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Determination of *p*-hydroxyphenylglycine by reaction with *o*-phthalaldehyde using a flow-injection fluorimetric procedure

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Abstract

In the present study, new flow injection procedures for the determination of *p*-hydroxyphenylglycine using either photometric or fluorimetric detection are proposed. The methods are based on the reaction of the amino acid with *o*-phthalaldehyde and 2-mercaptoethanol. The calibration graphs based on peak area were linear in the ranges 20–300 ng ml^{-1} with fluorimetric detection and 5–60 μg ml^{-1} using the photometric mode. The detection limit calculated for the fluorimetric procedure was 10 ng ml^{-1}. The method was applied to the determination of the free *p*-hydroxyphenylglycine present in industrial pharmaceutical samples of a Dane salt. © 1997 Elsevier Science B.V.

Keywords: *p*-Hydroxyphenylglycine; *o*-Phthalaldehyde; Fluorescence; Flow injection

1. Introduction

Some time ago Dane et al. [1] reported that, in the presence of methanolic potassium hydroxide, amino acids react with β-dicarbonyl compounds to give the potassium salts of the corresponding azomethines, some of these salts being sufficiently stable to be suitable for use as an amino protective group in peptide synthesis. Consequently, these so-called Dane salts have been used in the synthesis of numerous antibiotics [2–8]. This is the case of the Dane salt obtained by reaction of *p*-hydroxyphenylglycine (HPG) with methyl or ethyl acetoacetate in isopropyl alcohol and alkaline medium, which is used in the pharmaceutical industry. A common problem in quality control laboratories is the quantification of the free amino acid present in the salt. The method generally used is based on the reaction between HPG and *o*-phthalaldehyde (OPA) and ethanethiol (ET), resulting in a compound which is detected spectrophotometrically at 340 nm after waiting for 20 min. Derivatization of natural amino acids with OPA is simple and has been extensively studied using amino acid analysers or liquid chromatography [9,10]. Other method for determination of HPG is based in a cupric ion-sensitive electrode [11].

In this study, new flow injection (FI) fluorimetric and photometric methods based on the reaction between HPG, OPA and 2-mercaptoethanol (ME) have been optimized. The fluorimetric detection procedure lowered the detection limit of

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the amino acid compared with the photometric detection mode. In addition, the thiol-containing reagent ME, which is used instead of ET, is less toxic and the reaction kinetics are faster, thus simplifying the experimental procedure. Because the development of the reaction product is a strongly time-dependent process, FI methodology is employed to automate the reaction, since it permits a controlled dispersion of the sample and, consequently, a reproducible analytical signal. The proposed method provides a less expensive and more simple procedure with considerably reduced analysis time when compared with liquid chromatographic methods, in addition being more selective and sensitive than the manual photometric detection method. Consequently, the procedure here described could be useful for the routine quantitative analysis of HPG in the pharmaceutical industry.

2. Experimental

2.1. Instrumentation

Kontron (Zurich, Switzerland) SFM 25 fluorescence detector set at wavelengths of 340/440 nm (excitation/emission) and a Pye Unicam (Cambridge, UK) PU8625 UV/visible spectrophotometric detector set at 340 nm were used. Recording and integration of the peaks were performed using a PC integration pack (Kontron). The flow injection system consisted of a Gilson (Worthington, OH) Minipuls HP4 peristaltic pump; an Omnifit (Cambridge, UK) injection valve; Hellma (Jamaica, NY) 176.052-QS (fluorimetric) or 178.012-QS (photometric) flow cells; 0.8 mm i.d. PTFE tubing and various end fittings and connectors (Omnifit). The temperature of the reactor coil was controlled by a laboratory-made electronic thermostat.

2.2. Reagents

All the solutions were prepared using chromatographic grade methanol (Romil, Loughborough, UK) since Dane salts are rapidly decomposed by the action of water, releasing the free amino acid HPG. A fluorogenic reagent containing 0.005 M *o*-phthalaldehyde (Fluka, Buchs, Switzerland), 0.005 M 2-mercaptoethanol (Sigma, St. Louis, MO) and 0.003 M sodium tetraborate (Merck) was prepared in methanol. This solution was kept in a dark bottle at 4°C. *p*-Hydroxyphenylglycine was kindly supplied by DSM-Deretil (Almería, Spain); a 500 μg ml^{-1} stock solution was also prepared in methanol and kept at 4°C; working standard solutions were prepared by dilution with methanol just before use. Dane salt samples ([*N*-(3-methoxy-1-methyl-3-oxo-1-propenyl)-D-(-)-α-amino-*p*-hydroxy-phenyl acetate potassium salt], Dan-oxi®) were also supplied by DSM-Deretil and solutions were prepared in methanol.

2.3. Procedure

A schematic diagram of the two channels flow manifold is shown in Fig. 1. The sample containing HPG (100 μl) was injected into a channel through which flowed methanol containing 3 mM tetraborate, and mixed with the fluorogenic reagent (containing OPA and ME) by means of a T-piece. The resulting solution flowed through a reactor coil (1.6 m × 0.8 mm i.d.) thermostated at 40°C and then passed into the flow cell for the fluorescence (340/440 nm) or absorbance (340 nm) to be recorded. Both streams were pumped at the same flow rate by a peristaltic pump with a total flow rate of 0.4 ml min^{-1}. Calibration graphs were obtained by plotting peak area against HPG concentration.

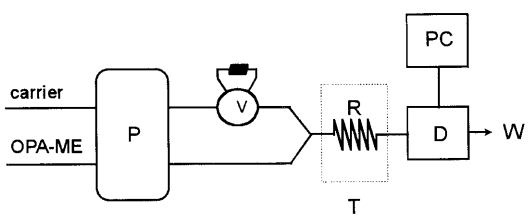

Fig. 1. FI manifold for the determination of *p*-hydroxyphenylglycine. (P) peristaltic pump, total flow-rate 0.4 ml min^{-1}; (V) injection valve, sample-loop 100 μl; (R) reactor coil, 1.6 m × 0.8 mm i.d.; (T) thermostat at 40°C; (D) fluorimeter, 340/440 nm (excitation/emission) or photometer, 340 nm; (PC) personal computer; (W) waste.

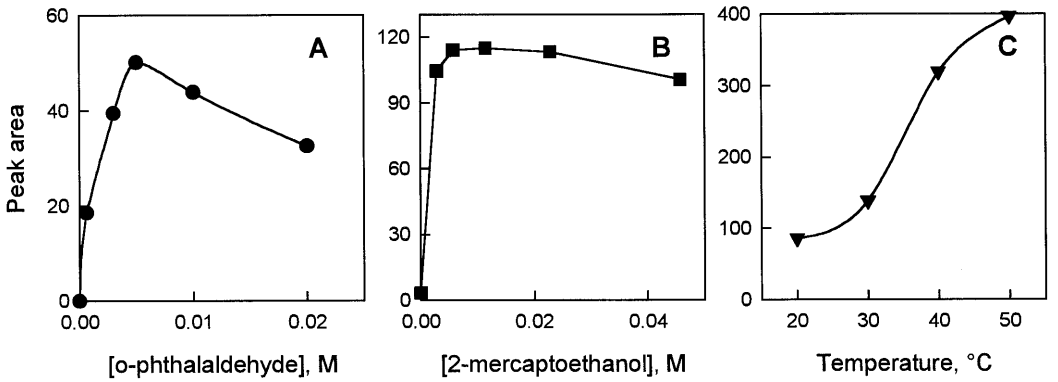

Fig. 2. Influence of the chemical variables using the fluorimetric procedure. A: variation of the OPA concentration. B: variation of the 2-ME concentration. C: influence of the temperature. HPG injected 0.3 μg ml^{-1}.

For determination of the free amino acid in industrial samples of a Dane salt (Dan-oxi®), 0.1 g of the powdered sample was dissolved in 25 ml methanol. An aliquot of 0.5 or 1.0 ml was subsequently diluted up to 10 ml with methanol.

3. Results and discussion

As indicated above, the manual method generally used in quality control laboratories for the determination of HPG in Dane salts is based on their reaction with OPA and a thiol-containing compound, usually ET. For routine control purposes, ET can be replaced by ME, which is less toxic, and the procedure can be automated by FI methodology. The high fluorescence quantum yield of the reaction product allows the sensitive fluorimetric detection mode to be used.

3.1. Optimization of the fluorimetric procedure

The experimental parameters were optimized to obtain maximum fluorescence, i.e. maximum sensitivity in the chemical analytical procedure. The reaction occurred in a basic medium containing 0.003 M sodium tetraborate dissolved in methanol. The influence of OPA concentration was studied between 6.3×10^{-4} and 0.02 M, while the concentration of ME was maintained at 0.01 M. Fluorescence rapidly increased up to 0.005 M (Fig. 2a) and then decreased at higher concentrations; hence, 0.005 M was chosen as optimal. When ME concentration was varied in the 2.9×10^{-3}–0.045 M range, concentrations above 0.01 M led to a decrease in fluorescence and a 0.005 M value was selected (Fig. 2b). An increase in temperature had a favourable effect on the reaction (Fig. 2c). However, a temperature of 40°C was selected because higher temperatures led to the formation of bubbles.

The influence of the FI variables is shown in Fig. 3. Increasing the total flow rate from 0.2 to 1.1 ml min^{-1} led to a decrease in fluorescence (Fig. 3a). Low flow rates improved fluorescence because the time during which the amino acid and the fluorogenic reagents reacted was greater. A 0.4 ml min^{-1} value (0.2 ml min^{-1} for each channel) was selected as a compromise between a good signal and an adequate sampling frequency. The influence of the reactor coil length was studied between 0.8 and 4.1 m. As expected (Fig. 3b), the fluorescence increased up to 1.5 m and remained constant up to 2.5 m, while greater lengths produced a decrease in fluorescence probably due to sample dispersion. An optimal value of 1.6 m was selected. The effect of the sample loop size was examined in the 30–300 μl range. Although fluorescence continuously increased (Fig. 3c), the peaks appeared distorted above 200 μl and, consequently, a 100 μl volume was selected.

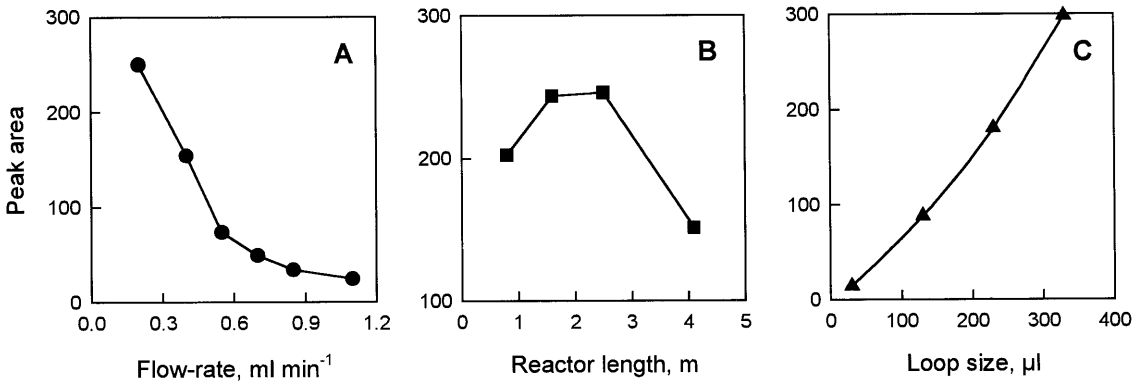

Fig. 3. Effect of FI variables using the fluorimetric procedure. A: flow-rate. B: reactor coil length. C: sample loop size. Experimental conditions: 5×10^{-3} M OPA, 5×10^{-3} M ME and 3×10^{-3} M sodium tetraborate in methanol; HPG injected 0.3 μg ml^{-1}.

3.2. Optimization of the photometric procedure

The experimental variables were again studied to obtain maximum sensitivity. As was to be expected, the concentration of OPA necessary to obtain suitable absorbance signals was higher than that needed for the fluorimetric procedure. Consequently, it was varied between 0.01 and 0.30 M, while the concentration of ME was maintained at 0.01 M. Absorbance increased up to 0.07 M (Fig. 4a) and this was chosen as optimal. The influence of ME concentration was studied between 2.9×10^{-3} and 0.035 M (Fig. 4b) and a 0.005 M value was selected. The temperature of 40°C and the FI variables were maintained at the values previously optimized for the fluorimetric procedure.

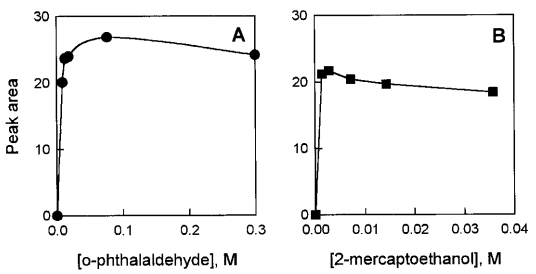

Fig. 4. Influence of the chemical variables using the photometric procedure. A: Variation of the OPA concentration. B: 2-ME concentration. HPG injected 15 μg ml^{-1}.

3.3. Calibration, interferences and applications

Calibration of HPG was carried out by plotting concentration against peak area for both detection modes. Linearity was obtained between 20 and 300 ng ml^{-1} using the FI-fluorimetric procedure and between 5 and 60 μg ml^{-1} using the FI-photometric method. The detection limits calculated on the basis of 3σ were 10 ng ml^{-1} and 3 μg ml^{-1}, respectively. The precision and accuracy of this method was demonstrated by repetitive analyses and the average R.S.D. calculated for ten replicate determinations of 0.1 μg ml^{-1} of HPG (fluorimetric procedure) was $\pm 4.1\%$.

Interferences caused by some common products used in the pharmaceutical industry were studied by injecting solutions containing HPG (0.2 μg ml^{-1}) and different amounts of these compounds. No interferences were found for lactose, glucose, saccharose, maltose, sorbitol or starch at [interferent]/[HPG] ratios up to 100/1. Higher concentrations were not assayed. The tolerance limit was taken as the concentration causing an error of no more than $\pm 3\%$ in HPG recovery.

To assess the reliability of the proposed method, several industrial samples of a Dane salt (Dan-oxi®) were analysed using the FI-fluorimetric procedure described here and the manual photometric method based on the use of ET. The resulting correlation graph (Fig. 5) shows that there were no significant differences between the two procedures.

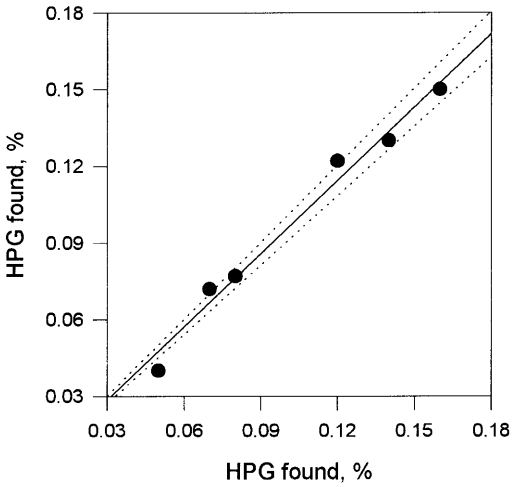

Fig. 5. Correlation graph for the determination of HPG in industrial samples of a Dane salt. Values on the abscissae axis were obtained by the manual photometric method and values on the ordinate axis were obtained by the FI-fluorimetric procedure. Solid line indicates the regression line. Broken lines indicate the confidence intervals at the 95% confidence level.

4. Conclusion

Automation of the reaction between *p*-hydroxyphenylglycine, *o*-phthalaldehyde and mercaptoethanol by flow injection methodology and the use of fluorimetric detection considerably improves the sensitivity and simplicity of the procedure. Analysis time is considerably reduced compared with that needed in manual methods and the thiol-containing reagent is less toxic than the ET commonly used. The procedure is useful for the routine quantitative analysis of the free amino acid in industrial samples of Dane salt. For those laboratories which do not have a fluorimeter available, the photometric method, although less sensitive, is also suitable for quality control purposes.

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