.

System suitability test

All the system suitability parameters were within the range and satisfactory as per the ICH guidelines. The % coefficient of variation (CV) for system suitability test was in the range of 1.0 ± 0.0316 for RT of NL, 1.18 ± 0.0512 for RT of VL and 0.23% for the area ratio (analyte area/IS area) of atorvastatin. Full results of the system suitability test are shown in Table 1.

Selectivity

Evaluation of selectivity was performed by testing six batches of K_2EDTA blank plasma. The analysis of extracted blank plasma showed that the developed method has good selectivity for both drugs and IS. Representative chromatograms are shown in Figure 3 of standard blank and blank with IS sample using pooled plasma.

Matrix factor evaluation

The matrix effect plays a vital role in the assessment of pharmacokinetic studies. It was expressed as an IS-normalised matrix factor and it varied from 0.90 to 0.99, which was close to 1, which indicates that there was no analytical signal suppression or enhancement in plasma samples. Results are shown in Table 2.

Precision and accuracy

The intra-day and inter-day accuracies of plasma samples were assessed and excellent mean % accuracies for NL and

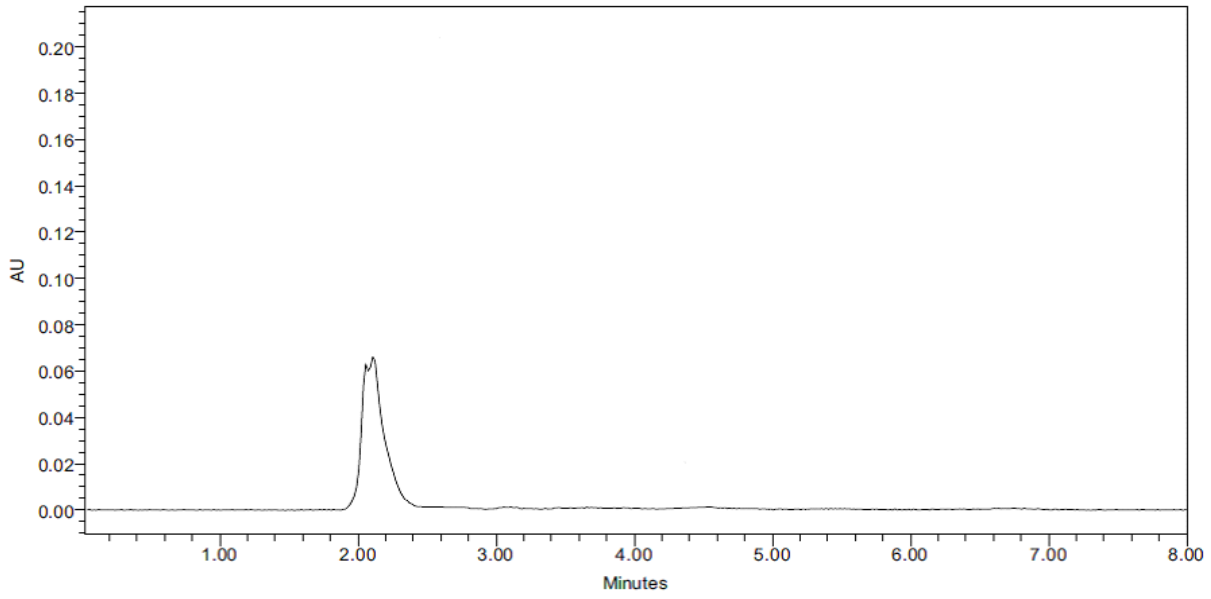

Figure 3. Extracted standard blank sample.

Table 1. Observation of optimised method chromatogram.

System suitability parameters	Atorvastatin	Nebivolol	Valsartan
RT	2.597	3.189	4.374
Area	97,579	5671	11,302
USP plate count	9792.8	9135.0	8011.3
USP tailing	1.4	1.4	1.1
USP resolution	-	4.6	6.9
Area ratio	-	0.05686	0.3545
SD	-	0.0316	1.00
% CV	-	0.0512	1.18

Table 2. Matrix factor evaluation of nebivolol and valsartan.

Parameters	Nebivolol		Valsartan	
	HQC	LQC	HQC	LQC
n	18	18	18	18
Mean	7.9592	1.4937	6400.5556	1190.4444
SD	0.07785	0.01768	44.02035	30.91016
% CV	0.98	1.18	0.69	2.60
% Mean accuracy	99.49	99.58	100.01	99.20
No. of QC failed	0	0	0	0

Table 3. Intra-day precision and accuracy of nebivolol and valsartan.

Parameters	Nebivolol				Valsartan			
	HQC	MQC	LQC	LLOQ QC	HQC	MQC	LQC	LLOQ QC
n	6	6	6	6	6	6	6	6
Mean	7.9513	4.9662	1.4943	0.4985	6403.1667	4017.5000	1196.000	396.5000
SD	0.05211	0.10071	0.01707	0.01320	10.60974	107.61924	11.6666	8.06846
% CV	0.66	2.03	1.14	2.65	0.17	2.68	0.97	2.03
% Mean accuracy	99.39	99.32	99.62	99.70	100.05	100.44	100.44	99.13

Table 4. Inter-day precision and accuracy of nebivolol and valsartan.

Parameters	Nebivolol				Valsartan			
	HQC	MQC	LQC	LLOQ QC	HQC	MQC	LQC	LLOQ QC
n	6	6	6	6	6	6	6	6
Mean	7.9238	4.9362	1.4907	0.4977	6394.3333	3999.0000	1194.8333	393.5000
SD	0.08962	0.08039	0.01770	0.01799	15.83246	70.11419	11.72035	6.05805
% CV	1.13	1.63	1.19	3.61	0.25	1.75	0.98	1.54
% Mean accuracy	99.05	98.72	99.38	99.53	99.91	99.98	99.57	98.38

Table 5. Batch-to-batch precision and accuracy of nebivolol and valsartan.

Parameters	Nebivolol				Valsartan			
	HQC	MQC	LQC	LLOQ QC	HQC	MQC	LQC	LLOQ QC
n	18	18	18	18	18	18	18	18
Mean	7.9577	4.9657	1.4917	0.4981	6398.5000	3993.7222	1194.17	396.1667
SD	0.07478	0.08347	0.01596	0.01470	11.92254	79.93071	11.1596	7.10634
% CV	0.94	1.68	1.07	2.95	0.19	2.00	0.93	1.79
% Mean accuracy	99.47	99.31	99.44	99.61	99.98	99.84	99.44	99.04

VL were obtained with range varying from 99.32% to 99.70% and from 99.13% to 100.44% for intra-day accuracy and from 99.31% to 99.61% and from 99.84% to 99.98% for inter-day accuracy, respectively. The precision (% CV) of the analytes in the plasma samples was calculated and found to be 0.66%–2.65% and 0.17%–2.68% for intra-day accuracy and 0.94%–2.95% and 0.19%–2.00% for inter-day accuracy, respectively. Results are shown in Tables 3–5.

Linearity

Calibration was found to be linear over the concentration range of 0.5–10 ng/mL for NL and 400–8000 ng/mL for VL. The coefficient of determination (r^2) value was found to be consistently greater than 0.999 in all the cases shown in Figure 4a and b. Linearity of results shows an excellent correlation between the peak area ratios for each concentration of analytes; results are shown in Table 6.

Recovery

The overall % mean recoveries for NL and VL were found to be 97.96% and 98.11%, respectively. The recoveries were obtained for NL and VL at three QC concentration levels. The overall % mean recovery for atorvastatin (IS) was found to be 99.00%. The results are shown in Table 7.

Stabilities

Long-term stock solution stability for NL

The % mean stability of six replicates of LQC and HQC samples (1.5 and 8 ng/mL) was analysed after 9 h of storage at room temperature on the laboratory bench. These were calculated and found to be 99.51% for LQC and 99.92% for HQC. The result is shown in Table 8.

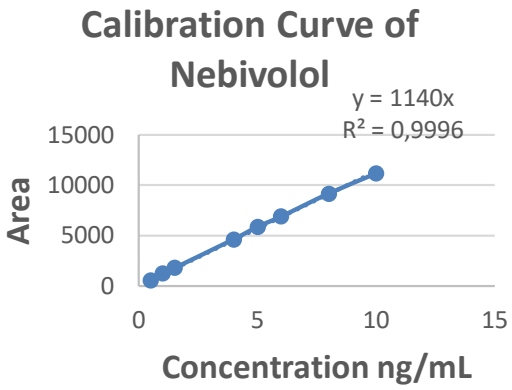

Figure 4a. Calibration Curve of Nebivolol.

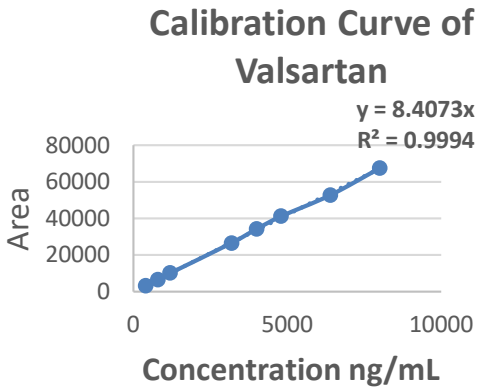

Figure 4b. Calibration Curve of Valsartan.

Table 6. Observation table for linearity of nebivolol and valsartan.

Nebivolol			Valsartan		
Final conc. in ng/mL	**AUC**	**Area ratio**	**Final conc. in ng/mL**	**AUC**	**Area ratio**
0.5	570	0.006	400	3415	0.0350
1	1235	0.013	800	6724	0.0689
1.5	1785	0.018	1200	10,160	0.1041
4	4643	0.048	3200	26,678	0.2731
5	5880	0.060	4000	34,216	0.3506
6	6902	0.071	4800	41,162	0.4217
8	9176	0.094	6400	52,728	0.5402
10	11,175	0.115	8000	67,420	0.6910
Parameters	**Nebivolol**		**Valsartan**		
Correlation coefficient	0.998		0.9994		
Regression equation	y = 1140x		8.4073x		
Linearity range	0.5–10 ng/mL		400–8000 ng/mL		

Table 7. Recovery study of nebivolol and valsartan.

Parameters	**Nebivolol**						**Valsartan**					
	Unextracted (HQC)	**Extracted (HQC)**	**Unextracted (MQC)**	**Extracted (MQC)**	**Unextracted (LQC)**	**Extracted (LQC)**	**Unextracted (HQC)**	**Extracted (HQC)**	**Unextracted (MQC)**	**Extracted (MQC)**	**Unextracted (LQC)**	**Extracted (LQC)**
n	6	6	6	6	6	6	6	6	6	6	6	6
Mean	9445	9253	5965	5782	1886	1857	54,658	53,310	35,376	34,761	10,665	10,507
SD	59.17	70.20	53.57	66.05	12.32	31.02	657.59	400.50	416.36	210.54	143.61	70.53
% CV	0.62	0.75	0.89	1.14	0.65	1.67	1.20	0.75	1.17	0.88	1.34	0.67
% Mean recovery	97.96		96.93		98.46		97.54		98.26		98.52	
Overall % mean recovery	97.784						98.112					

Table 8. Long-term stock solution stability of nebivolol and valsartan.

Parameters	Nebivolol		Valsartan	
	HQC	LQC	HQC	LQC
n	6	6	6	6
Mean	7.9938	1.4927	6391.6667	1193.5000
SD	0.01950	0.02082	21.42584	20.08731
% CV	0.24	1.39	0.34	1.68
% Mean accuracy	99.92	99.51	99.87	99.46

Table 9a. Matrix samples' stability of nebivolol and valsartan at -20°C ± 5°C for 37 days.

Parameters	Nebivolol				Valsartan			
	HQC (comparison sample)	HQC (stability sample)	LQC (comparison sample)	LQC (stability sample)	HQC (comparison sample)	HQC (stability sample)	LQC (comparison sample)	LQC (stability sample)
n	6	6	6	6	6	6	6	6
Mean	7.9930	7.8872	1.4872	1.4898	6394.5000	6391.1667	1193.1667	1188.5000
SD	0.02481	0.15394	0.02729	0.01393	23.57753	18.50856	20.48821	18.98157
% CV	0.31	1.95	1.83	0.94	0.37	0.29	1.72	1.60
% Mean accuracy	99.91	98.59	99.14	99.32	99.91	99.86	99.43	99.04
% Mean stability	98.68		100.18		99.95		99.61	

Long-term stock solution stability for VL

The % mean stability of six replicates of LQC and HQC samples (1200 and 6400 ng/mL) was analysed after 9 h of storage at room temperature on the laboratory bench. These were calculated and found to be 99.51% for LQC and 99.92% for HQC. The result is shown in Table 8.

Matrix samples' stability at -20°C ± 5°C and -70°C ± 5°C after 37 days of storage

The % mean stability of NL was found to be 100.18% (LQC) and 98.68% (HQC) at 20°C ± 5°C and 99.65% (LQC) and 100.33% (HQC) at 70°C ± 5°C. The % mean stability of VL was found to be 99.61% (LQC) and 99.95% (HQC) at 20°C ± 5°C and 99.51% (LQC) and 100.23% (HQC) at 70°C ± 5°C. The results are shown in Tables 9a and b.

DISCUSSION

After studying literature about NL and VL, the method development was started according to hydrophobic and hydrophilic interaction of the drugs. The absorbance spectra of both drugs were measured, overlayed and the wavelength of optimal sensitivity of determination was chosen (280 nm). The IS was selected based on the presence of chemical properties similar to the compounds of interest. Atorvastatin was chosen as the IS. After selecting the wavelength, the next step was the selection of the extraction procedure by observing the % recovery. The protein precipitation method was selected as the extraction procedure for better recovery. After selection of the extraction method, the method development process started with a flow rate of 1.0 mL/min at a column temperature 30°C, mobile phase 0.1% orthophosphoric acid (pH 2.2):acetonitrile (50:50), column Agilent C18 (150 mm × 4.6 mm, 5 µm) and injection volume 50 µL. In these parameters, VL was not eluted. So, further trial was carried out. In the second trial, the mobile phase and its ratio were changed to 0.01 N potassium dihydrogen phosphate (pH 3.0):acetonitrile (55:45) and other chromatographic conditions were the same as first trial. In the second trial, the resolution between atorvastatin and NL was unacceptable. So, third trial was carried out using 0.1% orthophosphoric acid (pH 2.2):acetonitrile (60:40) mobile phase and Symmetry C18 (150 mm × 4.6 mm, 5 µm) column. VL was not eluted in this trial. In the fourth trial, mobile phase 0.01 N potassium dihydrogen phosphate (pH 3.0):acetonitrile (70:30) and column Agilent C18 (150 mm × 4.6 mm, 5 µm) were used. In

Table 9b. Matrix samples' stability of nebivolol and valsartan at -70°C ± 5°C for 37 days.

Parameters	Nebivolol				Valsartan			
	HQC (comparison sample)	HQC (stability sample)	LQC (comparison sample)	LQC (stability sample)	HQC (comparison sample)	HQC (stability sample)	LQC (comparison sample)	LQC (stability sample)
n	6	6	6	6	6	6	6	6
Mean	7.9880	8.0145	1.4953	1.4902	6386.5000	6401.5000	1194.5000	1188.6667
SD	0.02458	0.05858	0.02273	0.01646	22.29574	21.37054	17.14351	14.17980
% CV	0.31	0.73	1.52	1.10	0.35	0.33	1.44	1.19
% Mean accuracy	99.85	100.18	99.69	99.34	99.79	100.02	99.54	99.06
% Mean stability	100.33		99.65		100.23		99.51	

this trial, NL United States Pharmacopeia (USP) plate count was low and RTs were unnecessarily high. So, in the next trial, 0.01 N potassium dihydrogen phosphate (pH 3.0):acetonitrile (60:40) was used as the mobile phase and Symmetry C18 (150 × 4.6 mm, 5 µm) was used as the column. All peaks eluted with good peak shape and RT. The tailing tests were also passed. So, this method is used as an optimised method for the separation of NL and VL from human plasma. After optimisation was completed, the method was evaluated for various validation parameters as per the ICH M10 guideline.

CONCLUSION

A simple, accurate and precise method was developed for the estimation of NL and VL in human plasma using atorvastatin as the IS. RTs of NL and VL were found to be 3.146 and 4.346 min, respectively. The % CV of NL and VL was found to be 1.0% and 0.58%, respectively. Per cent recoveries were obtained as 98.28% and 98.11%, respectively. The linearity concentrations were in the range of 0.5–10 ng/mL for NL and 400–8000 ng/mL for VL. The lower limits of quantification were 0.5 ng/mL for NL and 400 ng/mL for VL, which reached the levels of both drugs possibly found in human plasma. Further, the reported method was validated as per the ICH guidelines and found to be well within the acceptable range. The proposed method is simple, rapid, accurate, precise and appropriate for pharmacokinetic studies and therapeutic drug monitoring in clinical laboratories.

LIST OF ABBREVIATIONS

ABBREVIATIONS	FORMS
NL	Nebivolol
VL	Valsartan
RP	Reverse phase
HPLC	High-performance liquid chromatography
BA	Bioavailability
BE	Bioequivalence
API	Active pharmaceutical ingredient
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet
QC	Quality control
RT	Retention time
USP	United States Pharmacopeia
CV	Coefficient of variation
IS	Internal standard
SD	Standard deviation

References

[1] Neil O (2006) The Merck Index an encyclopedia of Chemical and Biological.14th ed. *Merck & Co. Inc1*. 48: 379.

[2] Nadeem S, Asif H, Lakshita C, Shamsher A, Moloy M, Parminder SB (2011) Pharmacological and Pharmaceutical Profile of Valsartan: A Review. *J. of Appl. Pharm. Sci.* 01(04):12-19

[3] Goodman and Gilman's (2001) The Pharmacology Basis of Therapeutics. New York: *Medical publishing Division*, pp 249-260.

[4] Indian Pharmacopoeia (2010), Vol. III; Controller of Publication: New Delhi, 1759.

[5] Indian Pharmacopoeia (2010),, Vol. III; Controller of Publication: New Delhi, 2257.

[6] Pratapa P.Prasad, Ching-Ming Yeh, Peter Gurrieri, Robert Glazer, and James McLeod (2002) Pharmacokinetics of Multiple Doses of Valsartan in Patients with Heart Failure, Journal of cardiovascular pharmacology40:801-807

[7] Vaibhav rajoriya & Varsha kashaw (2017) RP-HPLC Method for the simultaneous estimation of Nebivolol hydrochloride & valsartan, analytical chemistry letters,7:4,520-530

[8] Shinde V, Sufiyan Ahmad, V.M Shastri, Yogesh Patil (2017) analytical method development and validation for the simultaneous estimation of Nebivolol and Valsartan by RP-HPLC in bulk and tablet dosage forms, Journal of pharmacy research 11:5:472-478

[9] Seetharaman R, Lakshmi KS (2014) Development and Validation of a Reverse Phase Ultra Performance Liquid Chromatographic Method for Simultaneous Estimation of Nebivolol and Valsartan in Pharmaceutical Capsule Formulation. J Chromatograph SeparatTechniq 5: 229. doi:10.4172/2157- 7064.1000229

[10] NekkalaK, V Shanmukha KJ, Ramchandran D, Ganji R and Ganta S (2014) Method development and validation of stability indicating RP-HPLC method for simultaneous estimation of Nebivolol HCL and valsartan in bulk and its pharmaceutical formulations.Amer J of Adv Drug Del 2:.5:624-637

[11] Bhatia MS, Kokil SU(2009) Simultaneous estimation of Nebivolol HCL & valsartan using RP-HPLC in capsule dosage form, Ind. J of Pharm Sci71:.2:111-114

[12] Madhvi V, Siddhartha BK, Pratibhan C (2014) Simultaneous estimation of Nebivolol and Valsartan in tablet dosage form by RP-HPLC, Int J of Pharm and pharm Sci., 6:1:278-282

[13] Modiya JS, Pandya BC, Channabasavraj KP (2010) Simultaneous Estimation of Nebivolol Hydrochloride and Valsartan in Bulk and Capsule Dosage Form by Simultaneous Equation Method, Int J of ChemTech res 2:3:1387-1390.

[14] Sonawane LV, Poul BN, Usnale SV, Waghmare PV, Surwase LH (2014) Bioanalytical Method Validation and Its Pharmaceutical Application- A Review. Pharm Anal Acta 5: 288. DOI: 10.4172/2153-2435.1000288

[15] ICH guideline M10 on bioanalytical method validation, 13 March 2019, European medicine Agency EMA/CHMP/ICH/172948/2019. https://www.ema.europa.eu/en/ich-m10-bioanalytical-method-validation.

[16] Darkunde SL, Borhade RN (2017) Bioanalytical Method Validation: A Quality Assurance Auditor View Point. Asian J of Pharm Tech and Innov 5:59-60

[17] Tijare LK, Rangari NT, Mahajan UN (2016) A review on bioanalytical method development and validation. Asian J of PharmClin Res9:6-10

[18] Method development and validation skills and tricks (2019) :3 (https://lubrizolcdmo.com/technical-briefs/analytical-method-development-and-validation/)

[19] Latha E, Sailaja B, Padmavati S (2014) Bioanalytical Method Development and Validation of by HPLC. J of Med and PharmInnov. 1:1-9