Fluorometric Determination of D-Penicillamine with 9-Fluorenylmethyl Pentafluorophenyl Carbonate

Seung-Yeon Byeon, Jung-Kap Choi and Gyurng-Soo Yoo
College of Pharmacy, Chonnam National University, Kwang-Ju, Korea
(Received May 25, 1990)

형광 유도체화제 9-풀루오레닐메틸 펜타풀루오로페닐 카르보네이트를 이용한 D-페니씰라민의 형광분광학적 분석

변승연 · 최중갑† · 유경수
전남대학교 약학대학
(1990년 5월 25일 접수)

A sensitive fluorometric method using 9-fluorenylmethyl pentafluorophenyl carbonate (FMPC) as the fluorescent labeling agent was developed to determine D-penicillamine (D-PA). The fluorophore had excitation and emission wavelengths of 260 nm and 313 nm, respectively. After derivatization, the fluorescent product was separated, and quantified by spectrofluorometry. The derivative was highly fluorescent and stable. Optimum condition for the reaction was investigated. A linear response was obtained over the range of 4.0×10^{-7}-5.0×10^{-6} M with the correlation coefficient of 0.999 (n = 6). The procedure described was successfully applied to the determination of the dosage forms of capsule with the recovery of 98.62 ± 0.57% (150 mg), 98.36 ± 0.57% (250 mg).

Keywords—D-penicillamine, FMPC, derivatization, fluorometry.

D-penicillamine (β,β-dimethylcysteine) (D-PA) is a hydrolytic product of penicillin with various modes of action. It is widely used for the treatment of Wilson's disease[1], heavy-metal poisoning[2] and rheumatoid arthritis[3]. Despite of its frequent use, the pharmacological effects of D-PA have not been sufficiently investigated. This is partly due to the lack of sensitive and selective methods of its analysis[4].

For the determination of D-PA, properties of its functional groups are used. Several analytical methods for D-PA have been developed such as colorimetry[5,6] and amino acid analyzer method[7,8]. However these methods have some limitations such as non-specificity, low sensitivity, complicated manipulations of the samples which render their application in the analysis of pharmacokinetic samples inappropriate. Fluorescence technology offers a number of distinct advantages to the pharmaceutical anlysis: specificity and ultra-sensitivity.

Since D-PA does not have native fluorescence, the detection of the drug requires to derivatize one of the functional groups of D-PA with a suitable labeling agent. Many reagents previously reported as being suitable for the derivatization of sulfhydryl-containing amino acids were investigated. These included o-phthalaldehyde[9], dinitro-fluorobenzene and dansyl aziridine[4]. However, derivati-

†Correspondence

zation of sulfhydryl group with each of these reagents requires weakly alkaline media for the reaction (pH 8.0-9.0), which is the condition known to catalyze D-PA disulfide formation[10].

In addition to sulfhydryl group, D-PA contains both a carboxyl group and an amino group. While many analytical methods using derivatization of the sulfhydryl group were developed, few method for amino group was investigated. 9-fluorenylmethyl chloroformate (FMOC-Cl), a reagent originally introduced as a blocking agents of amino group in peptide synthesis, has been employed for amino acid analysis[11-13]. However, it was also react with water to yield the corresponding fluorescent alcohol as a hydrolysis product[11].

Recently, another amino protecting agent, 9-fluorenylmethyl penta-fluorophenyl carbonate (FMPC) was introduced[14]. Therefore, the derivatized D-PA with FMPC may be useful for a analytical purpose. This paper reports the sensitive spectrofluorometric analysis of D-PA based on its derivative formation with FMPC.

EXPERIMENTAL

Instruments

Fluorescence measurements were made with a Perkin-Elmer LS-5 luminescence spectrometer with a 8.3 W Xenon lamp, Quantum counter (Rhodamine B) and 10 × 10 mm quartz cell. Excitation and emission wavelengths were set at 260 and 313 nm and the slit bandwidths were 15, 20 nm, respectively. The condition of instrument was standardized with the solution of 1.0×10^{-5} M quinine sulfate in 0.1 N sulfuric acid of which relative fluorescence intensity showed 240.0.

Perkin-Elmer Lamda 5 UV/VIS spectrophotometer was used for the absorbance determination. pH was measured with a Corning pH meter model 7 at 25 °C.

Reagents and Chemicals

FMPC and D-PA were purchased from Aldrich Co. (Milwaukee, WI, U.S.A.). Other chemicals and solvents were of reagent grade. Deionized-distilled water was used throughtout the procedure.

Preparation of Stock Solutions

Borate buffer was prepared from boric acid solution (1.0 M) adjusted with 2.0 N sodium hydroxide solution to pH 4.0-12.0. The reagent was prepared by dissolving 415 mg of FMPC in 100 m*l* acetonitrile to give a concentration of 1.0×10^{-2} M and stored at room temperature. Further dilutions with acetonitrile were made to obtain the desired concentrations. A D-PA stock solution was prepared each day in deionized-distilled water to give a final concentration of 1.0×10^{-3} M. The stock solution was further diluted with deionized-distilled water to given solutions containing 5.0×10^{-7} M-2.0×10^{-5} M of D-PA.

Assay Procedure

A 2 m*l* aliquot of the assay solution was transferred into a stoppered test tube and 0.5 m*l* borate buffer, 2 m*l* of the reagent and 0.5 m*l* of acetonitrile were added. The solubility of the reagent in distilled water was low, necessitating the presence of acetonitrile in the reaction mixture. The mixture was incubated in circulating water bath at 70 °C for 40 min. Upon cooling at the room temperature, the reaction mixture was extracted with 25 m*l* of diethyl ether to remove the excess reagent and by-product. The extraction was repeated once. The aqueous solution including the D-PA derivative was then separated from organic phase. The 1.5 m*l* of aqueous layer was diluted by two times and the fluorescence intensity was measured. The blank test was performed following the proposed method.

Calibration Curve

The stock solution of D-PA was diluted stepwise with deionized-distilled water to give a series of concentrations suitable for the construction of the calibration curve in the range 5.0×10^{-7}-2.0×10^{-5} M of D-PA. 2 ml of each solution was used for the derivative formation with FMPC as described in the assay procedure. A calibration curve was obtained by plotting fluorescence intensity *vs.* D-PA concentration.

Determination of D-PA in Dosage Forms

The method was applied to the determination of

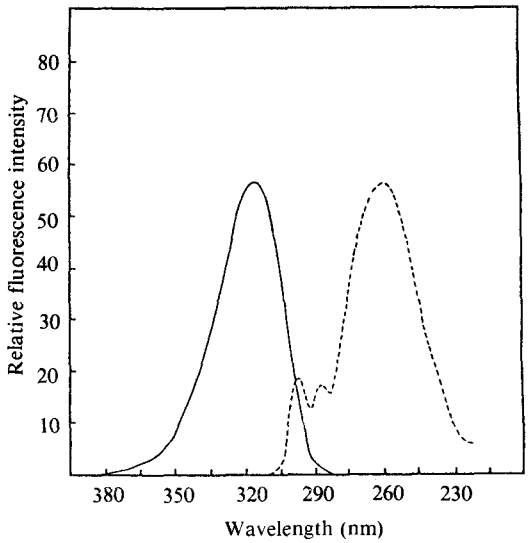


Figure 1—Fluorescence excitation and emission spectra of the derivative (—: emission, ---: excitation).

D-PA in two commercial preparations. Contents of twenty capsules were emptied and bulked. After the average weight per capsule was calculated, a quantity equivalent to 10 mg of D-PA was accurately weighed and extracted in deionized-distilled water of 100 m*l*. The solution was filtered through filter paper and diluted by 100 times with deionized-distilled water to give solution containing 6.7×10^{-6} M of D-PA. The filtrated solution was analyzed according to the proposed assay method.

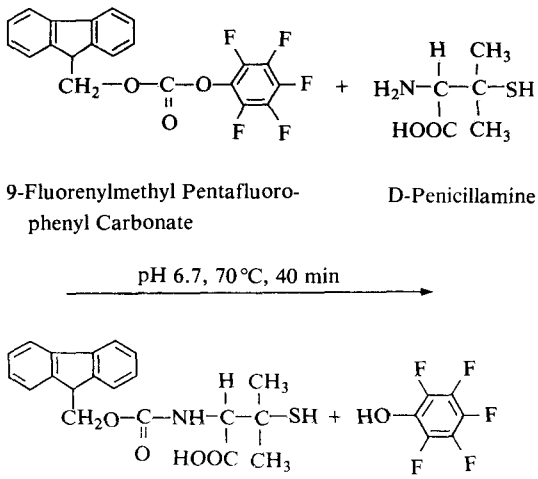
RESULTS AND DISCUSSION

Fluorescence Excitation and Emission Spectra of the Derivative

The fluorescence excitation and emission spectra of the reaction product of D-PA with FMPC were identical with those of FMPC (Fig. 1). The wavelengths of maximum fluorescence excitation and emission of reaction product were 260 nm and 313 nm, respectively. The labelled D-PA had fluorescence properties compatible with fluoren moiety.

Exected Reaction Product

Based on the fact that FMPC is a protective reagent of the amino group for peptide synthesis and



Scheme 1—Expected reaction product.

various 9-fluorenylmethyloxycarbonyl amino acids are synthesized from FMPC and amino acids through the peptide bond formation[15], it is expected the amine substituent of D-PA reacts with FMPC to form 9-fluorenylmethyl pentafluorophenyl penicillamine (Scheme 1).

Assay Conditions—1) Effect of pH

The effect of PH on the reaction of D-PA with FMPC was tested in the range of 4.0-12.0 using various buffers. The reaction was dependent on pH. As shown Fig. 2, a constant and maximum fluorescence intensity was obtained in the range of pH 6.5-7.0 using the borate buffer. Since the reaction of FMPC with D-PA proceeds under mildly acidic pH, the use of this reagent offers better condition for the derivatization of the D-PA than the other reagents such as OPA, NBD chloride, dansyl aziridine which require alkaline reaction media (pH 8.0-9.0).

2) Effect of Reaction Time and Temperature

The relationship between fluorescence itensity and the reaction time and temperature was studied. The reaction was carred out at 60 °C, 70 °C and room temperature. The reaction was not occur at room temperature and increased with the increase of the temperature. As shown in Fig. 3, the fluorescence intensity was maintained maximal after 30

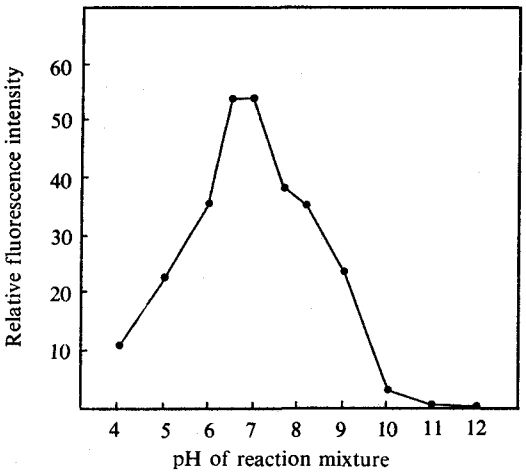


Figure 2—Effect of pH on the derivatization reaction of D-PA with FMPC.

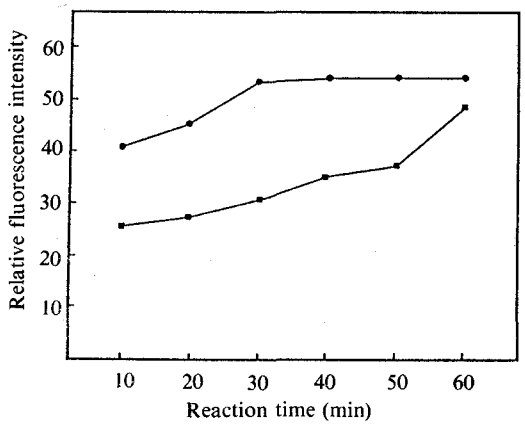


Figure 3—Effect of time and temperature on the derivatization reaction of D-PA with FMPC (●: 70°C, ■: 60°C).

min at 70° for 40 min.

3) Reaction Molar Ratio between FMPC and D-PA

The effect of the molar ratio of FMPC to D-PA was examined by varying the amount of FMPC to D-PA. As shown in Fig. 4, when FMPC was present more than 300 fold, a constant and maximum fluorescence intensity was obtained. S/N ratio was almost fixed according to increasing concentration of FMPC. Therefore, 300 fold excess of FMPC was used for the reaction.

4) Selection of Solvent for Separation

The separation of the derivative with different

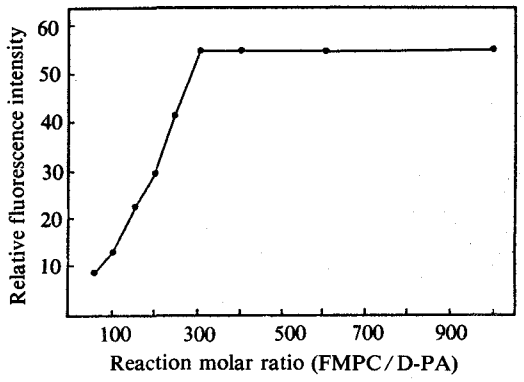


Figure 4—Effect of molar ratio on the derivatization reaction of D-PA with FMPC.

solvents was studied. Among several solvents, diethyl ether was selected since it removed the excess reagent and the hydrolyzed by product efficiently. Nonpolar solvent such as pentane did not work properly.

5) Stability of D-PA Solution and Derivative

Since thiols are readily oxidized[16], the stability of D-PA in solutions was studied by Rabenstein[17]. In that experiment, while D-PA in disodium EDTA was stable for 20 days. D-PA in distilled water was stable for 2 days at room temperature. Therefore D-PA solution was prepared using deionized disilled water daily. The derivative was stored at room temperature testing the stability of product at the regular interval up to 12 hours. The fluorescence obtained under the proposed derivatization conditions was stable for at least 12 hrs at room temperature (Fig. 5).

6) Calibration Curve and Precision

The calibration curve was made following the standard assay procedure. A linear relationship between the concentration of D-PA and the fluorescence intensity was obtained in the range of 4.0×10^{-7}-5.0×10^{-6} M in the final assay solution (Fig. 6). The precision of the proposed method was determined by analyzing six replicate samples, each containing 30.0×10^{-7} M in the final test solution. At this concentration level, the relative standard deviation was 0.40%. Linear regression analysis of fluorescence intensity *vs.* D-PA concentration gave a slope of 1.3395, an intercept of 0.2830, and a corre-

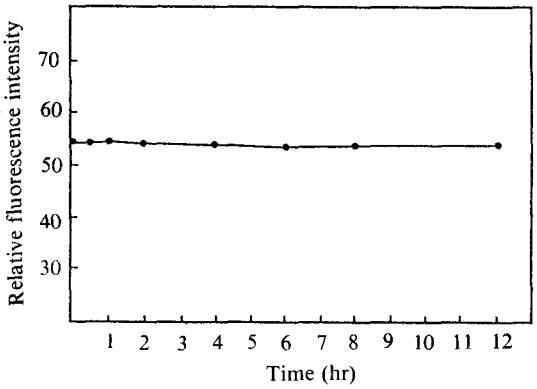


Figure 5—Stability of the derivative formed by the reaction of D-PA with FMPC.

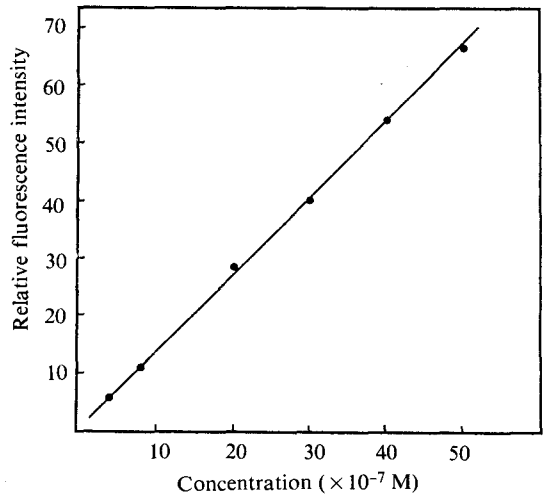


Figure 6—Calibration curve of D-PA standard.

lation coefficient, r, of 0.999 (n = 6) (Table 1).

Application to Dosage Forms

The suggested method was applied to the quantitative determination of D-PA in capsules (Table 2). The concentration of D-PA was calculated using slope and intercept constants derived from linear regression analysis of the calibration curve data, [Concn. = (R F I-Intercept)/Slope]. The results obtained indicate that the method is suitable for routine quality control analysis. The recent USP determination consists of a colorimetric titration with mercuric acetate[18]. The method either requires complex sample preparation or uses the highly toxic mercuric acetate which presents hazard to the analyst and the environment[19]. Therefore the proposed method has added advantages of sensitivity, simplicity and safety over the recent USP method.

Table I—*Estimated linearity of D-PA derivative by spectrofluorometry.*

Theoretical Concn. ($\times 10^{-7}$ M)	R F I*	Calculated Concn. ($\times 10^{-7}$ M)	Relative Error (%)
4.0	5.7	4.04	1.00
8.0	10.7	7.78	-2.75
20.0	27.5	20.32	1.60
30.0	40.1	29.73	-0.90
40.0	54.3	40.33	0.83
50.0	67.0	49.81	0.38
r	0.999 (n = 6)		
intercept	0.2830		
slope	1.3395		

* RFI: Relative Fluorescence Intensity.

Table II—*The results of fluorometric analysis of D-PA in capsules.*

Dosage form (Capsuke)	Theoretical ($\times 10^{-7}$ N)	Found* ($\times 10^{-7}$)	Recovery (%)	RSD (%)
150 mg	26.81	26.44 ± 0.15	98.62	0.57
250 mg	26.81	26.37 ± 0.15	98.36	0.57

* Mean ± SD based on triplicate determinations of each sample.

ACKNOWLEDGEMENT

This work was supported by a grant from Korea Health Foundation.

REFERNECES

1) J.M. Walshe, Penicillamine, a new oral therapy for Wilson's disease, *Am. J. Med.*, **21**, 487 (1956).
2) J.E. Boulding, R.A. Baker, The treatment of metal poisoning with penicillamine, *Lancet*, **2**, 985 (1985).
3) A.H. Vapaatalo, I.B. Linden and J. Paran-

tainen, in O. Lovgren (Ed.), New aspects of rheumatoid inflammation, Astra-Syntex Scandinavia AB, Sodertalje, 180 (1980).

4) E.P. Lankmayr, K.W. Budna and K. Muller, F. Nachtmann and F. Rainer, Determination of D-penicillamine in serum by fluorescence derivatization and liquid column chromatography, *J. Chromatogr.*, **222**, 249 (1981).
5) J. Mann and P.D. Mitchell, A simple colorimetric method for the estimation of D-(-)-penicillamine in plasma, *J. Pharm. Pharmacol.*, **31**, 420 (1979).
6) P.J. Vollmer, J. Lee and T.G. Alexander, Comparison of methods for determining penicillamine in bulk drug and formulations, *J. Assoc. Off. Anal. Chem.*, **63**, 1191 (1980).
7) B. Fowler and A.J. Robins, Methods for the quantitative analysis of sulphur-containing compounds in physiological fluids, *J. Chromatogr.*, **72**, 105 (1972).
8) A.O. Muijsers, R.J. van de Stadt, A.M.A. Henrichs and J.K. van der Korst, Determination of D-penicillamine in serum and urine of patients with rheumatoid arthritis, *Clin. Chim. Acta*, **94**, 173 (1979).
9) H. Nakamura and Z. Tamura, Fluorometric determination of thils by liquid chromatography with postcolumn derivatization, *Anal. Chem.*, **53**, 2190 (1981).
10) J.O. Miners, I. Fearnley, K.J. Smith and D.J. Birkett, Analysis of D-penicillamine in plasma by fluorescence derivatization with N-[*p*-(2-benzoxazolyl)-phenyl] maleimide and HPLC, *J. Chromatogr.*, **275**, 89 (1983).
11) S. Einarsson, B. Josefsson and S. Lagertvist, Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography, *J. Chromatogr.*, **282**, 609 (1983).
12) S. Einarsson, Selective determination of secondary amino acids using precolumn derivatization with 9-fluorenylmethyl chloroformate and reversed-phase HPLC, *J. Chromatogr.*, **348**, 213 (1985).
13) S. Einarsson, S. Folestad, B. Josefsson and S. Lagerkvist, High-resolution reversed-phase liquid chromatography system for the analysis of complex solutions of primary and secondary amino acids, *Anal. Chem.*, **58**, 1638 (1986).
14) Istvan Schon, Lajos Kisfaludy, 9-Fluorenylmethyl pentafluorophenyl carbonate as a useful reagent for the preparation of N-9-fluorenylmethyloxycarbonyl amino acids and their pentafluorophenyl esters, *Synthesis*, 303 (1986).
15) P.C. Jocelyn, "Biochemistry of the SH group", Academic Press, New York, N.Y., 121 (1972).
16) P.C. Jocelyn, "Biochemistry of the SH group", Academic Press, New York, N.Y., 94 (1972).
17) Rolf Saetre and D.L. Rabenstein, Determination of penicillamine in blood and urine by HPLC, *Anal. Chem.*, **50**, 276 (1978).
18) The United States Pharmacopeia, Penicillamine, Mack Publishing Co., Easton, PA, 21th rev., 791 (1980).