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Spectrophotometric determination of cysteine and/or carbocysteine in a mixture of amino acids, shampoo, and pharmaceutical products using *p*-benzoquinone

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Abstract

A simple, sensitive, and selective method has been developed for determination of cysteine (Cys) or carbocysteine (carboCys) in pharmaceutical products, shampoos and a mixture of amino acids. The results showed the reaction between *p*-benzoquinone (PBQ) and Cys occurs through the sulfhydryl group. Previous derivatization or extraction is not necessary before the assay is carried out. The method is based on the fact that the product of reaction between PBQ and Cys absorbs at 352 and 500 nm or PBQ and carboCys absorbs at 500 nm. Beer's law is followed in the range 0–40 μg/ml for Cys and 0–150 μg/ml for carboCys. The product of reaction PBQ-Cys is stable for 2 h with absorption bands at 352 and 500 nm. In the presence of amino acids, PBQ is highly selective to Cys. Several substances such as amino acids, urea, salts, and dipeptide did not interfere with the proposed method. A recovery of about 100% is observed for both Cys and carboCys, when the method is applied to determine Cys in a mixture of amino acids resembling blood plasma, shampoo, and pill food as well as carboCys in pharmaceutical products.

Keywords: *p*-Benzoquinone; Spectrophotometry; Cysteine; Carbocysteine; Pharmaceutical products

1. Introduction

Cysteine (Cys) plays an important role in several biological processes [1]; Cys or compounds of Cys are widely used in many pharmaceutical products. Determinations of Cys or compounds of Cys are commonly used in clinical investigation, pharmaceutical industry, and research.

Among the many methods for determination of Cys [2,3] or compounds of Cys, chromatographic methods are widely used. In spite of HPLC [4–6] or gas-chromatographic methods [7,8] which are very popular with equipment easily available in many laboratories, several disadvantages can be cited. In all chromatographic methods, the samples should pass through derivatization and extraction of the products of reaction before their microinjection into the column. Those methods

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use expensive reagents and equipment, and a significant period of time for whole assay.

In a previous paper [9], the use of *p*-benzoquinone (PBQ) for simultaneous determination of total proteins and amino acids was reported. It was observed that the spectrum of the product of reaction between Cys and PBQ (PBQ-Cys) was very different from the other proteic amino acids. An investigation of the reaction PBQ-Cys was undertaken and it was possible to obtain a specific reaction medium in which PBQ was selective for Cys in a mixture of amino acids.

The present paper describes the utilization of PBQ for spectrophotometric determination of Cys using the absorption bands at 352 and 500 nm, in pill food, shampoo, and a mixture of amino acids resembling blood plasma [10], as well as determination of carbocysteine (carboCys) using the absorption band at 500 nm in two pharmaceutical products (Mucoflux and Mucolitic). In all samples analysed, a good agreement between the nominal values and assay values was obtained.

2. Materials and methods

2.1. Materials

Ultraviolet and visible spectrophotometry were carried out on Varian DMS-80 and Shimadzu UV-1203 spectrophotometers.

All reagents were of analytical reagent grade. The pill food and shampoo were purchased from Calendola Pharmacy. Mucoflux (Merck) and Mucolitic (Laboratory Wyeth-Whitehall Ltda.) were purchased at a pharmacy in Londrina city (PR, Brazil). CarboCys and *N*-acetyl-cysteine (analytical reagent grade) were a gift from Dr João R.F. Teixeira of Merck and Dr Marta Y.Y. Satake of Zambon Laboratórios Farmacêuticos Ltda, respectively.

2.1.1. p-Benzoquinone (Sigma)

PBQ was purified by sublimation and a 0.1 M solution in DMSO was prepared, kept in an airtight flask and used for 5 days after its preparation.

2.1.2. Cysteine (CAAL) and carbocysteine

Cys and carboCys solutions (1.5 g/l) were prepared with distilled, deionized water and used as standards in all assays.

2.1.3. Buffer

The following 0.1 M solutions were used as buffer: $Na_2B_4O_7$–H_3BO_3, pH 8.0; Na_2HPO_4–NaH_2PO_4, pH 6.0 and 7.0; CH_3COONa–CH_3COOH, pH 3.0, 4.0 and 5.0.

2.1.4. Mixture of amino acids

An amino acid solution was prepared that contained a mixture of amino acids (Ala, Arg, Asn, Glu, Gln, Gly, Hys, Ile, Lys, Met, Pro, Ser, Tre, Tyr, Val) in proportions similar to those observed in blood plasma [10]. This solution was separated in aliquots and stored at -20°C.

2.2. Sample preparations

2.2.1. Standard samples

2.2.1.1. Cysteine. By dilution of the 1.5 g/l standard solution of Cys, a calibration curve was constructed using the following concentrations: 0.0, 7.5, 15.0, 22.5 and 30.0 μg/ml, and the volume was adjusted to 4.8 ml with acetate buffer, pH 3.0.

2.2.1.2. Cysteine with sodium dodecyl sulfate (SDS). Cysteine with SDS was used for assay of Cys in shampoo. The calibration curve was obtained as above and SDS was added to all tubes until the final concentration was 160 μg/ml.

2.2.1.3. Carbocysteine. By dilution of the 1.5 g/l standard solution of carboCys, a calibration curve was constructed using the following concentrations: 0.0, 30.0, 60.0, 90.0 and 120.0 μg/ml; the volume was adjusted to 4.8 ml with phosphate buffer, pH 7.0.

2.2.2. Pill food

A pill was ground and dissolved in 100 ml of distilled, deionized water. A 100-μl aliquot of this solution was transferred to a test tube, and the volume was adjusted to 4.8 ml with acetate buffer, pH 3.0.

2.2.3. Shampoo

A 200-μl aliquot of shampoo was transferred to a 5.0-ml volumetric flask and the volume was made up with distilled, deionized water. A 100-μl aliquot of this solution was transferred to a test tube, and the volume was adjusted to 4.8 ml with acetate buffer, pH 3.0.

2.2.4. Amino acids mixture

To 250 μl of the amino acids mixture, 710 μl of acetate buffer, pH 3.0 were added.

2.2.5. Mucoflux or Mucolitic

A 100-μl aliquot of Mucoflux or Mucolitic was transferred to a test tube and the volume was made up to 1.0 ml with distilled, deionized water. A 75-μl aliquot of this solution was transferred to a second test tube, and the volume was adjusted to 4.8 ml with phosphate buffer, pH 7.0.

2.3. Cys standard curve and Cys assay

To each tube of sample of pill food, shampoo, and standard Cys was added 200 μl of 0.1 M of PBQ, and for the sample of amino acids mixture, 40 μl of 0.1 M PBQ was added . The tubes were shaken, incubated at 37°C for 5 min, cooled to room temperature, and the absorbances at 352 and 500 nm were read against the blank.

2.4. CarboCys standard curve and carboCys assay

To each tube of sample of Mucoflux, Mucolitic, and standard carboCys was added 200 μl of 0.1 M of PBQ, shaken and incubated at 37°C for 10 min. The tubes were then cooled to room temperature and the absorbances at 500 nm were read against the blank.

3. Results and discussion

The reaction between quinones and amines, amino acids or proteins has sometimes been reported as a charge transfer complex [11,12] or substituted quinone compounds [13–16]. These discrepancies could be attributed to differences in the reaction conditions employed.

Table 1 shows that the reaction between PBQ and a pool of amino acids or PBQ and carbocysteine occurs when the pH is increased; in this case, the nitrogen of amino group is probably involved in the reaction as observed by several authors [11–16]. As pointed out by Ratajczak and Orville-Thomas [12], first a charge transfer complex is formed, then depending on conditions of reaction a mono- or di-substituent compound is formed. Several authors [13,14,16] obtained mono- or di-

Table 1
Maximum absorbance of the products of the reaction between *p*-benzoquinone (PBQ) and cysteine, pool of amino acids[a] (pool AAs) or carbocysteine (carboCys) at various pH values

PH	Maximum absorbance (nm)		
	Cys (30 μg/ml)[b]	Pool Aas[b] (30 μg/ml each)	CarboCys[c] (22.5 μg/ml)
8.0[e]	–	461–373	495–385[d]
7.0[f]	291	500–357–279	500–350[d]
6.0[f]	507[d]–343[d]–289	500–279	495[d]
5.0[g]	516–327[d]–284	275	–
4.0[g]	513–355–293	288	–
3.0[g]	500–352–293	288	–

[a] AAs used: Trp, Tre, Ser, Lys, Leu, Phe, Tyr, Val.
[b] Samples heated at 37°C for 20 min with 2.0 mM PBQ.
[c] Samples heated at 37°C for 10 min with 4.0 mM PBQ.
[d] Bands not well defined.
[e] Boric acid/borax buffer (0.1 M).
[f] Monobasic/dibasic sodium phosphate buffer (0.1 M).
[g] Acetic acid/acetate buffer (0.1 M).

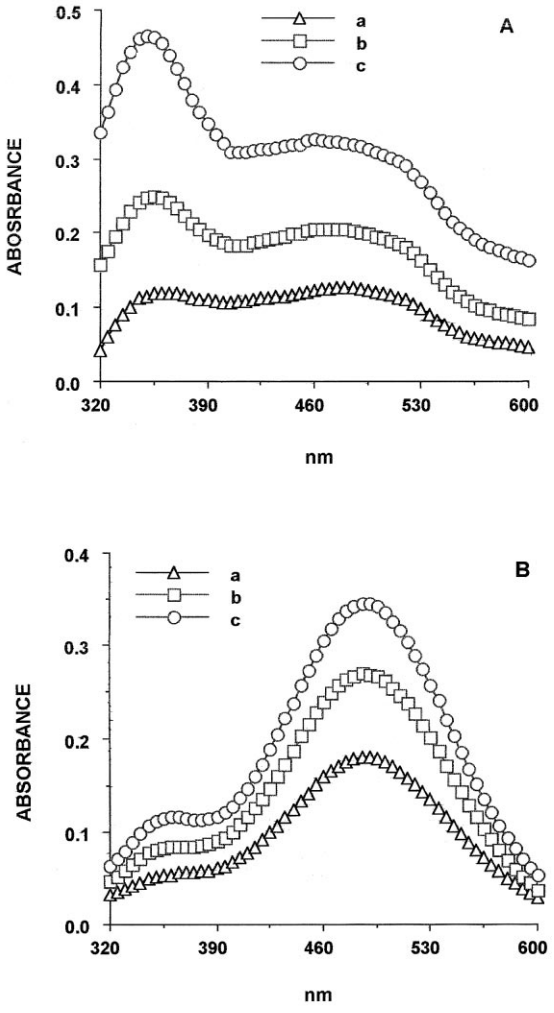

Fig. 1. (A) Absorbance spectra of the product of reaction between 7.5 (a); 15.0 (b) or 22.5 (c) μg/ml cysteine and 4.0 mM *p*-benzoquinone (PBQ); 5 min of heating at 37°C in acetate buffer 0.1 M, pH 3.0. (B) Absorbance spectra of the product of reaction between 60 (a); 90 (b) or 120 (c) μg/ml carbocysteine and 4.0 mM PBQ; 10 min of heating at 37°C in phosphate buffer 0.1 M, pH 7.0.

substituent compounds as the product of reaction between amines/amino acids and PBQ. Iskander and Medien [16] obtained a mono-substituent compound when an excess of PBQ reacts with Gly, which showed strong absorption at 500 nm and weak absorption at 340 nm. On the other hand, when an excess of Gly reacts with PBQ, the product of reaction had a strong absorption at 340 nm. As the product of reaction carboCys-PBQ (with an excess of PBQ) showed a strong absorption at 500 nm we probably also obtained a mono-substituent compound. The reaction between PBQ and Cys occurs when the pH is decreased probably because the sulfhydryl group is involved. We also tested carboCys (Table 1) and *N*-acetyl-cysteine (data not shown); for *N*-acetyl-cysteine, we observed the same behavior as observed for Cys, since the amino group is blocked by acetyl, thus the reaction was through the sulfhydryl group; for carboCys, we also observed the same behavior as observed for the pool of amino acids, since the sulfhydryl group is blocked by carboxy, then the reaction was through the amino group. At moderate temperatures in acidic solution with a high ratio of quinone/thiocompounds (Cys, thiourea, β-thiopropionic acid), several authors [17] obtained mono-substituent compounds with the sulfhydryl group attached to the ring of the quinone. As our blocking experiments showed, reaction between PBQ and Cys/*N*-acetyl-cysteine was through the sulfhydryl group, thus we probably also obtained a mono-substituent compound. The buffer components and the pH used here did not interfere with the reaction PBQ-pool of aas/carboCys/Cys. However, when borate buffer was used with pH higher than 9.0, polymerization of PBQ occurred interfering with the method.

Fig. 1 shows the absorbance spectra of the product of reaction between Cys or carboCys and PBQ. For the product of reaction between Cys and PBQ, there are two bands, one at 352 nm and another at 500 nm, both bands follow Beer's law in the range 0–40 μg/ml Cys (Table 2) and therefore both can be used for analytical purposes. In this case, the reaction PBQ-Cys occurred through the sulfhydryl group. On the other hand, the product of reaction between carboCys and PBQ shows one band at 500 nm that followed Beer's law in the range 0–150 μg/ml carboCys (Table 2) and can be used for analytical purposes. The reaction carboCys-PBQ occurred through the amino group.

Table 2 shows the straight line equations and range of linearity for the products of reaction between PBQ and Cys or carboCys in various

Table 2
Straight line equations and range of linearity (Beer's Law) for the products of reaction between *p*-benzoquinone (4.0 mM) and cysteine or carbocysteine in various media[a]

Medium	Straight line equation (Y (absorbance) $= mX$ (μg/ml) $+ b$)	Range of linearity (μg/ml)
Cysteine[b]		
Acetate buffer 0.1 M, pH 3.0	$Y = 0.01029X + 0.025$ at 500 nm (6)	0–40
(with 160 μg/ml of SDS)	$Y = 0.01558X - 0.016$ at 352 nm (7)	0–40
Acetate buffer 0.1 M, pH 3.0	$Y = 0.01230X + 0.013$ at 500 nm (25)	0–40
	$Y = 0.02180X - 0.045$ at 352 nm (25)	0–40
Acetic acid 1.0 M	$Y = 0.01530X - 0.021$ at 500 nm (2)	0–40
	$Y = 0.02200X - 0.113$ at 352 nm (2)	0–40
Acetic acid 0.5 M	$Y = 0.01510X + 0.078$ at 500 nm (2)	0–40
	$Y = 0.02550X - 0.019$ at 352 nm (2)	0–40
Acetic acid 0.1 M	$Y = 0.00920X - 0.063$ at 500 nm (2)	0–40
	$Y = 0.01770X - 0.053$ at 352 nm (2)	0–40
Acetic acid 0.01 M	$Y = 0.00770X + 0.006$ at 500 nm (2)	0–40
	$Y = 0.01750X - 0.085$ at 352 nm (2)	0–40
Hydrochloric acid 0.5 M	$Y = 0.00290X + 0.008$ at 500 nm (2)	0–40
	$Y = 0.00820X + 0.009$ at 352 nm (2)	0–40
Hydrochloric acid 0.1 M	$Y = 0.00870X + 0.114$ at 500 nm (2)	0–40
	$Y = 0.01470X + 0.034$ at 352 nm (2)	0–40
Hydrochloric acid 0.01 M	$Y = 0.01390X + 0.104$ at 500 nm (2)	0–40
	$Y = 0.01840X - 0.045$ at 352 nm (2)	0–40
Carbocysteine[c]		
Borax buffer 0.1 M, pH 8.0	$Y = 0.00952X + 0.001$ at 500 nm (3)	0–40
Phosphate buffer 0.1 M, pH 7.0	$Y = 0.00266X + 0.018$ at 500 nm (7)	0–150

[a] Number of experiments in parentheses.
[b] Samples heated at 37°C for 5 min.
[c] Samples heated at 37°C for 10 min.

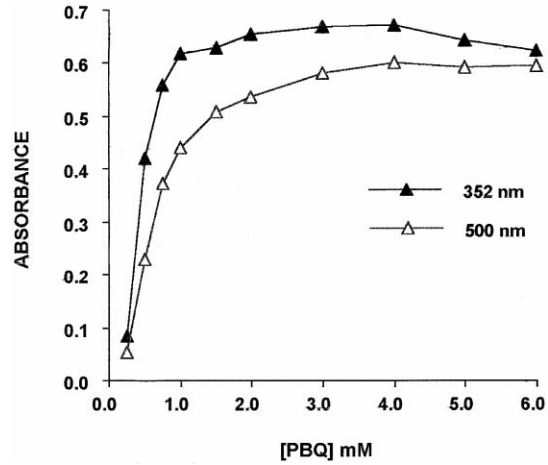

Fig. 2. Dependence of absorbance on the concentration of *p*-benzoquinone (PBQ). The samples of cysteine (30 μg/ml) were heated with different amounts of PBQ in acetate buffer, pH 3.0, at 37°C for 20 min.

media. For determination of Cys in the samples with absorbances at 352 and 500 nm, acetate buffer 0.1 M (pH 3.0) was used because one of the best specific absorbances and the best correlation coefficient of the straight line were obtained with that medium. Higher specific absorbances for Cys, shown in Table 2, were obtained in the pH range 2–3. The samples with 0.01 M acetic acid and 0.5 and 0.1 M hydrochloric acid were out of that range and their specific absorbances were lower. Only one buffer medium (0.1 M acetic acid) showed specific absorbance as low as the media buffer outside the good range. In spite of 0.1 M phosphate buffer (pH 7.0) showing minor specific absorbance at 500 nm, when compared with borax buffer pH 8.0 at 500 nm (Table 2), it was used for determination of carboCys, because the product of reaction was more stable at pH 7.0 than at pH 8.0 and the straight line equations were better. For the reaction PBQ-carboCys, an increase in specific absorbance was obtained when the pH was increased because the amino group is free. The correlation coefficients for all the straight lines shown in Table 2 were at least 0.98.

Fig. 2 shows the effect of different concentrations of PBQ on the absorbance of Cys. For both absorption bands (352 and 500 nm), the absorbance increased almost linearly with the in-

crease in PBQ concentration up to 1.0 mM. Above 3.0 mM, there is only a small increase in absorbance. Shifts of 352 or 500 nm bands were not observed at any of the PBQ concentration tested (data not shown). PBQ (4.0 mM) was used which gave a ratio of 16 of PBQ/Cys in mol for the curve with the highest concentration of Cys. This concentration seems to be sufficient because Beer's law was followed up to 30 μg/ml of Cys.

We also tested the effect of heating time at 37°C on absorbance of products of reaction between PBQ (4.0 mM) and Cys or carboCys (data not shown). The temperature was standardized at 37°C because on cold days (15°C), the reaction is slow. Heating at 37°C provide an easier way to control and to standardize the time of reaction. It was observed that there was only a small increase in absorbance for Cys after 5 min of heating; for carboCys heating, it should last for 10 min.

After heating, the absorbance at 352 nm of the product of reaction between PBQ and Cys showed a smaller increase up 20 min and after that, the stability lasted for 2 h (Fig. 3). At 500 nm, a slight decrease in the absorbance was observed after 30 min of heating (Fig. 3). Thus, time after heating is not a critical factor for the measurement of Cys with PBQ at 352 or 500 nm. On the other hand, the product of reaction between PBQ and carboCys was not as stable as the PBQ-Cys product (data not shown), so after heating, the absorbance must be measured promptly.

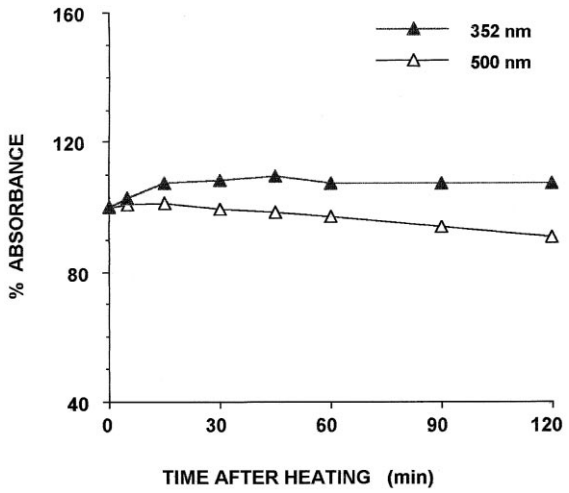

Fig. 3. Stability of the absorbance of the products of reaction between *p*-benzoquinone (PBQ) and cysteine (30 μg/ml). The samples were heated at 37°C for 5 min in 0.1 M acetate buffer, pH 3.0, with 4.0 mM PBQ and their absorbances at zero time were taken as 100%.

Table 3 shows the effect of selected potential interfering compounds on Cys determination. For both bands (352 or 500 nm), Cys determination with PBQ was not susceptible to interference by the pool of amino acids (7.5 μg/ml), salts (30 mM), cystine, dipeptide, glucose and urea (30 mM). Fatty acids, triglycerides, and cholesterol did not react with PBQ but they seriously interfered with Cys assay because they increased the turbidity of the solution, so these substances should be removed before Cys assay is carried out.

The absorbance spectra of 160 μg/ml SDS plus 4.0 mM PBQ and 4.0 mM PBQ, using buffer pH 3.0 as blank, showed a decrease in the absorbance of PBQ with SDS (data not shown). Although PBQ seems to be consumed by SDS, there are not differences between the spectrum of PBQ with or without SDS. This consumption of PBQ by SDS could explain why the Cys recovery shown at Table 3 is low. Thiourea was also studied (Table 3). It was shown to interfere with the Cys assay at concentrations higher than 11.4 μg/ml because the product of reaction between PBQ and thiourea shows two bands, one strong at 275 nm and other at 420 nm.

This method using PBQ was applied to the determination of Cys in pill food, shampoo and a mixture of amino acids resembling blood plasma [10], as well as carboCys in Mucoflux and Mucolitic (Table 4). The results show that determination of Cys can be carried out with the band at 352 nm as well as at 500 nm, because the results shown in Table 4 for both bands are not statistically different. It was necessary to add SDS to the standard curve for determination of Cys in shampoo, because it interferes with the Cys assay. In all samples studied (Table 4), the results showed a good agreement between the nominal and assay value using the proposed method. The recoveries are from 97.0 to 104.4% for all results shown in Table 4.

Table 3
Effect of selected potential interfering compounds on cysteine determination[a]

Cys (150 μg) + interfering compounds	Addition	Absorbance at 352 nm as percentage of control	Absorbance at 500 nm as percentage of control
Cysteine	Buffer	100.0 ± 3.2	100.0 ± 3.2
KNO_3	300 mM	124.2 ± 0.4	145.6 ± 0.5
	30 mM	101.7 ± 1.2	103.5 ± 1.6
$MgCl_2$	300 mM	130.0 ± 0.2	105.8 ± 4.5
	30 mM	105.4 ± 0.6	111.1 ± 1.5
NaCl	300 mM	126.3 ± 3.1	121.0 ± 2.7
	30 mM	100.0 ± 4.1	103.2 ± 1.6
$CaCl_2$	30 mM	101.4 ± 0.3	106.7 ± 0.5
BSA	600 μg/ml	75.1 ± 1.5	76.0 ± 0.9
	60 μg/ml	82.8 ± 1.3	84.4 ± 2.1
Amino acids[b]	75 μg/ml	93.4 ± 1.3	83.0 ± 0.9
	7.5 μg/ml	102.5 ± 1.6	101.7 ± 1.7
Glucose	150 mM	96.0 ± 2.5	101.2 ± 1.8
	15 mM	91.6 ± 4.1	96.8 ± 3.7
Fatty acids[c]	1000 μg/ml	271.8 ± 1.0	542.5 ± 0.9
	100 μg/ml	187.9 ± 0.4	186.5 ± 0.6
Triglycerides	100 μg/ml	142.0 ± 0.2	117.1 ± 1.0
	10 μg/ml	120.5 ± 0.4	98.9 ± 1.9
Cholesterol	200 μg/ml	365.1 ± 0.8	407.2 ± 0.6
	20 μg/ml	205.4 ± 0.8	130.3 ± 1.1
TEA	10 mM	209.8 ± 0.2	24.9 ± 2.1
	1.0 mM	77.5 ± 3.1	66.6 ± 3.1
Ethanol 100%	100 μl/ml	87.6 ± 1.0	90.4 ± 0.6
	10 μl/ml	103.7 ± 1.4	101.7 ± 1.7
Ascorbic acid	1000 μg/ml	–	7.9 ± 3.1
	100 μg/ml	108.0 ± 3.7	94.5 ± 3.2
EDTA	100 μg/ml	95.5 ± 0.3	89.3 ± 0.8
	10 μg/ml	100.9 ± 3.2	98.2 ± 3.8
Sodium tungstate	135 mg/ml	274.6 ± 2.2	65.0 ± 3.8
	13.5 mg/ml	288.7 ± 6.6	86.3 ± 2.9
Glycylglycine	100 μg/ml	103.3 ± 1.0	95.5 ± 0.7
	10 μg/ml	102.5 ± 2.9	101.6 ± 2.9
Cystine	100 μg/ml	99.9 ± 2.6	96.7 ± 3.3
	10 μg/ml	103.9 ± 3.1	101.7 ± 3.4
Urea	300 mM	88.1 ± 2.2	90.7 ± 1.5
	30 mM	95.5 ± 1.7	99.5 ± 2.5
Sodium dodecyl sulfate	160 μg/ml	89.8 ± 5.2	79.2 ± 4.7
Thiourea	11.4 μg/ml	105.3 ± 4.0	78.1 ± 6.6
	114.0 μg/ml	247.4 ± 2.5	87.4 ± 1.1

[a] The results are presented as mean ± S.E.M. of four determinations. Each well contained 150 μg of cysteine and the indicated concentration of the addition in a total volume of 5.0 ml.
[b] The concentration of each of the proteic amino acids.
[c] Pool of oleic and palmitic acids.

4. Conclusion

The results show that the reaction between PBQ and Cys occurs through the sulfhydryl group, the product of reaction PBQ-Cys is stable, and PBQ is highly selective to Cys in a mixture of amino acids. The proposed method is sensitive for most applications, is not interfered with by many substances, and is not expensive. The proposed method for determination of Cys or carboCys is

Table 4
Determination of cysteine or carbocysteine in pharmaceuticals, shampoo or a mixture of amino acids[a]

Sample	Source	Nominal value (mg)	Found (mg)	
			500 nm	352 nm
Pill food[b]	Calendola Pharmacy	80.0	77.8 ± 2.0 (4)	78.6 ± 3.3 (4)
Shampoo[b]	Calendola Pharmacy	25.0	24.7 ± 0.6[d] (6)	25.1 ± 0.5[d] (6)
			21.6 ± 0.7[e] (7)	20.9 ± 0.6[e] (7)
Mixture of amino acids[b] (10^{-3})	–	40.0	39.0 ± 1.4 (6)	38.8 ± 0.8 (7)
Mucoflux[c]	Merck S..A.	50.0	51.5 ± 1.4 (5)	–
Mucolitc[c]	Laboratory Wyeth-Whitehall Ltda.	50.0	52.2 ± 2.0 (6)	–

[a] The samples were heated at 37°C with 4.0 mM of p-benzoquinone. The results are presented as mean ± S.E.M. Number of assays in parentheses. Composition of the samples: Pill food: methionine, 200 mg; cysteine, 80 mg; cystine, 25 mg; collagen, 25 mg; calcium pantothenate, 25 mg; vitamin B-6, 10 mg; vitamin B-2, 1 mg; vitamin E, 3 mg; biotin, 0.2 mg and excipients. Shampoo: cysteine, 25 g; sodium dodecyl sulfate, 200 g and 1 l of water. Mixture of amino acids (Ala, Arg, Asn, Glu, Gln, Gly, Hys, Ile, Lys, Met, Pro, Ser, Tre, Try, Tyr, Val) with composition and concentration similar to blood plasma [10]. Mucoflux: carbocysteine, 250 mg; excipients and water 5 ml. Mucolitic: carbo-cysteine, 250 mg; excipients and water 5 ml.
[b] Acetate buffer 0.1 M, pH 3.0, 5 min of heating.
[c] Phosphate buffer 0.1 M, pH 7.0, 10 min of heating.
[d] Standard curve with 160 μg/ml of sodium dodecyl sulfate.
[e] Standard curve without sodium dodecyl sulfate.

simple to carry out, because it is not necessary to perform any derivatization or extraction as occurs in chromatographic methods [4–8] and good recoveries were obtained in all applications.

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