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Article

The Use of Solid Phase Reactors as the Source of a Catalytic Solution. The Indirect Flow-Injection Spectrophotometric Determination of Amino Acids

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A determinação indireta de aminoácidos é realizada em um sistema de injeção de fluxo utilizando-se um reator de fase sólida que contém sais cúpricos, imobilizados em esferas de resina de poliéster. Uma substância farmacêutica é forçada através do reator e os íons cúpricos liberados (complexados pela substância farmacêutica) agem como catalisadores da reação subseqüente entre Fe(III) e tiosulfato de sódio. A curva de calibração é linear no intervalo de concentrações 0,1-0,3 μg L^{-1} para glicina, com um desvio padrão relativo de 2,3% e uma velocidade de passagem de 28 amostras por hora. Foi analisada a influência de substâncias estranhas e o método foi aplicado à determinação de glicina em duas formulações farmacêuticas diferentes.

The indirect determination of amino acids is carried out in a flow-injection assembly by means of a solid-phase reactor containing cupric salts, immobilized in polyester resin beads. A pharmaceutical substance is forced through the reactor and the released cupric ions (complexed by the pharmaceutical substance) act as a catalyst for the subsequent reaction between Fe(III) and sodium thiosulfate. The calibration graph is linear over the range 0.1-3.0 μg mL^{-1} glycine, the RSD was 2.3%, and the sample throughput was 28 h^{-1}. The influence of foreign substances was studied and the method was applied to the determination of glycine in two different pharmaceutical formulations.

Keywords: *Cu(II) catalyst, spectrophotometry, glycine, amino acids, flow analysis, solid-phase reactors*

Introduction

Solid phase reactors are now firmly established in continuous-flow applications. Their use in FI assemblies offers some advantages over the homogeneous solution systems that have been illustrated in several papers[1,2,3]. In fact, FI solid phase reactors afford a variety of applications and facilitate sample derivation. So far they have been used for the purification of both sample and reagent solutions, and for the *in situ* preparation of unstable reagents[4].

Several methods have been proposed for the determination of amino acids as relevant indicators of nutritional requirements. The direct titrimetric determination of amino acids in multivitamin formulations has been officially recommended[5]. However, most of the published methods for the determination of amino acids are based on the derivation of the analyte, which allows an increase in sensitivity and facilitates the separation step in real samples, which usually contain a mixture of several amino acids. In this way, fluorimetric[6], polarographic[7], and chromatographic methods (TLC[8], GC[9,10] and HPLC[11-13]) have been proposed.

Some methods based on the FI methodology have been developed. Glycine and cysteine can be fluorimetrically determined by means of prior oxydation with potassium iodate in the presence of 2-mercaptoethanol[14]. Indirect determinations of amino acids in pharmaceutical formulations have been carried out using solid-phase reactors[15,16] and atomic absorption for the detection of the metallic

cation liberated from the reactor. Sensors for industrial process monitoring using enzyme-cartridge flow-injection analysis[17,18], electrodialysis with amino acid oxydation to enhance selectivity in FI for L-amino acids[19], and bienzyme sensors based on electrically wired peroxidase[20], have improved the analytical strategies for amino acids.

This paper deals with a new strategy for exploiting the use of solid phase reactors for the indirect determination of pharmaceutical substances. The proposed manifold includes a reactor containing copper carbonate immobilized on a polymer resin. On passage through the reactor, the sample forms a complex and releases the metal ion; then the released cupric ion acts as the catalyst in a subsequent reaction between Fe(III) and thiosulfate ion. The product of this reaction (ferrous ion) is spectrophotometrically monitored (at 510 nm) by reaction with 1,10-phenanthroline (hence forth referred to as phen).

Experimental

Reagents

Aqueous solutions of glycine (Probus) at pH 9.5 were potentiometrically adjusted by dripping diluted NaOH (Panreac). The other aqueous solutions were ferric nitrate (Panreac), sodium thiosulfate (Panreac), phen in 0.05 mol L^{-1} HCl (Panreac). The solid-phase reactor was prepared according to a previously published procedure[15] with $CuCO_3.Cu(OH)_2.2H_2O$ (Panreac), polyester resin solution AL-100-A (Reposa) containing the cobalt activator and adding methylketone (Akzo) as the catalyst. All reagents used were of analytical grade, unless otherwise stated.

Apparatus

The proposed FIA manifold is depicted in Fig. 1. The assembly was provided with a Minipuls 2 peristaltic pump (Gilson) and a 5041 injecting valve (Rheodyne). The complex formed was monitored at 510 nm by a Lambda 16 (Perkin-Elmer) spectrophotometer, provided with a 18 μl flow-cell (Hellma). The PTFE tubes were of 0.8 mm ID, except the solid-phase reactor which was prepared with 1.5 mm ID PTFE tubes.

Procedures

578 μL of aqueous alkaline glycine solution at pH 9.5 (potentiometrically adjusted by dripping diluted NaOH) was injected into the carrier solution of NaOH (pH 9.5, flow-rate 2.6 mL min^{-1}). The injected solution was forced through the solid-phase reactor (10 cm long, 1.5 mm ID, and particle size 150-200 μm), and then merged with a 7.0 x 10^{-4} mol L^{-1} Fe(III) solution. The subsequent mixture with a 2.58 x 10^{-3} mol L^{-1} $Na_2S_2O_3$ solution produced Fe(II), which was spectrophotometrically determined at 510 nm by means of reaction with a 0.5% (w/v) phen solution.

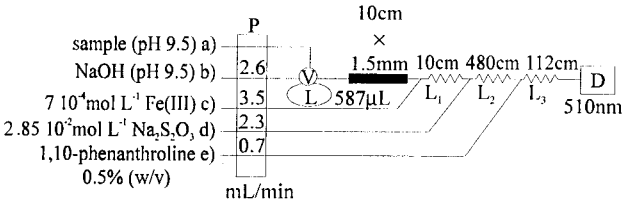

Figure 1. The flow-injection assembly for the determination of amino acids. P: peristaltic pump; V: injection valve; L: sample loop; D: detector.

Results and Discussion

Figure 2 shows the experimental setup and solutions used in preliminary experiments with a number of pharmaceutical substances that released cupric ion from the reactor by a displacement reaction. The cupric ion, released from the solid-phase reactor by complexation with the pharmaceutical substance, acts as a catalyst for the reaction between Fe(III) and thiosulfate ion. The injection of a phen solution and absorbance readings at 510 nm provided the amount of Fe(II) present. Experiments were carried out by alternately circulating solutions of the pharmaceutical under study through channel *d* at pH 8.5 and an aqueous stream at the same pH. The most favorable results were obtained with adrenaline, chlorpromazine, dipyrone, pyramidon, and glycine (Table 1). These pharmaceutical substances were used in further experiments where the reagent (cupric salt) was removed from the reactor in order to ascertain whether the presence of free pharmaceutical substance in the reactor affects the reaction between Fe(III) and thiosulfate. These tests revealed that adrenaline provides the most promising results (under the working conditions used, the pharmaceutical substance was readily oxidized by Fe(III)).

Experiments performed with three amino acids provided results as good as those previously obtained with adrenaline. In fact, the calibration graphs for glycine, histidine, and proline were linear (with correlation coefficients of *ca.* 0.998) and had similar slopes. The throughput was close to 250 samples h^{-1}.

Notwithstanding the excellence of these results, we tested alternative configurations in order to reduce pharmaceutical substance consumption. In fact, the previous assembly required a high volume of sample to flush the reactor on switching between samples of different concentrations; otherwise, carry-over would have been quite sig-

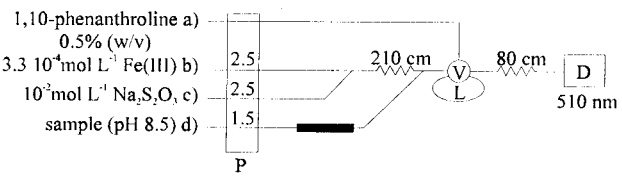

Figure 2. The flow-injection assemblies from previous studies on the influence of chemical and FIA parameters.

Table 1. The effects of pharmaceuticals on the release of Cu(II) from the reactor by displacement reaction.

Pharmaceutical Substance	Concentration (μ g mL^{-1})	Absorbance(*)
Glycine	5.0	+157.6
Phenformin	10.5	+10.2
Penicillin	10.3	+0.4
Adrenaline	12.4	+248.3
Terramicine	10.0	+17.9
Doxycycline	10.8	+17.9
Metformin	11.3	+16.7
Ondansetron	10.0	+3.1
Phenylbarbituric Ac.	11.0	+2.7
Nortriptyline	11.6	+1.4
Pyramidon	12.2	+59.6
Captopril	10.7	-9.1
Dipyrone	12.0	+45.7
Ephedrine	10.5	+30.7
Atropine	11.3	+0.7
Prometazine	10.5	+12.2
Isoniazid	10.0	+10.3
Paracetamol	10.6	+7.0
Reserpine	10.1	+6.3
Procaine HCl	10.0	-0.5
Moroxydine HU	10.0	+3.0
Sulfamethoxypiridazine	9.1	+6.3
Pentobarbital Na	10.1	+8.3
Trimethoprim	9.1	-1.7
Pyridoxine HCl	12.3	-2.4
Nicotinamide	10.6	+0.3
Levamisol HCl	8.9	-3.8
Folcodine	12.2	-3.5
Hidroclorotiazide	9.8	-0.7
Phenylefrine HCl	8.8	-0.3
Etoformin HCl	10.8	+4.6
Chloropromazine	10.2	+64.0
Amitriptyline	9.0	+7.7
Perfenacine	7.6	+4.6
Sulfamethoxazole	10.3	-2.1
Pirazinamide	12.9	-2.4
Pilocarpine HCl	11.0	+1.2

(*) % of absorbance over the blank (analyte solution) absorbance.

nificant. In addition, the new experiments were aimed at increasing the sensitivity and decreasing the detection limit by diminishing the blank signal.

A series of preliminary experiments were carried out in order to characterize and control the influence of several experimental variables on the chemical system. The influence of the reactant concentration was studied by keeping it constant in the circulated solutions and changing the flow-rate of the streams. The flow-rates of the Fe(III) and thiosulfate streams (identical for both) were altered and the influence of the presence of the catalyst at variable concentrations of ferric and thiosulfate ions was studied (the glycine flow-rate was kept constant and the amino acid was replaced with water to evaluate the blank signal). The absorbance decreased with increasing flow-rate of the Fe(III) and thiosulfate streams. The highest absorbances were obtained at low flow-rates (lower than 1 mL min^{-1}) of both Fe(III), sulfate and aqueous glycine, which suggests that the redox reaction is favored by long residence times. However, the differences between the signals revealed that the best results were achieved with intermediate catalyst flow-rates (about 3 mL min^{-1}) and low Fe(III) and thiosulfate flow-rates. In other words, a compromise must be made between residence and contact times, and an effective catalyst concentration. Obviously, the longer the residence time, the greater the extent of reaction development, and hence the greater the detector response. However, the effect of interferences may be increased. The differences between the signals for the blank (distilled water) and the sample (pharmaceutical substance solution) were also illustrative of the reaction kinetics in the absence of a catalyst.

We studied the development of the reaction using the stopped-flow technique. For this, we carried out a series of experiments changing the phen concentration in the presence and absence of the catalyst. The flow was stopped for 10 min and 1,10-phenanthroline concentrations in the range of 0.02-0.5% (w/v) in 0.05 mol L^{-1} HCl were circulated. In these experiments, the reagent column and injection valve in Fig. 2 were removed from the assembly, and a 1,10-phenanthroline solution of variable concentration was circulated through channel *d*.

There were two experimental batches: one without a catalyst and one with a catalyst (using the same assembly but including Cu(II) concentrations between 0 and 3.11 mg L^{-1} in the ferric solution). These experiments allowed us to draw the following conclusions: (*a*) the absorbance decreased markedly with decreasing phen concentration; (*b*) the more concentrated (0.1, 0.5%) ligand solutions gave much greater slopes in the absorbance *vs.* time plots; (*c*) the absorbance increased sharply with an increase in the concentration, both in the presence and absence of a catalyst (absorbance values in the latter case were quite high anyway); (*d*) a Cu(II) concentration of 3.11 mg L^{-1} quantitatively converted Fe(III) to Fe(II) ion at time zero; (*e*) under

these conditions, a Cu(II) concentration as low as 6 μg L^{-1} could be detected.

The influence of other experimental variables was studied using the manifold depicted in Fig. 3. Channel *a* was used with and without an intercalated solid phase reactor. In the absence of the reactor, the channel was used to circulate Cu(II) at a variable concentration; in its presence, various amino acids at different concentrations were passed. By comparing the signals obtained in both cases, the conversion efficiency of the reactor and the extent of the decomposition of the Cu(II)-amino acid complex were simultaneously determined.

The poor performance of the amino acids in the previous assembly was the likely result of the low quantitativeness of the reaction taking place in the solid phase reactor or of the Cu(II)-amino acid complex being too stable to allow Cu(II) to act as a catalyst. In order to ascertain the reason, the manifold depicted in Fig. 4 was assembled. The Cu(II)-glycine complex was merged with the acidic Fe(III) stream to release Cu(II) by displacement of H^+ ion. Two types of transient signals were obtained by circulating a Cu(II) stream through channel *a* in the absence of the solid phase reactor, and an amino acid stream in the presence of the reactor. The results were similar to the previous ones; however, they allowed us to ascertain that the fact that absorbances could not be higher was not the result of poor mixing (in the previous assembly, three streams converged on the same point), nor of a high stability of the Cu(II)-aminoacid complex. Rather, the sensitivity was limited by the quantitativeness of the reaction between the amino acid and the immobilized cupric salt.

Increased reactor lengths up to 10-15 cm (Fig. 4) resulted in increased signals. The effect of longer lengths was offset by the increased sample dispersion. The peak absorbances obtained at reactor lengths of 10 and 15 cm were 0.138 and 0.139, respectively.

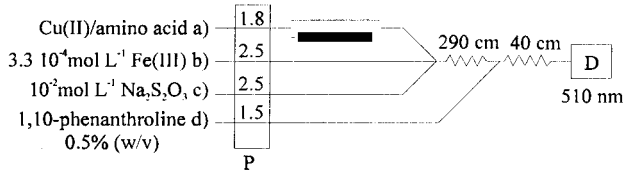

Figure 3. The flow-injection assemblies from previous studies on the influence of chemical and FIA parameters.

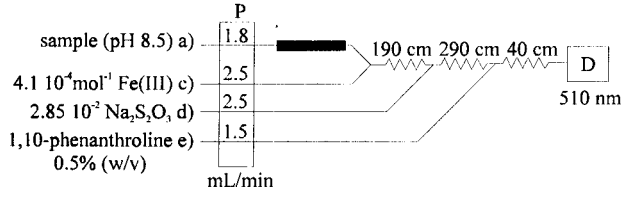

Figure 4. The flow-injection assemblies from previous studies on the influence of chemical and FIA parameters.

Placing the solid phase reactor in the loop of the injection valve (Fig. 5) was indicated in principle, since it resulted in no sample dispersion during the reaction. In addition, the time the solution and solid bed remained in contact was controlled by switching the valve on and off without the need to stop the flow, which would have required automatic control and detracted from the reproducibility, owing to the typical flow-rate oscillations observed within the first few moments after a peristaltic pump is started. Nevertheless, the many channels included in this manifold caused problems that are usually absent from simpler configurations. Thus, switching the valve on and off caused peak distortions arising from changes in the hydrostatic pressure; the effect varied with column length, which could not be increased at will. The peak for the blank standard could not be minimized. Also, conditioning after the analyte concentration was changed took a fairly long time since the system was not periodically flushed with carrier.

Placing the solid phase reactor between the injection valve and the merging point with ferric solution resulted in a decrease in the blank transient signal; the continuous flow of the carrier solution through the reactor avoids the conditioning or washing steps required in the other manifolds. This manifold was tested by preparing and injecting different concentrations of glycine (up to 8 μg mL^{-1}; five replicates for each point), and resulted in a wider linear graph. This assembly was selected for further work.

The selected manifold (Fig. 1) was optimized in terms of all of the influential chemical and FIA variables involved in the process using the univariate method. The results are summarized in Table 2. The optimization sequence was the same as described in the table, and the experiments involved injecting 280 μL of a 5 μg mL^{-1} glycine solution.

The selection of the optimum value that could be obtained for each tested parameter was the one which gave the best compromise of peak height-peak base width-rsd (%).

In optimizing the pH, transient signals were corrected for the baseline at pH 11.5, where no signal was observed. Possibly, the lack of a response at pH 11.5 was due to the *in situ* precipitation of the released Cu(II). The optimal pH, 9.5, was that resulting in the best compromise between the maximum absorbance and the baseline at pH 8.5.

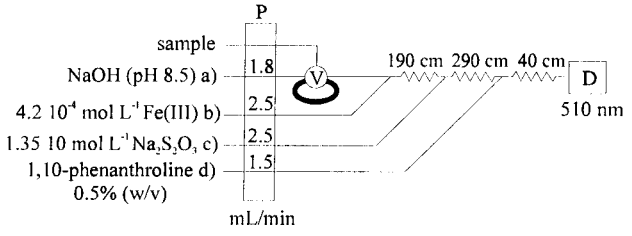

Figure 5. The flow-injection assemblies from previous studies on the influence of chemical and FIA parameters.

Table 2. The range of optimized variables and selected values.

Parameter	Tested Range	Selected Value
1,10-phenanthroline (%) (w/v)	0.10-1.00	0.5
pH carrier	6.0-11.5	9.5
$Na_2S_2O_3$ (g L^{-1})	1.40-7.40	6.40
Fe(III) (mol L^{-1})	(2.0-10.0) x 10^{-4}	7.0 x 10^{-4}
Coil reactor length (L_3) (cm)	32-202	112
Coil reactor length (L_2) (cm)	90-570	480
Coil reactor length (L_1) (cm)	10-280	10
Sample volume (μL)	90-729	578
Solid-phase reactor length (cm)	5-25	10

The Fe(III) concentration resulted in a critical variable, and then it was re-optimized with the selected FIA assembly at three different glycine concentrations: 0.5, 1.0, and 2.0 μg mL^{-1}. The Fe(III) concentration range was from 2.0 x 10^{-4} to 10.0 x 10^{-4} mol L^{-1}. The optimum value, 7.0 x 10^{-4} mol L^{-1}, was also relative to the baseline since an increase in the Fe(III) concentration also accelerated the uncatalysed reaction, thereby increasing the blank signal.

Analytical figures of merit

The calibration graph was linear from 0.1 to 3.0 μg mL^{-1} glycine, and fitted the equation, $A = -0.0093 + 0.3380\ C$, where A denotes the absorbance and C the glycine concentration, in micrograms per milliliter. The correlation coefficient was 0.9957. We ran calibration graphs for other a-amino acids, the equations for which are shown in Table 3. A comparison of the slopes of the graphs with those for previously reported methods[15,16] reveals that the proposed system is 20 times more sensitive in one case and 7 times more in the other; hence, the application range is necessarily short in a spectrophotometric technique with a narrow applicability "window" such as this.

The day-to-day reproducibility was determined by running five calibration graphs for glycine on different days. The average of the slopes obtained was 0.3380, and the relative standard deviation (RSD) was 4.36%. The repeatability of the process was calculated using 16 replicates of an injection of 1 μg mL^{-1} glycine. The mean peak height and RSD thus obtained were 0.3719 AU and 2.3%, respectively. In the same experiment, the throughput was estimated to be 28 samples h^{-1}.

Table 3. Calibration equations with the optimal flow-injection assembly for different a-amino acids.

Amino Acid	Equation (c in μg mL^{-1})	Corr. Coeff.
Alanine	y = 0.0196 + 0.2633 c	0.9936
Proline	y = 0.0170 + 0.1589 c	0.9952
Cysteine	y = -0.0868 + 0.2608 c	0.9993
Histidine	y = -0.0567 + 0.1492 c	0.9943

The influence of foreign substances, some of them accompanying glycine in pharmaceutical formulations, was studied by preparing solutions containing 2.46 μg mL^{-1} of the amino acid and different concentrations of the potential interferent. Results obtained (relative error in %) were as follows: 0.9 for acetyl acetic acid at 5 μg mL^{-1}; 3.8 for caffeine at 50 μg mL^{-1}, and 5.2 for NaCl at 50 μg mL^{-1}. High errors were calculated for ascorbic and citric acid at 5 μg mL^{-1}, but by preparing concentrations similar to or smaller than that of glycine (which is case of the pharmaceutical formulations) the errors were always smaller than 3%.

Finally, glycine was determined in two different formulations: Okal (tablets, contents according to label claim: acethyl salicylic acid 500 mg, glycine 100 mg and caffeine 30 mg) and Actilevol (ampoules). A few Okal tablets were ground in an agate mortar and an aliquot of *ca.* 0.35 g of the resulting powder was accurately weighed and dissolved in distilled water. The resulting mixture was filtered and brought to 500 mL with distilled water; ten milliliters of the resulting solution were leveled to 100 mL; aliquots of 5 mL were adjusted to pH 3 with 0.1 mol L^{-1} HCl and the acetyl salicylic acid was removed by extraction with diethyl ether. The second formulation, Actilevol (ampoules, composition according to label claim: ascorbic acid 500 mg, hemathoporphyrin 6 mg, glycine 500 mg, concentrated live extract 20 mg, id yeast 100 mg, sodium citrate 300 mg, ethanol 0.05 g) was processed by diluting an ampoule to 10 mL with distilled water. This stock solution was used to prepare several dilutions from 10% to 20% (w/v). The results obtained were quite consistent with the certified values, namely: 102.5 mg/tablet for Okal (certified content 100 mg/tablet, relative error 2.5%) and 507.9 mg/ampoule (certified content 500 mg/ampoule, relative error 1.6%). The results obtained were compared with those obtained

with a reference method [16], in which the determination of glycine was carried out by AAS using the same solid-phase reactor without a derivation reaction. The results obtained were: Okal (99.85 mg/tablet) and Actilevol (497.1 mg/ampoul).

Conclusions

The proposed FIA configuration uses a solid phase reactor that provides a dilute, reproducible stream of constant concentration that acts as a catalyst in a subsequent reaction. The product of such a reaction is used to indirectly obtain the concentration of the species released by the metal ion acting as the catalyst.

The proposed method allows monitoring procedures for organic compounds based on atomic absorption measurements (of the released metal ion) to be converted into molecular absorption procedures, which are much more commonplace in pharmaceutical analysis. In addition, the use of a catalytic procedure is a possible way to improve the detection limits and sensitivity of the method.

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