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An experience with the colorimetric testing of amlodipine in physiological fluids: interference in drug assay by phytochemicals

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Abstract

This study aims to examine the possible interactions between amlodipine (AML) and methanol extract of Aframomum melegueta seeds (AMSE). Methods: Using the potassium ferricyanide/FeCl3 (FeCl3/K4(Fe(CN)6)) technique, amlodipine concentrations of 2.5, 5.0, 7.5, 10, 12.5 and 15 µg/mL were tested in vitro with or without AMSE. Absorbance measurements were taken at 393.1, 455.6, and 774.8 nm, and the solution was then subjected to wavelength scanning in the 380–950 nm range. Conclusions: The presence of Aframomum melegueta seeds (AMSE) in biological fluids and AML solutions considerably hindered the reaction of FeCl3/K4(Fe(CN))6. The maximum interference was seen at 774.8 nm, and a concentration of 50 µg/mL AMSE resulted in a 2.5 µg/mL rise in absorbance, which is 1.5 times higher than when it was not present. The sample's spectra showed two extra peaks at 393.1 and 455.6 nm, with unit increments of just 0.07 and 0.16 nm, respectively, when AMSE was present. At these two wavelengths, Beer-Lambert's law was met by the concentration-absorption relationship. It was decided not to follow Beer-Lambert's rule after the AML concentration reached 15 µg/mL at 774.8 nm. This research concludes that the components of the Aframomum melegueta seed methanol extract may interact with one another in AML testing techniques. In addition, measurements of concentration at either 393.1 or 455.6 nm have been shown to be reliable. When measuring drugs in populations where the use of herbal treatments is likely to occur concurrently, this should be taken into consideration. Amlodipine, Aframomum melegueta, Colorimetry, Interference

INTRODUCTION

Amlodipine (AML) Besylate is a dihydropyridine calcium channel blocker clinically used as an antihypertensive, anti-angina drug [1,2], as well as a peripheral arterial vasodilator [3,4]. Its chemical name is 3-ethyl-5-methyl (4RS)-2-(2-aminoethoxymethyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methylpyridine-3,5-dicarboxylate benzenesulfonate (Figure 1) [5].

In an attempt to carry out a pharmacokinetic study of amlodipine (AML) in rodents using potassium ferricyanide/FeCl3 (FeCl3/K4(Fe(CN)6)) colorimetric method [4], absorbance values obtained showed wide variations such that measured AML concentrations did not conform to any reasonably expected pattern consistent with pharmacokinetic characterization [5]. Several studies have revealed potential interactions of herbal constituents with amlodipine. For example, the antihypertensive effect of amlodipine was augmented by *Lepidium sativum* and *Curcuma longa* extract [6]. In another study, green tea and cumin increased plasma concentration of amlodipine and prolonged antihypertensive effect [7]. A major key issue is not about the efficacy of herbal preparations, but the fact that the preparations are almost always marketed as food supplements in order to avoid rigorous and stringent regulatory requirements. This designation as food supplements implies that such substances are as safe for general consumption as ordinary food is. In addition, wide use of herbal remedies may not only be due to resource

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limitations but also to apparent support offered by those who tout these preparations as very safe. Justification for safety is based on natural sources of the preparations which are believed to be devoid of any harm or complications [8]. Moreover, it is always very convenient to forget that plant kingdom is a veritable, high-capacity chemical factory and that herbal components used as therapeutic remedies contain vast amounts of chemical substances that interact with chemical reagents used for clinical drug concentration measurements in biological fluids [8]. As a result, this study was aimed at investigating the pharmacokinetics of AML in the presence of AMSE.

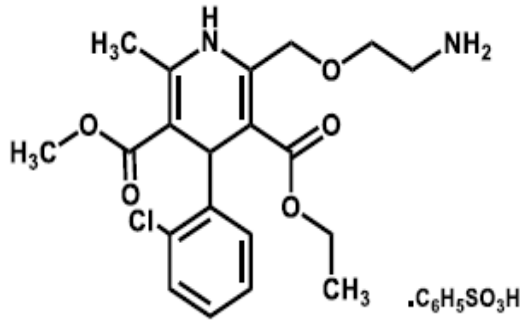

Figure 1: Structure of Amlodipine besylate, (2- ((2-Aminoethoxy)-methyl)-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5- pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzene sulfonate

EXPERIMENTAL

Preparation of reagents

A 0.4 % Ferric chloride (Sigma-Aldrich Laborchemikalien GmbH. D-30926, Seelze, Germany) was prepared by dissolving 200 mg of FeCl3 in 30 mL of 1 N hydrochloric acid and final volume made up to 50 mL with the same solvent. Thereafter, 0.2 % potassium ferricyanide (Sigma-Aldrich Laborchemikalien GmbH Seelze, Germany), was prepared by dissolving 100 mg of the salt in 30 mL of distilled water and final volume made up to 50 mL with the same solvent. Tyrode solution (a physiological salt solution isotonic to *in vivo* intestinal tissue milieu containing sodium chloride, 8 g; potassium chloride, 0.2 g; calcium chloride dihydrate, 0.24 g; magnesium chloride hexahydrate, 0.1 g; glucose 1 g per Liter) was prepared following standard guidelines [9].

Preparation of plant extract

Dried *A. melegueta* fruits were bought from local markets in Abia State, Nigeria, and were further dehydrated to a consistent weight in a 40 oC oven. The pods were opened to release seeds which were ground into fine powder using a laboratory manual grinder. After that, 100 g powder was extracted in a soxhlet extractor of 500 mL capacity with petroleum ether followed by methanol. Methanol extract was evaporated at a reduced temperature of 40 oC and stored in a desiccator till further use. The percent yield of methanol extract was calculated to be 6.7 %.

***In vitro* intestinally absorbed assay of AML**

In order to study interactions between AMSE and amlodipine, gastrointestinal local environment where drugs are usually absorbed was reproduced *in vitro* by use of everted intestinal sacs immersed in biological fluid (Tyrode solution).

Tyrode-filled everted intestinal sacs were incubated for 30 mins in 500 mL beakers containing either test drug solution alone (AML; 100 µg/mL) or in the presence of 50 µg/mL AMSE. After incubation, tissues were damped dry and fluid from the serosal side of sacs was drained into labeled sample bottles. Contents of sample bottles were assayed for AML using the potassium ferricyanide/Ferric chloride colorimetric assay method [9] with slight modification for sample volume.

Standard curve for AML besylate solution assay

A standard curve for AML besylate (NORVASC, Pfizer Inc.) was constructed by using the colorimetric method for the determination of AML besylate [4].

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Standard concentrations of amlodipine 2.5, 5, 7.5, 10, 12.5 and 15 µg/mL respectively were prepared from stock solution of 100 µg/mL and used in the construction of the standard curve. Amlodipine concentrations were determined using the same protocol and absorbencies of AML solutions were read at 393.1, 455.6 and 774.8 nm against blank reagent.

Spectrophotometric scan of AML solution with or without AMSE

A full spectrophotometric wavelength scan was performed for standard concentrations of AML after being subjected to colorimetric protocol. Specific scans of selected AML concentrations in the presence and absence of AMSE were also conducted using a Spectrovis® Plus spectrophotometer (range: 380 - 950 nm; resolution: ~2.5 nm optical resolution, 570 wavelengths, 1 nm reporting Intervals), with Logger Pro ® Software (Vernier International, 5026 Calle Minorga, Sarasota, FL 34242 USA) running on an Intel Pentium® PC. Data were recorded in wavelength values expressed in nanometers (meters x 10-9) as abscissa and in optical densities, expressed as log Io/I (absorbance), as ordinates. Measurements of the extinction coefficients were taken at intervals of 1 nm from 380 - 950 nm.

Statistical analysis

Data were recorded as Mean ± SEM and statistical comparison was performed using the student's *t*-test. Calculated probability values were two-tailed and $p < 0.05$ was considered significant. All statistical tests were done using GraphPad Prism (GraphPad Prism version 5.01. GraphPad Software Inc.).

RESULTS

Standard curve and determination of AML

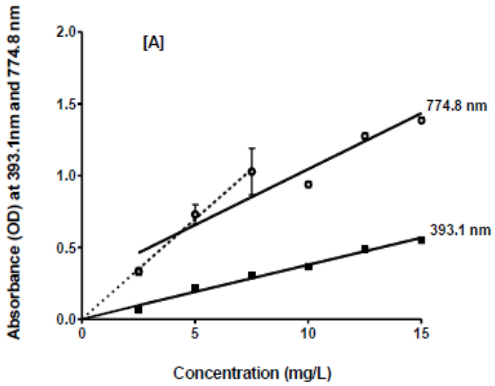
Standard concentrations of amlodipine of 2.5, 5, 7.5, 10, 12.5 and 15 µg/mL respectively were used to construct standard curves. Concentration-absorbance relationships obtained for AML are shown in Figure 2 A. Regression for the standard curve is represented by the linear Eq 1.

YABS = BX+C ……. (1)

where B = slope and C = intercept on the Y axis.
Linear regression parameters obtained for standard curves at different wavelengths are shown in Table 1. The intercept of the curve at 393.1 nm passed through the origin (zero) while that for 774.8 nm has a positive offset value at zero concentration (Figure 2 A). Also, residual values calculated for 393.1 nm fluctuate less around the mean regression line when compared with those for 774.8 nm (Figure 2 B). In both cases though, fluctuations were more pronounced at lower AML concentrations than at higher concentrations.

Table 1: Parameters of regression lines plotted for standard curves for AML at different wavelengths

Parameter	Wavelength (nm)	
	393.1	774.8
Slope (B)	0.038±0.00	0.078±0.01
Intercept (C)	0.003±0.01	0.268±0.08
Correlation coefficient (r^2)	0.98	0.83



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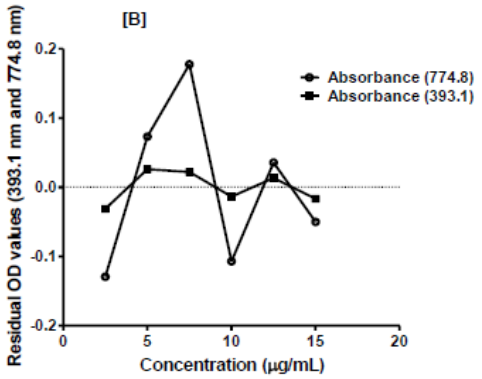

Figure 21: (A) Standard curves of AML plotted at 774.8 nm and 393.1 nm. Solid line for 774.8 nm wavelength was constructed using AML concentrations ranging from 2.5 µg/mL to 15 µg/mL. Dashed line represents curve when only concentration of up to 10 µg/mL was used to construct the standard curve at 774.8 nm wavelength. **(B)** Residual plots of AML at two wavelengths 774.8 nm and 393.1 nm

Wavelength scans of AML in the presence and absence of AMSE

Spectral scans of different standard solutions of AML over wavelengths spanning 380 - 950 nm at 1 nm intervals are shown in Figure 3. There were three distinct peaks precisely at 393.1, 455.6 and 774.8 nm, respectively and that these peaks showed progressing intensities from lowest to highest wavelengths. Beer's Law was obeyed at these three peaks based on standard concentrations. However, at 10 µg/mL, with 774.8 nm wavelength, absorbance values started showing signs of non-linearity which became progressively worse up to 15 µg/mL where the maximum noisy signal was recorded. At 393.1 and 455.6 nm wavelengths, there is an indication that Beer's Law will still be satisfied at AML concentrations far greater than 15 µg/mL although responses at 455.6 nm wavelength were far better than those at 393.1 nm wavelength (Figure 3 A).

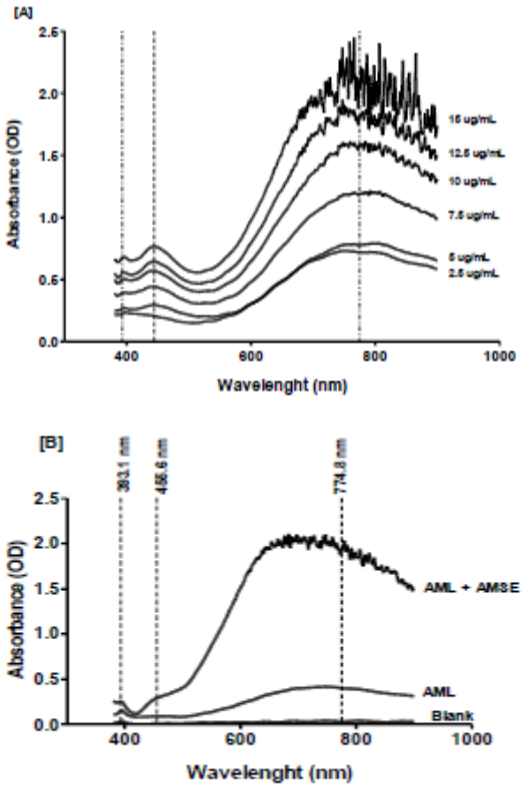

Figure 3: (A) Overlain spectra of wavelength scans of AML standard concentrations ranging from 2.5 to 15 µg/mL. Broken vertical lines indicate wavelengths at 393.1, 455.6 and 774.8 nm respectively. **(B)** Wavelength scans for 2.5 µg/mL AML in the absence and presence of AMSE. AML concentrations were determined according to the potassium ferricyanide/FeCl3 (FeCl3/K4(Fe(CN)6)) method
Influence of AMSE on absorbance of AML was tested and shown in Figure 3 B. Spectral scan for 2.5 µg/mL AML when measured alone was far lower than that obtained in the presence of 50 µg/mL of AMSE. This effect of AMSE is seen at three peaks in Figure 3 A although it is most pronounced at 774.8 nm. Absolute changes in absorbance due to the presence of AMSE at 393.1, 455.6 and 774.8 nm wavelengths are 0.07, 0.16, and 1.50 respectively.

DISCUSSION

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Doijad *et al* [4] reported a method for measurements of AML in biological fluids which involves use of a combination of potassium ferricyanide and ferric chloride. They did not provide any rigorous chemical justification for selection of two reagents except that they both contain particular and required functional groups present in the chemical structure of the drug. It was also reported that this method was optimized in terms of the concentration of reagents used as well as the sequence of addition of these reagents.

In preliminary studies on the pharmacokinetics of amlodipine in the presence of AMSE, peak absorbances were measured at 774.8 nm wavelength, which was similar to 775 nm used by developers of ferric chloride/ferricyanide assay method [4]. Measurements at this wavelength, however, deviated from linearity within concentration range of amlodipine used in the study (2 - 30 µg/mL). It was observed that beyond 10 µg/mL, concentration, and absorbance values of standard drug solutions were tilting towards a non-linear region (absorbance 1.5) beyond which Beer's law started to fail. Consequently, concentrations above 10 µg/mL in AML standard curve were bound to introduce non-linearity into the regression line meaning that such standard curve equation cannot be relied on for the determination of the concentration of any unknown amlodipine solution. An initial attempt at plotting standard curve with concentrations up to 15 µg/mL resulted in a regression line with large deviations of residuals around the mean.

Furthermore, to investigate what effect the absorbance values nearing the 1.5 mark were contributing to observed fluctuations in residuals, wavelength spectral scans (380 to 900 nm) of standard AML concentrations were performed. There was significant noise in absorbance values in scans from concentrations above 10 µg/mL, which worsened progressively up to15 µg/mL. Also, the presence of two other smaller peaks for AML at wavelengths of 393.1 and 455.6 nm respectively demonstrated compliance with Beer's law although with reduced intensity when compared to that at 774.8 nm. The plot of absorbance values at 393.1 nm wavelength against standard concentrations gave a better correlation with minimal residual fluctuations and also a regression line that passes through the origin. Surprisingly, excluding absorbance values for concentrations above 10 µg/mL from standard curve constructed at 774.8 nm gave a better correlation and linear regression line that also passed through the origin.

It is clear from this study that if the range of AML standard concentrations as reported by Doijad *et al* [4] had been used for constructing a standard curve without discrimination, concentration of AML in biological fluid measured would have given erroneous values with an offset corresponding to intercept of standard curve on absorbance axis. This will result in a positive AML concentration value in the absence of any drug in the solution. When concentrations of AML were determined using standard curve of AML measured at 774.8 nm, nature of the observed effect of AMSE was different from that seen when standard curve was plotted from concentration measurements at 393.1 nm. The reason for this anomaly is attributed to the sensitivity of spectrophotometer used, which was much more sensitive than that used by Doijad *et al* [4] such that at 12.5 µg/mL, AML was already showing absorbance values within a non-linearity range. The exclusion of concentrations beyond 10 µg/mL was able to give a standard curve with improved linearity and zero intercept on absorbance axis.

It was observed that color reactions and absorbance values for AML in presence of the extract were so high as to give unusual concentration values of AML far above what was reasonably expected. Wavelength scan of AML (2.5 µg/mL) in presence of AMSE (50 µg/mL) greatly enhanced the absorbance value of AML when compared to that measured in its absence, and while this effect was phenomenal at the 774.8 nm wavelength, it was far reduced at both 393.1 nm or 455.6 nm. Enhanced color reaction and absorbance values in the presence of AMSE is due to an extract component absorbing significantly at 774.8 nm after reacting with either or both chemical species in AML reagent (potassium ferricyanide and ferric chloride). Taking into consideration what is already known about the chemical reactions of ferric chloride, it seems reasonable to attribute this interference by AMSE to direct reaction of ferric chloride with components of

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AMSE. This is because ferric chloride has always been employed as a reagent for the identification of phenols and phenolic compounds such as flavonoids [10].

A dilute solution of ferric chloride has been known to interact with phenolic hydroxyl groups to produce color reactions that range from violet through blue to red and green, depending on the type of phenolic compound involved [11]. It is however not likely that this reaction formed the basis for use of FeCl3 in quantification of AML. This is because AML molecule does not possess phenolic hydroxyl group and as such is incapable of interacting with FeCl3 directly in order to produce classical blue color attributable to phenolics. Although the nature of chemical reaction of AML with components of the reagent was not specified by Doijad *et al* [4], however, an earlier study [12] had reported using similar reagents for evaluation of reducing power of tannins based on modification of the method of Gulcin *et al* [13]. According to these authors, Fe(CN)63- ions will be reduced to Fe(CN)64- ions when a reducing agent is added, thereafter, FeCl3 solution will then react with these ions to form Fe4(Fe(CN)6)3 complex while the Fe2+ present in the complex will be detected at 700 nm, thus, increased absorbance indicates increased reducing power. It therefore suggests that this reducing mechanism is responsible for absorbance of AML. In addition, the reduction of ferricyanide species is a result of the phenolic OH group of tannins which are essentially phenolic.

Phenolic hydroxyl groups of flavonoids, though capable of direct ferric chloride reaction with its OH group to produce classical color reaction between phenols and ferric chloride, may be acting as a reducing agent and reacting like AML with the same reagents. This is because flavonoids are reducing agents by nature and they act as antioxidants [14]. There were no absorbance peaks at a range of wavelengths at which direct ferric chloride-phenol reaction absorbs and even if there were, it probably must have been swamped or masked by a broad peak shown at wavelength 774.8 nm.

As for AML, accurate quantifications are still achieved and interference is avoided by taking measurements at 393.1 and 455.6 nm which are beyond the range where AMSE or any other phenolic components interfere.

CONCLUSION

Chemical reactions between components of AMSE and reagents for colorimetric determination interfere with the measurement of AML in biological fluids. This demonstrates the potential of such interactions from herbal components with measurements of other clinically used drugs, a possibility that should be kept in mind when carrying out drug measurements in populations where concurrent use of herbal remedies is highly probable.

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