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Comparative study between capillary electrophoresis and high performance liquid chromatography in 'guarana' based phytopharmaceuticals

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Abstract

The last years have seen a significant increase in the use of herbal medicines and their preparations all over the world. Adulterations with synthetic drugs are common problems with phytopharmaceutical products and this can potentially cause adverse effects. In consequence, it is important to determine the presence of synthetic drugs in herbal medicines to ensure their efficacy and safety. In this study, guarana derivatives were analyzed by capillary electrophoresis (CE), and the results were compared with those obtained by the HPLC technique. In order to obtain adequate fingerprints, and search for adulterants, caffeine was used as the marker compound. This separation method was applied to analyze the seed powder and commercial tablets of *Paulinia cupana* Mart. The methodology performance was evaluated in terms of specificity, sensitivity and precision. The results are in agreement with those obtained by the HPLC method. Furthermore, the analysis time of the CE method is up to two times shorter than the respective parameter in HPLC and solvent consumption is more than 100-fold less.

Keywords: Herbal medicines; Phytopharmaceuticals; Capillary electrophoresis; HPLC; Gurana; Adulterants

1. Introduction

During many years, medicinal plant products have been available for treatment of diseases in both developing and developed countries [1]. Phytopharmaceuticals are complex products, and their inherent biological variation is due to different growth, harvest, drying and storage conditions. Therefore, cultivation of the plants under standardized conditions is desirable. The polarity of the solvent, the mode of extraction, and the instability of constituents may influence the composition and quality of the extracts and must therefore be kept constant. Depending on the type of preparation sensoric features, moisture, ash, physical constants, solvents residues and adulterations have to be checked to prove identity and purity [2].

Although herbal remedies are often perceived as being natural and consequently safe, they are not free from adverse effects. Adulteration, substitution, contamination, misidentification, lack of standardization, incorrected preparation and/or dosage and inappropriate labeling are the most common problems with medicinal herb [3,4]. Substitution or adulterations with more toxic herbs or synthetic drugs may occur erroneously or deliberately [5,6]. Cases of clinical complications and adverse effects resulting from the synthetic drugs in herbal medicine have been reported [7,8].

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Guarana (*Paullinia cupana* Mart.) is a Brazilian native plant, with seeds being the only part suitable for human consumption. Most of the guarana seeds derivatives are used in concentrates and soft drinks and as ingredients of a variety of phytopharmaceutical products. Guarana seeds contain high concentrations of caffeine [9] generally range from 3 to 6% (dry weight) [10], as well as theophylline and theobromine in very small amounts; they also contain large quantities of tannins [11]. The presence of the minor alkaloids theophylline and theobromine in greater concentrations suggest the adulteration of the vegetal drug with another parts of the plant and/or the deliberate adding of these drugs [12].

Guarana has shown to be an aphrodisiac and stimulant over the cardiovascular and nervous system; most of these properties are attributed to its caffeine content [13,14]. Various studies have demonstrated the pharmacological activity of caffeine and related compounds such as theophylline. Both are also presented as medicinal products for enhancing analgesic and bronchodilatory effects, respectively [15–17]. However, not much information is available on the potential risk to human health. Some in vitro bioassays were carried out for cytotoxical activity and they suggest that prolonged use or high doses could be harmful to human health [18].

To check and prove the quality of herbal preparations, adequate analytical methods have to be applied to the quantitative determination of the constituents with known therapeutical activity [19–22]. Capillary electrophoresis (CE) is a technique that is suitable for a wide range of analytical problems. It offers a number of advantages, especially short analysis time, high separation efficiency and low sample and solvent consumption [23]. Thus, performance of CE as a quantitative separation technique is very attractive.

HPLC is an established analytical method widely used for routine analyses in the pharmaceutical industry [24–27]. Therefore, the objective of this study is to develop a CE method for the quantification of constituents in 'guarana' derivatives and to compare it with HPLC as a means of validating the CE assay. Comparisons of elution order, UV spectra, method sensitivity and precision between reversed phase (RP) HPLC and capillary zone electrophoresis (CZE) will be present in this study.

2. Experimental and methods

2.1. CE apparatus and operating conditions

A Beckman P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising an IBM personal computer and P/ACE System MDQ Software. The fused-silica capillaries were obtained from MicroSolv Technology Corporation and had the following dimensions: 67 cm total length, 50 cm effective length, 75 μm i.d., 375 μm o.d.

The CE operation parameters were as follows: the detection was performed at 212 nm, the capillary temperature was maintained at 25 °C, voltage was set at 25 kV and a positive polarity. Samples were pressure-injected at the anodic side at 0.5 psi for 5 s.

To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with sodium hydroxide 0.1 M for 2 min, followed by water for 2 min, then equilibrated with the running buffer for 4 min. An Orion 940 pHmeter equipped with a glass-combined electrode was used for the electrolyte pH measurement.

Electroosmotic flow (EOF) determination was performed by using acetone as an EOF marker. The background electrolyte (BGE) used consist of 20 mM sodium tetraborate buffer, pH 9.2.

2.2. HPLC apparatus and operating conditions

The experiments were performed with a Beckman HPLC system coupled with a PC-IBM compatible computer which consisted of a solvent delivery module (model Beckman Gold 126), a diode array UV–Vis detector (model Beckman Gold 168) and a Gilson Rheodyne injecting valve fitted with a 20 μL sample loop. Data acquisition was performed with the System Gold software. The absorption wavelengths were set at 254 nm for detecting drug components. A Beckman 5 μm Ultrasphere® (Phenomenex, CA, USA) C-18 column (250 × 4.6), commercially available was used in all experiments. The mobile phase consisted of methanol–water solvent (70:30); the water solvent consist of ultrapure water, pH 3.5 adjusted with phosphoric acid. Other chromatographic conditions included 0.9 mL min^{-1} flow-rate and ambient column temperature.

2.3. Reagents and chemicals

The water used in all studies was ultrapure water (18 MΩ cm) obtained from a Barnstead Easy pure RF compact ultrapure water system. The structure and formulae of the compounds studied are shown in Fig. 1. Caffeine, theobromine and theophylline were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphoric acid (grade pro-analysis) and methanol (HPLC grade) were from Merck (Buenos Aires, Argentina) and sodium tetraborate ($Na_2B_4O_7{\cdot}10H_2O$) was acquired from Mallinckrodt (Saint Louis, USA). All other reagents and solvents were of analytical grade quality.

All the solutions were degassed by ultrasonication (Testlab, Argentina) Running electrolytes and samples were filtered through a 0.45 μm Titan Syringe filters (Sri inc., Eaton Town, NJ, USA).

2.4. Phytopharmaceutical samples

Samples analyzed consisted of two different commercial tablets containing *P. cupana* seed powder. Both tablet marks were purchased from a local pharmacy (sample 1) and an

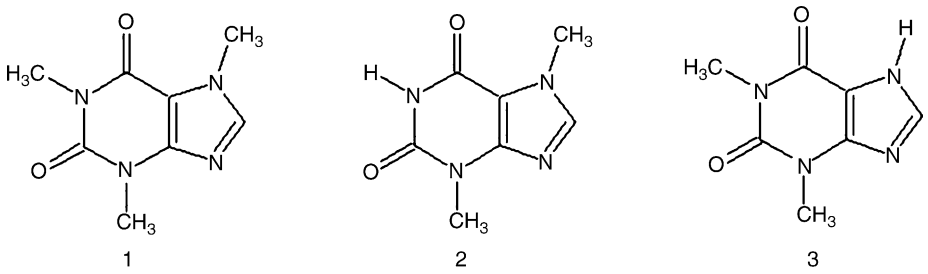

Fig. 1. Chemical structures of (1) caffeine (1,3,7-trimethylxanthine); (2) theobromine (3,7-dimethylxanthine) and (3) theophylline (3,7-dimethylxanthine).

herbal shop (sample 2) in San Luis, Argentina and manufactured by Natufarma (Buenos Aires, Argentina) and Herbalife International of America Inc. (Los Angeles, USA), respectively. Guarana seed powder (sample 3) was supplied from a local pharmacy and manufactured by Parafarm® (Buenos Aires, Argentina).

2.5. Sample preparation

Twenty tablets of each commercial mark were finely powdered and the equivalent of one tablet was accurately weighed (0.24 g) and dissolved with 50 mL mixture methanol–water (70:30), pH = 3.5 adjusted with phosphoric acid. These solutions were sonicated for 15 min and vortex mixing at 5 min interval to avoid aggregation of the powdered sample. After centrifugation (3500 rpm for 5 min), supernatants were collected, filtered and diluted in a 50 mL volumetric flask.

Stock standard solutions of caffeine (CF), theobromine (TB) and theophylline (TP) were prepared dissolving an accurately weighed aliquot of the drugs in methanol–water mixture. The final concentrations of the standards were: 20 mg mL^{-1} of CF, 0.8 mg mL^{-1} of TB and 2 mg mL^{-1} of TP. A combined standard solution containing caffeine, theobromine and theophylline were prepared by suitable diluting the standard solutions of each drug and the resultant solution was made up to 100 mL.

2.6. Validation

Method validation was carried out according to the ICH Guidelines [28] for pharmaceutical industry. The present method was validated for caffeine, theobromine and theopylline. The procedure required the assessment of migration time and peak area reproducibility, detector response linearity with sample concentration, sensitivity and accuracy.

3. Results and discussion

3.1. Method development

In order to propose a specific and accurate way of analyzing guarana based pharmaceuticals by using capillary zone electrophoresis (CZE), it is essential to find the best experimental conditions in which the analytes can be separated from each other. The optimization was performed using a synthetic mixture of CF, TB and TP. The following parameters were consecutively optimized: sample conditioning, pH, composition and concentration of the BGE, sample and capillary temperatures, and other electrophoretic parameters such as separation voltage, injection mode and length, etc.

3.1.1. Effect of pH

The BGE pH plays an important role for improving selectivity in CE especially for closely related compounds, because it affects both the overall charges of the solute and the EOF.

The effect of the buffer pH was investigated within the range of 8.0–10.0 at a fixed buffer concentration, adjusted by 0.1 mol l^{-1} NaOH and 0.1 mol l^{-1} HCl. It was found that when the pH was increased, resolution also increased, while time analysis decreased. At pH 9.2 baseline separation was achieved.

3.1.2. Effect of buffer composition and concentration

Buffer concentration has also a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. Different BGEs have been tested, but the one producing the best results considering selectivity, reproducibility, baseline and current performance, was sodium tetraborate, pH 9.2.

Keeping other parameters constant (pH: 9.2, 25 kV, 25 °C) the buffer concentration was varied from 5–75 mM. Increases in migration times as well current were observed when the concentration of buffer increased. Resolution also increased for higher buffer concentrations, but no appreciable improvements were observed for buffer concentrations above 20 mM. So, a 20 mM sodium tetraborate buffer, pH 9.2 was selected as optimal. The sample injections were made in the hidrodinamic mode (0.5 psi) for a period of 5 s.

3.1.3. Separation performance

Fig. 2 shows the sample solution electropherograms obtained using the optimized experimental conditions. The migration times of CF, TB and TP were found to be 3.39, 3.64, and 5.05 min, respectively. This electrophoretic behavior is in complete agreement with that expected considering the following factors: acid–base equilibria and chemical nature

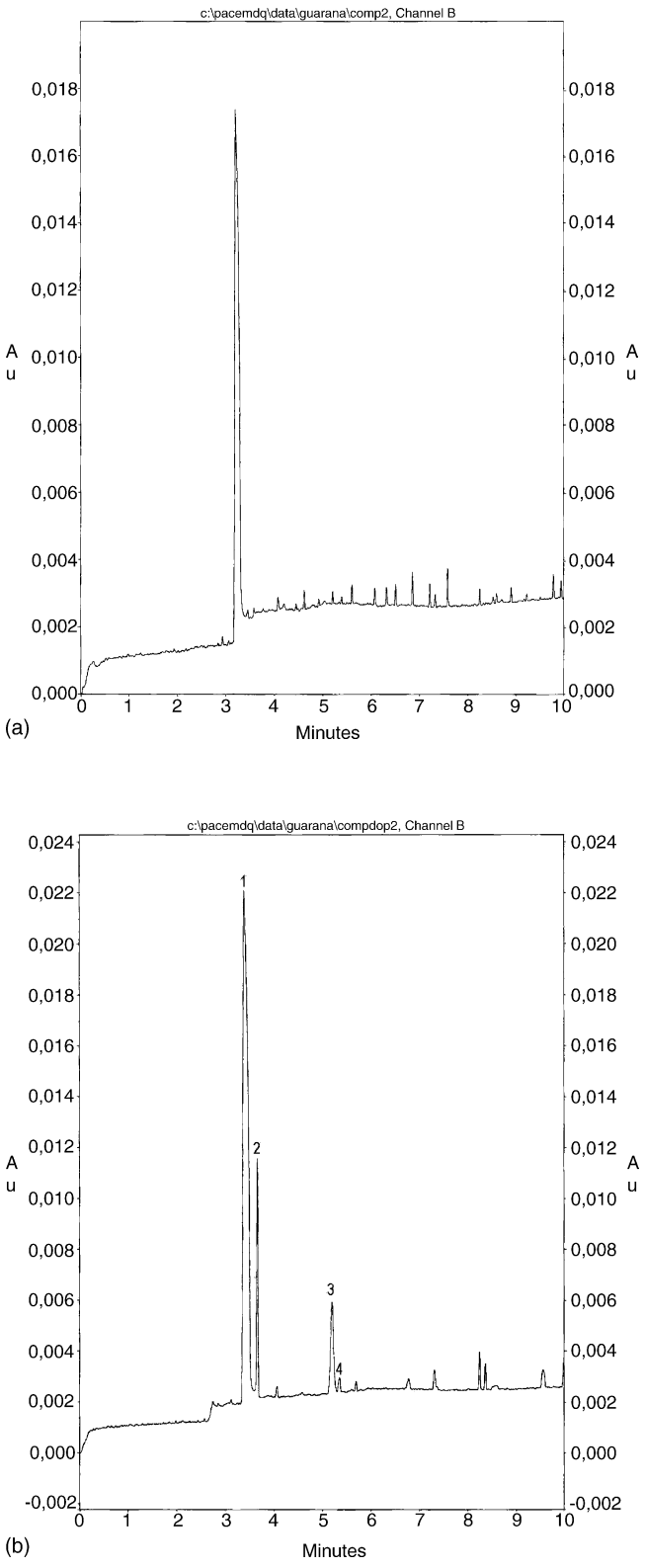

Fig. 2. (a) Electropherogram of a commercial guarana tablets. Conditions: 20 mM sodium tetraborate buffer, pH 9.2; capillary, 67 cm full lengh, 50 cm effective length, 75 μm i.d., 375 μm o.d.; hydrodynamic injection at 0.5 psi, 5 s; 25 kV constant voltage; detection by UV absorbance at 212 nm. (b) Electropherogram of a commercial spiked guarana tablets. Peak identification: (1) caffeine; (2) theobromine; (3) theophylline; (4) unknown compound.

of the purine alkaloids. The pK_a values are 14.0 for CF; 10.8 for TB and 8.6 for TP.

The elution order and time analyses were different from those obtained for HPLC separation. The retention times were 4.02, 5.74 and 7.78 min for TB, TP and CF, respectively; the relative retentions were governed by the different molecular sizes and hydrophobicities.

Elution of caffeine occurred just with the EOF at around 3.40 min. The migration times did not vary to any considerable degree during and between analyses (%R.S.D. less than 1% for the migration time of each peak). With respect to the selectivity, the resolution values were 1.50 for CF from TB and 3.00 for TP from peak 4. These values meet the acceptance criteria. From the analysis of Fig. 2b (original sample spiked with TP and TB) it can be seen two new peaks corresponding to the added compounds. Therefore, we can conclude that the original sample did not contain the TP and TB compounds.

Caffeine, theobromine and theophylline were baseline separated by CE in less than 6 min, giving separation efficiencies of up to 86062 experimental electrophoretic plates (N) for TB. The number of experimental N for CF and TP were approximately 9581 and 51788, respectively.

3.2. Method validation

3.2.1. Precision

The repeatability of the entire analytical procedure should be demonstrated by performing the recommended procedure at several analyte concentration levels [29]. The R.S.D. values were better than 0.5% for the migration time and 2.6% for the peak area. The repeatability of the CE system was evaluated performing successive injections (n = 10) of the combined solution. The R.S.D. values were better than 0.1% for the migration time and 1.4% for the peak area.

Intermediate precision was determined changing the following factors: the capillary, the day of operation, the operator and the sources of reagents or electrolytes. The factors changed were analyzed simultaneously, recovery studies were performed on three different days by three analysts, each one independently preparing solutions; also, capillary supplier variations were tested. The R.S.D. values were better than 0.8% for the migration time and 3.4% for the peak area.

Method precision was also demonstrated by comparing CE method with HPLC. A R.S.D. of 1.4% by peak area response ratio was obtained for the main compound (caffeine) in CE, and a R.S.D. of 1.1% in HPLC was obtained.

3.2.2. Linearity

Linearity was demonstrated by comparing CE and HPLC systems within the ranges: 0.40–200 μg mL^{-1} for CF, 1.20–20 μg mL^{-1} for TB and 1.70–50 μg mL^{-1} for TP. All plots of analyte concentrations versus average peak area response ratios have correlation coefficients better than 0.996. The results are presented in Table 1.

Table 1
Quantitative parameters of the analysis of caffeine, theobromine and theophylline by CZE

	Caffeine	Theobromine	Theophylline
Calibration range ($\mu g\,mL^{-1}$)	0.40–200	1.20–20	1.7–50
Slope of the calibration curve ± S.D. [a] (95%; $n = 6$)	$0.6 \times 10^4 \pm 3.3 \times 10^2$	$3.0 \times 10^4 \pm 1.0 \times 10^2$	$2.9 \times 10^4 \pm 1.6 \times 10^2$
Intercept ± S.D.[a] (95%; $n = 6$)	$-4839 \pm 3.3 \times 10^3$	$49855 \pm 1.3 \times 10^3$	$55309 \pm 4.5 \times 10^3$
Regression coefficient of the calibration (r^2)	0.996	0.998	0.996
Detection limit ($\mu g\,mL^{-1}$)	0.12	0.33	0.25
Quantitation limit ($\mu g\,mL^{-1}$)	0.40	1.10	0.83

[a] Standard deviation.

3.2.3. Limits of detection (LOD) and quantification (LOQ)

The amount of standard, which could be detected with a signal to noise ratio ≥3 was considered to be limit of detection (LOD). The limit of quantification (LOQ) was calculated as the analyte concentrations that give rise to peak heights with a signal-to-noise ratio of 10.

LOD and LOQ were determined by injecting standard combined solution at three different level concentrations for each analyte. The LODs for CF, TB and TP determined are shown in Table 1. Although optical path lengths and injected volumes are considerable smaller, the detection limits obtained for CE are in the same concentration order than those for HPLC. The latter could be ascribable to the higher efficiencies for CE.

3.2.4. Accuracy

The accuracy of the present method was evaluated by performing recovery experiments. The results are shown in Table 2. Accuracy was also assessed by comparing the results of the same test samples analyzed by the method undergoing validation and a method considered as a reference method. Cross-correlation between HPLC and CE has been used. Relationship between the CF, TP and TB contents obtained for the same phytopharmaceutical sample by the HPLC and the CE recommended procedure were obtained. The correlation graph gave the following statistics: $R = 0.988$; $n = 6$ (three replicates each).

3.3. Assay of a commercial product: recovery study

Once the conditions for separation and quantification were established, the CE method was applied to the determination of CF, TB and TP in commercial formulations. The electropherogram in Fig. 2 shows the separation of the active compounds in a commercial sample (sample 1).

The results of the assay for CF on the commercial products were: 13.10 and 32.75 $mg\,g^{-1}$ for samples 1 and 2, respectively (tablets). Also, a guarana seed powder sample was analyzed (sample 3) the result for CF was: 16.20 $mg\,g^{-1}$. Neither TP nor TB was detected in the samples under investigation.

Recovery test was performed adding known quantities of CF, TB and TP and following the recommended procedure for the alkaloids determination. The study was performed at three concentration levels: 80, 100 and 120% on sample 1. Each concentration was injected in triplicate; mean values ($n = 3$) were better than 96.7% in all the cases. The results are presented in Table 2. Mean recoveries of 99.5–99.8%

Table 2
Recovery test of the diluted tablet[a]

	Base value ($\mu g\,mL^{-1}$)	Quantity added ($\mu g\,mL^{-1}$)	Quantity found ($\mu g\,mL^{-1}$)	Recovery (%)[b]
Aliquot I–VI				
CF	–	0.00	62.00	–
TB	–	0.00	0.00	–
TP	–	0.00	0.00	–
Aliquot VII–IX				
CF	62.00	50.0	111.90	99.80
TB	0.00	5.00	5.05	101.00
TP	0.00	10.00	9.95	99.50
Aliquot X–XII				
CF	62.00	62.00	123.70	99.50
TB	0.00	10.00	9.93	99.30
TP	0.00	20.00	20.05	100.25
Aliquot XIII–XV				
CF	62.00	74.40	136.35	99.90
TB	0.00	13.00	12.95	99.60
TP	0.00	30.00	29.03	96.70

CF: caffeine; TB: theobromine; TP: theophylline.
[a] Sample 1, diluted tablet (0.24 g in 50 mL of assay solution).
[b] 100 × [(found-base)/added].

were obtained with the HPLC method. The recoveries, which were achieved for the CE method, were in the same range: 96.7–100.2%.

4. Conclusions

Caffeine level in the analyzed samples was within the normal concentrations. As expected for non-adulterated products neither theophylline nor theobromine was detected in the samples under investigation.

The study performed exhibits how CE can be a very effective analytical tool with great potential. With regard to time and expense of analysis the data supports the claim that CE methodology can compete with well-established techniques such as HPLC for the quantitative analysis of phytopharmaceutical samples. Indeed, cross validation studies gave highly satisfactory results. Under optimal conditions CE can achieve high efficiency, low cost per analysis, rapidness, adequate sensitivity and a minimum use of organic solvents.

This study has demonstrated a simple and rapid CZE procedure that makes possible the quantitative determination of drug substances and their related substances in a phytopharmaceutical dosage form.

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