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Validation of a colorimetric assay for the *in vitro* screening of inhibitors of angiotensin-converting enzyme (ACE) from plant extracts

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Abstract

A new method for the *in vitro* screening of plant extracts with potential angiotensin-converting enzyme (ACE) inhibitory activity is proposed. The method is based on the cleavage of the substrate hippuryl-glycyl-glycine by ACE and subsequent reaction with trinitrobenzenesulfonic acid to form 2,4,6-trinitrophenyl-glycyl-glycine, whose absorbance is determined at 415 nm in a microtitre plate reader. Rabbit lung dehydrated by acetone was employed as an enzyme source. Validation of the method showed satisfactory intra-day (CV = 7.63%) and inter-day precision (CV = 13.61%), recovery (97–102.1%), sensitivity (IC_{50} = 14.1 nmol/l) and linearity in the range 7.5–120 mmol/l of glycyl-glycine (r^2 = 0.9921). Besides, the method showed good correlation with a HPLC assay already established for the screening of ACE inhibitors (r = 0.9935 and 0.9034, respectively, for captopril solutions and for plant extracts). The method involves only inexpensive reagents and apparatus.

Keywords: Angiotensin-converting enzyme inhibitors; *In vitro* colorimetric assay; Screening; Plant extracts

Introduction

The angiotensin-converting enzyme (ACE), a component of the renin-angiotensin system, plays a key role in the homeostatic mechanism of mammals, contributing to the maintenance of the normal blood pressure and for the electrolyte balance, being involved in the regulation and control of the arterial pressure. The use of ACE inhibitors is well established as one of the therapeutic principles in the treatment of hypertension. Besides, ACE inhibitors are also employed for the prophylactic control of diabetic nephropathy and for the treatment of heart failure (Coates, 2003; Unger, 2002). The clinical use of ACE inhibitors is strongly recommended due to their ability in preventing and reversing functional and structural alterations commonly associated with hypertension (Coates, 2003; Varagic and Frohlich, 2002).

Natural products are recognized as important sources of ACE inhibitors; it is worth mentioning that the peptides from the venom of the Brazilian viper *Bothrops jararaca* were the template for the development of captopril and other synthetic antihypertensive drugs. Nevertheless, the potential of plant secondary metabolites as a source of new ACE inhibitors remains

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underexploited and only a limited number of plant species has been screened for this target (Braga et al., 2000; Hansen et al., 1995; Duncan et al., 1999).

There are a few methods described for the *in vitro* screening of inhibitors of ACE and the one proposed by Elbl and Wagner (1991) and further improved by Braga et al. (2000) and Hansen et al. (1995) is the most frequently employed assay. Despite showing satisfactory analytical parameters for the evaluation of complex matrices, such as plant extracts, this method presents some hindrances for the high throughput screening of samples, namely, the long time required for analysis and the high cost of the purified ACE employed in the assay. Based on this background, the main goal of the present work was to develop and validate a fast, sensitive, precise, accurate and inexpensive assay for the *in vitro* screening of plant species for ACE inhibiting activity.

Materials and methods

Chemicals

Glycyl-glycine (Gly Gly), hippuryl-glycyl-glycine (Hip-Gly-Gly), 2,4,6-trinitrobenzene sulfonic acid (TNBS), HEPES and sodium tungstate were purchased from Sigma (USA). Rabbit lung dehydrated by acetone was obtained from Continental Produtos Biológicos (Brazil) and captopril from Calbiochem (USA).

Buffers

Assay buffer: 297.5 mg of HEPES (50 mmol/l), 438.75 mg of NaCl (300 mmol/l) and 1420 mg of Na_2SO_4 (400 mmol/l) were added into a 25 ml volumetric flask (final concentration in parenthesis). They were dissolved in 20 ml of distilled water containing 50 μl of saturated NaOH solution; after adjusting the pH to 8.15 with 10% NaOH solution, the volume was completed with distilled water. Phosphate buffer (100 mmol/l): 340.2 mg of anhydrous potassium phosphate was dissolved in 20 ml of distilled water, adjusted to pH 8.5 with 10% NaOH solution and diluted to 25 ml.

Stock and working solution of rabbit lung dehydrated by acetone

The stock solution was prepared as previously described (Vermeirssen et al., 2002), by dissolving 2 g of the powder in 10 ml of phosphate buffer (50 mmol/l, pH 8.3). The stock solution was highly active and stable for at least 3 months, kept under refrigerator (2–6 °C). The working solution (1 g/10 ml) was prepared freshly before the assays, by diluting the stock solution in the phosphate buffer.

Substrate solution

Two hundred milligram of Hip-Gly-Gly was dissolved in 4 ml of 1 mol/l ammonium hydroxide solution. After complete dissolution, the volume was increased to 6.8 ml with distilled water (final concentration = 100 mmol/l).

TNBS solution

Two thousand and thirty microliter of TNBS was added to a 5 ml volumetric flask and the volume was completed with distilled water (final concentration = 60 mmol/l). The solution was stored at −20 °C, being used within 3 months.

Plant material and extract preparation

The plant material was collected in the state of Minas Gerais, Brazil, and the species were identified by Dr. J.A. Lombardi, Departamento de Botânica, ICB, UFMG, Belo Horizonte, Brazil. Voucher specimens of the collected species (number in parenthesis) are deposited at the BHCB Herbarium, ICB, UFMG, Belo Horizonte, Brazil: *Argemone mexicana* L. (Papaveraceae), leaves (BHCB 4752); *Buddleja stachyoides* Cham. & Schltdl. (Loganiaceae), leaves (BHCB 2291); *Cecropia glaziovii* Sneth. (Cecropiaceae), leaves (BHCB 4063); *Combretum fruticosum* Stuntz (Combretaceae), leaves and stems (BHCB 2292); *Croton antisyphiliticus* Mart. (Euphorbiaceae), leaves (BHCB 2293); *Hancornia speciosa* Gomes (Apocynaceae), leaves (BHCB 3565); *Leea rubra* Bl. Ex Spreng. (Leeaceae), aerial parts (BHCB 4416); *Mangifera indica* L. (Anacardiaceae), leaves (BHCB 4413); *Persea americana* Miller (Lauraceae), leaves and stems (BHCB 4412); *Phoenix roebelinii* O'Brien (Arecaceae), leaves (BHCB 3133); *Pyrostegia venusta* (Ker.) Miers (Bignoniaceae), stem (BHCB 4457) *Tabebuia serratifolia* (Vahl) Nicholson (Bignoniaceae), stem bark (BHCB 1357) and *Tulbaghia violacea* Harv. (Liliaceae), aerial parts (BHCB 4420).

After separately drying the distinct parts of the plants at 40 °C, for 72 h, the materials were powdered and portions (10 g) were extracted with ethanol (3 × 30 ml) under sonication (3 × 15 min). The solvent was vacuum removed in a rotavapor evaporator, at 50 °C, and the residues were kept at −20 °C. For screening, the extracts were dissolved with 20% MeOH and 80% HEPES to a concentration of 100 μg/ml.

Optimization of the assay conditions

The parameters listed in Table 1 were evaluated during method development, aiming to optimize the ACE activity.

Table 1. Parameters evaluated during method development

Parameters	Assayed values				
Concentration of the rabbit lung solution (g/10 ml)	0.2	0.6	1.0	1.4	2.0
	0.4	0.8	1.2		
pH of the phosphate buffer (100 mmol/l)	7.5	8.0	8.5	9.3	9.6
pH of the assay buffer	7.0	7.5	8.15	8.5	9.0
Concentration of the substrate solution (mmol/l)	7.5	15.0	30.0	60.0	
Temperature for enzyme incubation (°C)	25	30	37	40	45
Time of enzyme incubation (min)	20	25	30	35	40

Statistical analysis

The parameters evaluated during method development were analyzed by Tukey's test (Prism Graph Pad 3.0—Graph Pad Software, Inc., USA).

Colorimetric method for ACE inhibition assay

Ten microliter of the rabbit lung solution (1 g/10 ml) was added to an eppendorf flask containing 10 μl of the extract solution (100 μg/ml) to be tested, or 10 μl of 50 mmol/l phosphate buffer, pH 8.3 (negative control) or 10 μl of captopril solution (64 nmol/l) (positive control). The mixture was homogenized and pre-incubated for 5 min at 37 °C. The enzymatic reaction was started by adding 60 μl of the assay buffer and 30 μl of the substrate solution. After homogenization, the mixture was incubated for 35 min, at 37 °C. The reaction was stopped by the addition of 100 μl of sodium tungstate solution (100 g/l) and 100 μl of sulfuric acid (0.33 mmol/l); the eppendorf flask was mixed for 10 s, following addition of 1000 μl of distilled water. In the sequence, the mixture was centrifuged at 2000 rpm, for 10 min. An aliquot of the supernatant (75 μl) was placed on a microtitre plate and mixed with 100 μl of phosphate buffer (100 mmol/l, pH 8.5) and 5 μl of TNBS solution. The plate was kept in the dark, at room temperature, for 20 min. Later, its absorbance was read in a microtitre plate reader (BioRad, Model 550) at 415 nm against a blank solution prepared in a similar way, except for adding the sodium tungstate and the sulfuric acid solutions before the rabbit lung solution. Assays were performed in triplicates. Calculation of ACE inhibition, on percentile basis, was accomplished by the following equation:

$$\text{Inhibition (\%)} = \frac{100 - [A_I \times 100]}{A_C},$$

where A_I is the measured absorbance at 415 nm in the presence of an inhibitor and A_C is the absorbance of the blank solution.

HPLC method for ACE inhibition assay

The assays were carried out with some modifications (Braga et al., 2000; Hansen et al., 1995) of the original method (Elbl and Wagner, 1991). Assays were performed in triplicates.

Captopril concentration–response curve

The sensitivity of the method was assayed by a captopril concentration–response curve, prepared with solutions of 2.0, 4.0, 8.0, 16.0, 32.0 and 64.0 nmol/l. Each point is the mean ± SD of 10 replicates.

Method validation

The validation of the colorimetric ACE inhibition assay was performed according to the INMETRO guidelines (Inmetro, 2003) and included the evaluation of linearity, intra-day and inter-day precision, precision between methods, accuracy and correlation between methods. The linearity was assessed by plotting a calibration curve between Gly-Gly solutions of different concentrations (7.5–120.0 mmol/l) and their respective absorbances. Intra-day precision was estimated by the coefficient of variation (CV) obtained from the measurement of 10 replicates of the incubation solution containing 1 g/10 ml of rabbit lung solution and 30 mmol/l of the substrate solution. The inter-day precision was evaluated by the CV of 10 replicates, in 6 consecutive days, employing the same concentrations used in the intra-day study. The precision between the colorimetric and HPLC assay (Elbl and Wagner, 1991) was evaluated by statistical analysis (Fisher's test) of the ratio between the variances of the two methods, estimated from the IC_{50} concentration–response curves of captopril. The accuracy of the method was assessed by the recovery of Gly-Gly solutions (15, 30 and 45 nmol/l) spiked into the assay solution, containing 10 μl of the plant extract (100 μg/ml). Correlation between the colorimetric and HPLC methods was estimated by the Pearson coefficient, employing two sets of data: the percentage of inhibition obtained with

captopril solutions and with 13 plant extracts. The plant species were selected based on their traditional use as antihypertensive or diuretics and on a chemosystematic consideration.

Results and discussion

The substrate used in the present assay was Hip-Gly-Gly, previously employed for the colorimetric determination of ACE in serum (Neels et al., 1983). Its cleavage by the ACE produces Gly-Gly, which subsequently reacts with TNBS, forming TNP-Gly-Gly, detected at 415 nm (Fig. 1). The colorimetric assay proposed here for ACE inhibitors screening is based on the partial or total inhibition of the color product formation.

Evaluation of the rabbit lung solution as source of ACE

The absorbance of rabbit lung solutions of different concentrations was determined at 415 nm, wave length employed for detection in the colorimetric assay. The absorbances of the rabbit lung solutions and those of the blanks were significantly different ($p<0.05$) for all the assayed concentrations, as stated in Fig. 2a. In order to eliminate this interference on the assays, rabbit lung solution previously inactivated by sodium tungstate and sulfuric acid was added to the blanks. The concentration of 1 g/10 ml was chosen for the rabbit lung solution in the assays, once it gives an absorbance of 0.43 (Fig. 2b), which is recommended for colorimetric assays (Fell, 1986). The ACE activity of this solution was 33 U/l, determined by a calibration line obtained as a regression between ACE activity and the absorbance of TNP-Gly Gly ($r^2 = 0.9724$; $y = 0.08032 + 0.01055x$) (Fig. 3).

Optimal conditions for the enzyme reaction

The use of TNBS in coupling reactions with amines, amino acids and peptides has been previously reported (Satake et al., 1960; Snyder and Sobocinski, 1975; Okuyama and Satake, 1960). The reaction rate is dependent on the pH of the medium and the reaction

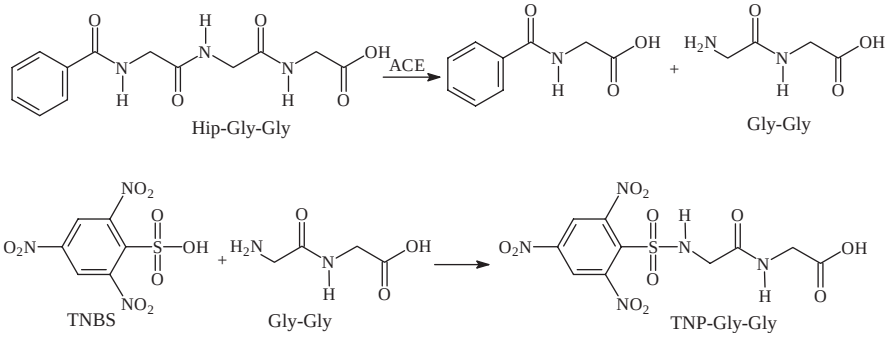

Fig. 1. Cleavage of the substrate Hip-Gly-Gly by ACE and reaction of Gly Gly with TNBS, forming the color derivative 2,4,6-trinitrophenyl-glycyl-glycine (TNP-Gly-Gly), which is determined at 415 nm.

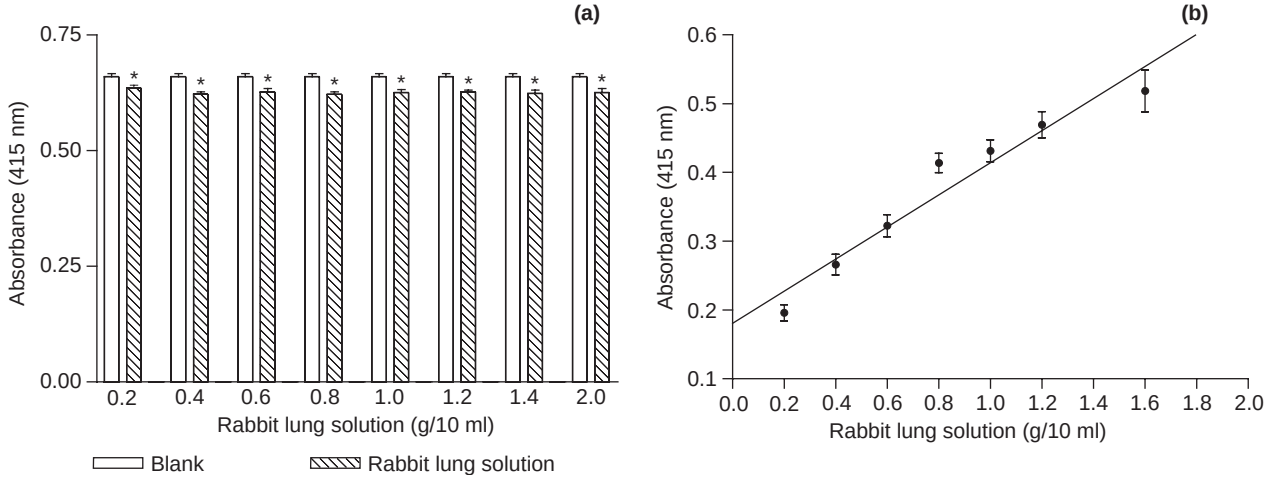

Fig. 2. Effect of the rabbit lung solution on the formation of the color product TNP-Gly Gly (a) and the corresponding calibration graph (b). The absorbances in (b) were determined against a blank containing inactivated rabbit lung solution.

is usually completed within 15–30 min (Satake et al., 1960; Snyder and Sobocinski, 1975; Okuyama and Satake, 1960). The influence of the pH value of the phosphate buffer on the reaction course is depicted in Fig. 4a, for the period of time employed in the assay (20 min). The absorbances did not vary significantly ($p > 0.05$) in the range of pH values evaluated. Despite this, pH 8.5 was adopted for the phosphate buffer in our method, the addition of the other reagents to carry out the assay results in a final pH ca. 8.0 being given, which is required for optimum enzyme activity (Okuyama and Satake, 1960).

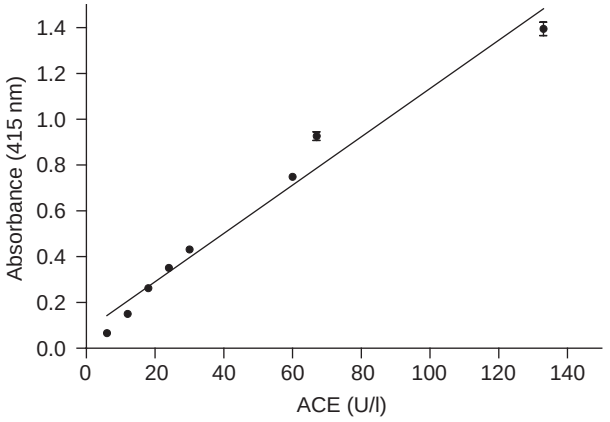

Fig. 3. Calibration graph as a regression between ACE activity and the absorbance of TNP-Gly-Gly. Points are means ± SEM of 10 replicates.

The ACE catalytic activity is influenced by the nature of the substrate; Hip-Gly-Gly shows the highest activity on a HEPES medium, at pH 8.15 (Dorer et al., 1976). Our results showed no significant difference in the product absorbances at pH values 7.00, 7.50 and 8.15 ($p > 0.05$) which, in turn, did differ from the others ($p < 0.05$) (Fig. 4b). These findings led us to select pH 8.15 for the HEPES solution, the same value reported in the previous colorimetric method (Neels et al., 1983).

It is known that substrates containing the dipeptides His-Leu or Gly-Gly in their terminal portions are cleaved by ACE (Yang et al., 1970). In the case of

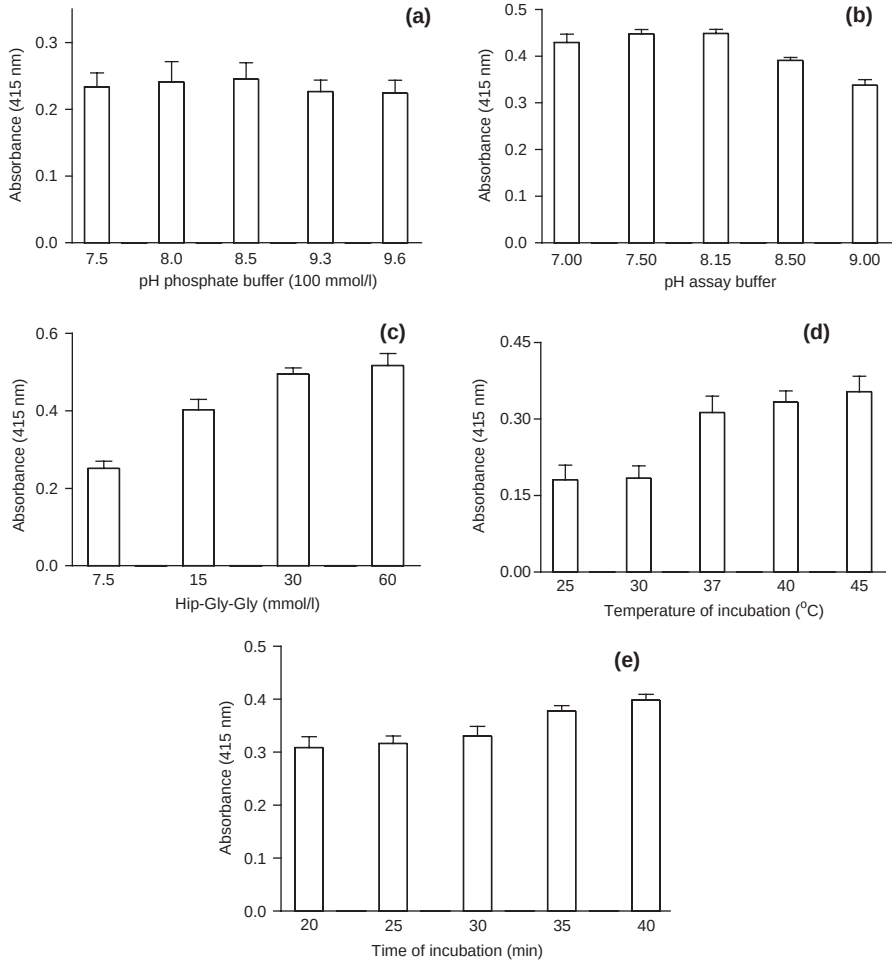

Fig. 4. Effect of the reaction conditions on the formation of the color product TNP-Gly-Gly. (a) pH of the phosphate buffer; (b) pH of the assay buffer; (c) substrate concentration; (d) incubation temperature; (e) incubation time. Data are expressed as means + SEM ($n = 6$).

Hip-Gly-Gly, the 30 mmol/l concentration is recommended for *in vitro* assaying the ACE activity (Yang et al., 1970; Weisser and Schloos, 1991; Neels et al., 1982). This substrate concentration was confirmed by our results, as stated in Fig. 4c. The absorbances obtained for Hip-Gly-Gly solutions of 30 and 60 mmol/l were significantly higher ($p < 0.05$) than the others (Fig. 4c) and thus 30 mmol/l of substrate was employed in our method.

Kinetics studies carried out with ACE demonstrated that 37 °C is the temperature of choice for optimum enzyme activity (Cushman and Cheung, 1971; Pihlanto-Leppälä et al., 1998). Among the temperatures assayed during method development, 37, 40 and 45 °C did not differ significantly ($p > 0.05$) (Fig. 4d). Based on this finding, we selected 37 °C for our method, considering that it mimics the physiologic temperature and is in agreement with results from kinetics studies (Cushman and Cheung, 1971; Pihlanto-Leppälä et al., 1998).

According to previous works, the activity of ACE remains constant over a period of 35 min and the incubation time recommended for enzyme optimum activity is within 30–90 min (Hansen et al., 1995; Duncan et al., 1999; Weisser and Schloos, 1991). The graph depicted in Fig. 4e shows no significant difference ($p > 0.05$) between the incubation times of 35 and 40 min which, in turn, are different from the others. For this reason, the incubation time of 35 min was chosen for our method.

Sensitivity of the assay

The sensitivity of the method was tested by determining the IC_{50} value for the reference ACE inhibitor, captopril, using a concentration–response curve. The IC_{50} value determined by our colorimetric method was 14.1 nmol/l, whereas the value reported for captopril was 23 nmol/l, obtained by a fluorimetric assay (Ondetti, 1988). As far as we know, there is no previous report on the IC_{50} value for captopril determined by a colorimetric assay, employing similar conditions. Considering the great variability of values reported for the IC_{50} of captopril, ranging from 11 to 23 nmol/l, the colorimetric method may be regarded more sensitive than the fluorimetric assay (Ondetti, 1988). It is recognized that IC_{50} values may vary due to the influence of several factors, including the conditions of the assay, the enzyme source, the calculation method and the relationship between the inhibitor and the enzyme source (Vermeirssen et al., 2002; Neels et al., 1982; Ariyoshi, 1993), thus explaining the differences observed between the fluorimetric and the colorimetric assays.

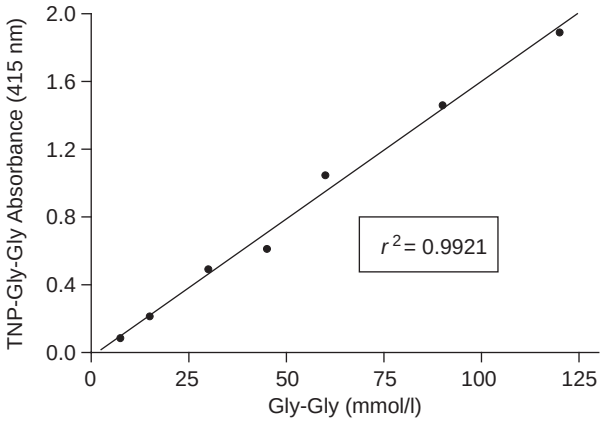

Fig. 5. Calibration graph as a regression between Gly-Gly concentration and the absorbance of TNP-Gly-Gly. Points are means ± SEM of 10 replicates.

Validation of the colorimetric assay

Linearity

The calibration curve (Fig. 5) was linear in the range 7.5–120.0 mmol/l of Gly-Gly ($r^2 = 0.9921$) and the linear regression equation is $y = -0.02403 + 0.01625x$.

Precision

The CV obtained for intra-day and inter-day assays were 7.63% and 13.61%, respectively. These values are within the range acceptable for results obtained from assays with multiple sources of variation, the limit of CV = 2–10% being recommended for intra-day assays and CV = 15–20% for inter-day assays (Chasin et al., 1998; Nigrinis, 1995).

Precision between methods

The precision between the colorimetric and the HPLC assay for ACE inhibiting activity does not differ significantly ($p > 0.05$), according to the Fisher test ($F = 1.92$) applied to the variance ratio of the two methods (Inmetro, 2003).

Accuracy

The recovery of Gly-Gly varied from 97.0% to 101.8% (Table 2), a satisfactory range in analytical terms, considering the complex composition of the matrix.

Correlation between methods

In order to investigate the correlation between the HPLC (Elbl and Wagner, 1991) and the new colorimetric assay for ACE inhibitory activity, we tested different concentrations of captopril by the two methods and a strong correlation was observed ($r = 0.9935$) (Fig. 6a). Additionally, 13 plant extracts were assayed by the two methods and submitted to correlation

Table 2. Recovery of Gly-Gly (% mean; $n = 6$)

Plant extract	Gly-Gly concentration (mmol/l)		
	15	30	45
Argemone mexicana L.	101.8	97.0	100.8
Cecropia glaziovii Sneth.	100.5	99.1	98.6
Mangifera indica L.	100.6	102.1	100.4

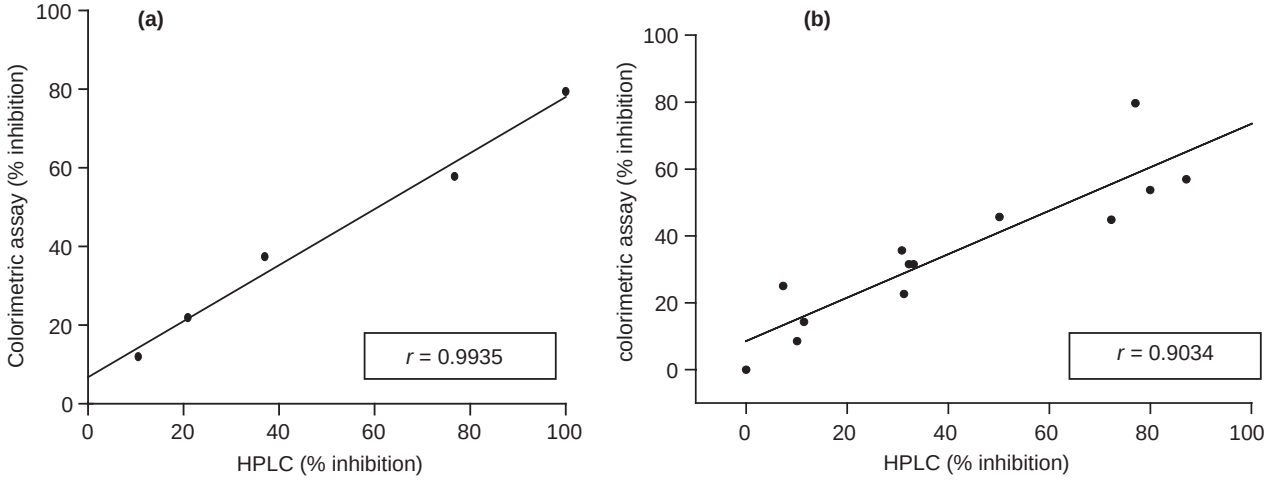

Fig. 6. Correlation between ACE inhibitory activity determined by the HPLC method and by the new colorimetric assay for (a) captopril solutions and (b) plant extracts. Points are means of triplicates.

Table 3. ACE inhibitory activity of plant extracts assayed by the colorimetric and HPLC methods (% mean; $n = 3$)

Plant extracts	ACE inhibition	
	HPLC assay	Colorimetric assay
Argemone mexicana L.	0.0	0.0
Buddleja stachyoides Cham. & Schltdl.	31.2	22.7
Cecropia glaziovii Sneth.	72.2	44.9
Combretum fruticosum Stuntz	79.9	53.8
Croton antisyphiliticus Mart.	32.2	31.6
Hancornia speciosa Gomes	50.1	45.7
Leea rubra Bl. Ex Spreng.	87.1	57.0
Mangifera indica L.	10.1	8.6
Persea americana Miller	7.3	25.1
Phoenix roebelinii O'Brien	77.0	79.7
Pyrostegia venusta (Ker.) Miers	33.1	31.6
Tabebuia serratifolia (Vahl) Nicholson	11.4	14.3
Tulbaghia violacea Harv.	30.8	35.7

analysis ($r = 0.9034$) (Table 3; Fig. 6b). These results allowed us to attest undoubtedly the correlation between the methods. Among the species assayed, *Argemone mexicana*, *Cecropia glazioui*, *Mangifera indica*, *Persea americana* and *Tulbaghia violaceae* were selected based on their traditional use as antihypertensive and/or diuretics, whereas a chemosystematic approach was adopted for the others.

Procyanidins and flavonoids are the major classes of natural products presenting *in vitro* ACE inhibitory

activity, isolated from plants selected by ethnopharmacological approach (Wagner et al., 1991; Wagner, 1998; Lacaille-Dubois et al., 2001; Actis-Goretta et al., 2003). Other classes of active compounds include phenylpropanes, xanthones, fatty acids, terpenoids, alkaloids and peptide amino acids (see references reviewed in Braga et al., 2000). The active extracts reported here will be further submitted to bioguided fractionation, aiming to isolate and identify the ACE-inhibiting compounds.

In conclusion, the work demonstrated that the proposed colorimetric assay is a suitable method for the screening of potential ACE inhibitors. The analytical parameters evaluated during the method validation were satisfactory and therefore it can be used as an assay for the primary screening of plant extracts. Besides, the time required for method execution is shorter than the HPLC assay and it involves only inexpensive reagents and a microtitre plate reader.

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