Identification and Quantification by HPLC-DAD of Furosemide as a Co-adulterant in Products of Natural Origin

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Abstract

The low cost added to easy access and expectation of low or no side effects make these products increasingly attractive. When a product of natural origin contains synthetic substances that are not declared in its formulation, the synthetic substance is characterized as adulteration. In order to identify and quantify adulterants in natural products, analytical methods have been developed and used as fundamental tools in the control of these products. Thus, two products of natural origin indicated for treatment of rheumatic and inflammatory diseases were analyzed to verify the presence of the co-adulterant furosemide. Co-adulterant presence in the products was tested using an Agilent® brand 1100 HPLC system with a quaternary pump, an automatic injector and a DAD detector, with a mobile phase composed of methanol/formic acid 0.2% 60/40 (v/v). HPLC-DAD indicates the presence of the undeclared furosemide compound in the original formulation of both analyzed samples. In sample A, 24 mg of furosemide per gram was found, while in sample B, 47mg per gram of product was obtained. The consumption of adulterated products may lead to risks such as drug interaction and intoxication, since active ingredients of synthetic origin are added without taking in consideration adjustments and quality of the raw material.

Keywords: Chromatography, Co-Adulterant, Furosemide, HPLC, Natural Products

1. Introduction

The use of medicinal plants as a form of healing and health maintenance in daily and cultural practices is part of ancient traditions around the world[1,2]. In the last decades, herbal products of medicinal interest, so-called "natural products", have gained prominence in this trade category.In 2002, approximately two thirds of the planet's population were using natural products[3].

In 2007, a study undertaken in the United States showed that 4 out of every 10 adults (40%) took some type of Complementary and Alternative Medicine (CAM) in the 12 months prior to the survey.Among them, 37.4% used DHA (docosahexaenoic acid), fish oil or omega 3, glycosamine (19.9%), Echinacea (19.8%), linseed oil (15.9%) and Ginseng (14.1%) in the 30 days prior to the research[4]. These products can be easily obtained, from the informal market such

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as the Internet too[5]. If synthetic drugs have many side effects listed in their package leaflets, so-called "natural products", on the contrary, give an idea of safety and effectiveness, which ultimately attracts more consumers, not only because of their natural origin, but also for the ease of purchase. Behind this presumed image of safety, these products may contain synthetic substances not stated in their formulation. This mislabeling phenomenon has been documented in several countries[6–8]. In order to potentiate the desired effect, several undeclared synthetic substances are added to natural products, putting the safety of thousands of people at risk[9]. Indications for use are the most varied, such as weight loss, sex stimulants, anti-inflammatories, analgesics, treatment of cardiovascular diseases, rheumatic diseases, antidiabetics, vitamin and food supplements, among others. In formulations indicated for weight loss, for example, synthetic substances belonging to the therapeutic classes of anorectics, anxiolytics, antidepressants, diuretics and laxatives have been found[6,10]. Another indication that drives the consumption of natural products is the treatment of rheumatic and inflammatory diseases.In these formulations, several substances belonging to different therapeutic classes, such as corticosteroids, benzodiazepines, analgesics, opioids, diuretics, non-steroidal anti-inflammatory drugs among others have been identified[11,12].

The presence of undeclared synthetic substances in the composition of a product constitutes the practice of adulteration, which violates the legislation of many countries[6]. In order to identify and quantify adulterants in natural products, analytical methods have been developed and used as fundamental tools in the control of these products[13,14]. Using sensitive and modern analytical techniques such as HPLC-DAD, HPLC coupled to mass spectrometry, gas chromatography, electrochemical and spectroscopic methods, among others, it is possible to efficiently detect these adulterants[15].

Thus, two products with indication for treatment of rheumatic diseases, pains and inflammations were analyzed to identify and quantify furosemide as a co-adulterant in the tested products using a High-Performance Liquid Chromatography (HPLC) with a Diode-Array Detector (DAD).

2. Materials and Methods

2.1 Samples and Standards

Two natural product samples in the form of pharmaceutical capsules were purchased via the internet. Sample A contained in its declared formulation extracts of pecan (*Carya illinoinensis*), yellow ipê (*Handroanthus albus*) and sucupira (*Pterodon emarginatus*). Sample B hadin its declared composition *Moringa oleifera* extract (leaves and stems) and "Canela de velho" (*Miconia albicans*). As aworking standard, 40 mg of Furosemide from Biossintética® (ACHÉ batch: 1809460, expiry date: 07/2020, content 90-110%) and the reagents HPLC Grade HPLC methanol Baker®: Y07C06, Formic Acid were used.

Samples were prepared from the mixture of 10 capsules of each product, respectively. After, about 1g of this mixture was diluted into 40mL of the mobile phase. Samples A and B were placed for 5 min in an ultrasonic bath.Subsequently, 4mL of this solution was transferred and completed to 10 mL with the mobile phase. After homogenization, samples were filtered on a 0.45μm PVDF membrane.

2.2 Chromatography Conditions

The samples and standards were analyzed using an Agilent® brand 1100 HPLC system with a quaternary pump, an automatic injector and a DAD detector. The software used for the processing of the samples was Empower® 3.

Three experimental tests were performed with the mobile phase and two with the preparation of the samples, which served as a basis for the development of the final method used to obtain the results of this work. First, the mobile phase was composed of acetonitrile/formic acid 0.2%, ratio of 70/30 (v/v); then the condition of replacing acetonitrile with methanol was tested in the same proportions as the previous. Subsequently, different organic solvent ratios (70/30 - methanol/ formic acid 0.2%; 65/35 - methanol/formic acid 0.2% and 60/40 - methanol/ formic acid 0.2%) were tested.For quantification of the samples, 1g of the product was first tested in 40 mL (0.025 g/ mL) of mobile phase, and later, a sample dilution step was

included, which resulted in the final concentration of 0.01 g of the product/ mL of mobile phase.

After the tests were performed, the chromatographic conditions established for the analysis was a mobile phase composed of methanol/formic acid 0.2% 60/40 (v/v). A Shimadzu® C18 250 x 4.6 mm 3 mm stationary phase chromatographic column was used at 40 °C with an injection volume of 10 μL.The flow rate was 1mL/min with detection of furosemide, by DAD, at the wavelength of 273 nm, corresponding to the analyte. The running time was 30min and the samples were kept at room temperature. For the concentration curve, a stock solution of 400 μg/ mL of mobile phase furosemide was prepared. Afterwards, the mixture was placed for 5 min in an ultrasonic bath and after reaching room temperature, the dilutions were carried out at concentrations of 0.5, 1.0, 2.5, 3.5, 5 and 10 ppm.

2.3 Statistical Analysis

For statistical analysis of linear regression results, the Least Squares Method (LSM) was used. Its validity as well as homoscedasticity, systematic error and linear model of the obtained data were analyzed by ANOVA (Analysis of variance).For the statistical analysis of the accuracy and precision of the results, the relative standard deviation tests were performed, using Excel software®.

3. Results

The chromatographic conditions used to obtain the results were chosen considering the best performance in the separation of the compounds and the quantification of the analyte in question present in the samples during the preliminary tests (data not shown).

The quantification of samples in mg/g of the product was done based on the concentration curve elaborated using furosemide as the standard. Each point of the curve was injected in duplicate with the 2.5 ppm concentration point being injected 4 times to verify the accuracy of the system by relative standard deviation (RSD%). The acceptance criteria follow the United States Pharmacopea (USP) physical tests <621>

RSD% ≤ 2% recommendation. The acceptance criteria for linear regression were established considering the requirements of resolution no. 166, dated July 24, 2017 providing for the validation of analytical methods and other measures[16]: the correlation coefficient must be greater than 0.99, the regression intercept should not differ statistically from zero and the slope of the regressionline should be statistically greater than zero and homoscedasticity of the data should be analyzed.

The analysis of the furosemide standard correspondent to 2.5 ppm revealed a major peak at 3.599 min (Figure 1). This retention time was used as an identification parameter of furosemide in the samples.
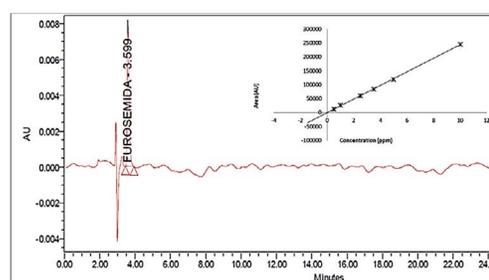

Figure 1. Chromatogram of standard Furosemide® 2.5ppm solution, and graphical profile of the concentration curve.

The visual analysis of the concentration curve of the graph was done prior to statistical analysis of the data and no abnormality was observed. Data was analyzed considering the 95% confidence interval (Table 1). The correlation coefficient obtained was greater than 0.99, the regression intercept found did not statistically differ from zero and the presented regression slope is statistically greater than zero. In addition, the homoscedasticity of the test was graphically evaluated, and no tendency profile was observed (data not shown).

Samples were injected in duplicate and the obtained chromatograms (Figures 2 and 3) presented a peak with a retention time very close to the furosemide standard in both analyzed products. This result indicates the presence of this undeclared compound in the original formulation (Table 2).
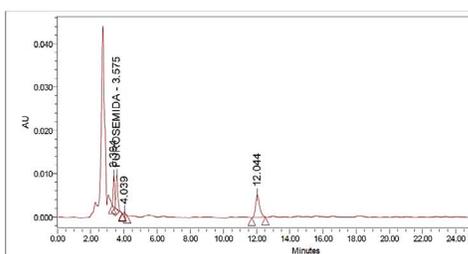
4. Discussion

The adulteration of natural products indicated for the treatment of rheumatic and inflammatory diseases is knownto occur through the addition of corticosteroids and non-steroidal anti-inflammatory drugs, among other synthetic substances that act on pain relief[17]. Adulterated natural products may present more than one synthetic substance, that act as co-adulterants. As a result of the variety of substances that can be found in cases of adulteration, establishing a relationship between all compounds becomes difficult[18]. In general, these substances are added in smaller amounts and may potentiate or pharmacologically mask the action of the main substance.

In the products analyzed in this study, the presence of furosemide, a fast-acting diuretic with short duration of onset, was identified. Specifically, it acts by blocking the NKCC2, Na+/ K+/ 2Cl- transporter located in the luminal cell membrane of the thick ascending limb of the loop of Henle[19]. In addition, furosemide also inhibits the reabsorption of other cations (magnesium and calcium) by reducing the positive potential that arises from the recycling of potassium. Its action results in the excretion of 25% of filtered sodium[20]. The use of furosemide and other diuretics in natural products indicated for pain and inflammation as co-adulterants has been described in Chinese medicine[18] and may be related to its potent diuretic effect, which leads to a systemic reduction of liquid retention caused by the possible presence of corticosteroids.

A second substance observed in the chromatograms of the analyzed samples caught our attention because it

Figure 2. Sample A chromatogram: pecan (*Carya illinoinensis*), yellow ipê (*Handroanthus albus*) and sucupira (*Pterodon emarginatus*)extracts.
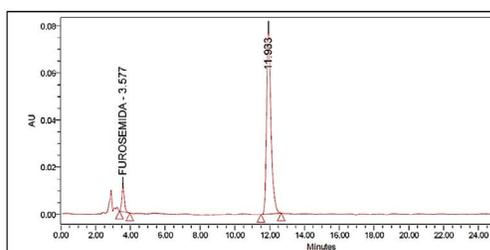

Figure 3. Sample B chromatogram: Moringa (*Moringa oleifera*) (leaves and stems) and "Canela de velho" (*Miconia albicans*)extracts.

Concentration of the adulterant was calculated based on the relation between chromatographic peak area and the analytical concentration curve. In sample A, 24 mg of furosemide per gram was found in the analyzed group, while in sample B, 47 mg per gram of the product was obtained. The mean of the obtained areas and quantification of furosemide found in samples can be found in Table 2.

Table 1. Statistical analysis (ANOVA) of linear regression at significance level of 5%

R2	Intercept	Slope (b)	Equation of the line	Value of F	RSD%
0.9995	-3945,6679; 3669,344925	24390.2764	y=24390 x=138.16	7631.284	1.35

Table 2. Relation between the peak area and concentration in mg/g of the product

Solution	RSD(%) RT	Average of the areas	Concentration (ppm)	Furosemide content/ g of product
Standard	0.003768	60757.25	2.5	------
Sample A	0.000226	58684	2.41	24mg
Sample B	0.000226	114380.5	4.70	47mg

is present in both products, in different concentrations, but both in comparison to the furosemide peak (Figures 2 and 3). In addition, furosemide concentration is higher when the second substance appears in a higher concentration (sample B), which reinforces the idea that furosemide is a co-adulterant in the analyzed products.

The bioavailability of 40 mg oral furosemide is on average 50%, but for each individual this condition can vary from 10 to 100% [21]. Considering the average of this bioavailability, the concentrations found in samples A and B represent a risk of: in sample A, every 1 gram of consumed product (two capsules) equals to 60% of the possible concentration absorbed from a furosemide pill; in sample B, every 1 gram of consumed product equals to 117.5% of the absorbed concentration of a 40 mg furosemide pill.

In view of the potent pharmacological action of furosemide, the incorporation of this compound through adulteration constitutes a crime with penalties foreseen by law and puts at risk the health of the user who is consuming a drug with medical prescription of unknown origin. The consumption of adulterated products may lead to risks such as drug interaction and intoxication, since active ingredients of synthetic origin are added without taking into consideration the adjustment and quality of the raw material[22].

Among the people that use these natural products, we can highlight 3 populations that present a high-risk exposure level: pregnant women, children and the elderly. Due to the limitation in the use of synthetic drugs during the gestation period, the use of herbal products may seem to be an alternative for the treatment of diseases and some complications that may arise during gestation. As the popularity of these products increases, the lack of safety becomes increasingly evident, considering the scarcity of studies that prove the safety and effectivenessof these products in pregnant women[23]. In this case the risks extend to the neonate, and problems such as malformations, birth complications and even death of the neonate should be considered as potential risks related to the use of natural products and in greater severity in cases where they have been adulterated[4].

A study in the United States showed that in 2007 37.2% of the interviewed children reported using echinacea (a plant indicated for the treatment of colds and flu, rhinitis and sinusitis) and 17.9% used combined herbal capsules[23]. On the other hand, the elderly makes up a group that, in addition to the physiological alterations, presents a large number of associated medications for the treatment of different diseases, culminating in polypharmacy, which combined with the use of natural products in an attempt to reduce side effects, may represent ahigh risk of drug interactions and even intoxication[18]. Because of the easeof commercialization, these risks are even greater and have become a concern of regulatory agencies.

Nevertheless, it is necessary to direct efforts to prevent, inspect more intensively and raise awareness among the population of the risks associated to the consumption of natural products of unknown origin and suspected of adulteration, which may cause irreparable damage to health and represent an imminent risk to public health.

5. Conclusion

Detecting adulteration was possible in two commercially available natural products by identifying undeclared furosemide by HPLC-DAD. In view of the obtained results we can conclude that the method was accurate, linear and precise. Considering the pharmacological action of furosemide, the presence of this substance suggests the presence of other analgesics and/or concomitant corticosteroids. This finding adds to several already published studies concerning the identification of unauthorized synthetic substances in natural products using instrumental methods. The quantification of this synthetic substance demonstrates the severity and exposure level of the population to the risk of intoxications and adverse reactions, which further reinforces the criticality and the necessity to address this issue from the point of view of public health.

6. Acknowledgement

7. References

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