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High performance liquid chromatographic determination of folic acid and its photodegradation products in the presence of riboflavin

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Abstract

A high performance liquid chromatographic procedure was developed to determine folic acid and its photodegradation products, *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid, and pteroic acid in the presence of riboflavin. The method involves reversed phase, paired-ion chromatography on μ-BondaPak C_{18} column using a UV detector (254 nm), and isocratic solvent system (at ambient temperature) comprising 0.017 M monobasic potassium phosphate, tetrabutyl ammonium hydroxide solution (20%, aqueous) and methanol (870:15:250, v/v). The range of quantitation for the individual compounds was found to be: *p*-aminobenzoic acid, $0.01-1.25 \times 10^{-5}$ M; pterine-6-carboxylic acid, $0.01-2.0 \times 10^{-5}$ M; *p*-aminobenzoyl-L-glutamic acid, $0.02-2.0 \times 10^{-5}$ M; pteroic acid, $0.02-2.5 \times 10^{-5}$ M; folic acid, $1.0-5.0 \times 10^{-5}$ M; riboflavin, $1.0-5.0 \times 10^{-5}$ M. Linear regression analysis of the data demonstrates adequate performance of the method in terms of accuracy and precision (R.S.D. 3%). The method is specific, rapid and convenient and has been applied to photodegradation studies of folic acid in the presence and absence of riboflavin. 1997 Published by Elsevier Science B.V.

Keywords: *p*-Aminobenzoic acid; *p*-Aminobenzoyl-L-glutamic acid; Folic acid; Pterine-6-carboxylic acid; Pteroic acid; Riboflavin; Reversed-phase liquid chromatography; Isocratic elution

1. Introduction

Folic acid is formulated with other B-vitamins including riboflavin in various dosage forms. These may be affected by light leading to the formation of several photoproducts. A number of high performance liquid chromatographic (HPLC) methods have been reported for the assay of folic acid [1–5] and its decomposition products or related compounds [6–9].

The identification and determination of folic acid and reduced folates has been performed by HPLC using isocratic elution with UV-detection [6,7]. A HPLC method for the separation and quantitation of the naturally occurring monoglutamate derivatives of folic acid, as well as several decomposition products of reduced folates has been developed that employs reversed-phase, ion-

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Table 1
Regression analysis of the calibration data

Compound	Range ($M \times 10^5$)	Slope ± S.D. (min)	Intercept (± S.D.)	Correlation coefficient
p-Aminobenzoic acid	0.01–1.25	2030.80 ± 99.02	−91.46 ± 82.10	0.9996
Pterine-6-carboxylic acid	0.01–1.25	8183.32 ± 374.61	+56.49 ± 426.49	0.9985
p-Aminobenzoyl-L-glutamic acid	0.02–2.00	5030.54 ± 390.42	−164.32 ± 485.92	0.9997
Pteroic acid	0.02–2.50	4664.20 ± 1043.79	+357.501 ± 1730.93	0.9926
Riboflavin	1.00–5.00	5792.80 ± 431.54	+198.75 ± 1281.05	0.9995
Folic acid	1.00–5.00	1821.30 ± 229.44	+151.60 ± 760.99	0.9973

pair chromatography on a C_{18} column eluted with a non-linear gradient of ethanol and detection at 280 nm [8]. A HPLC procedure based on post-column derivatization with fluorometric detection has also been reported for the determination of folacins and some degradation products [9].

In the present investigation, a HPLC method has been developed for the simultaneous determination of folic acid and its various photoproducts, *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid and pteroic acid in the presence of riboflavin, with a very simple chromatographic system. Riboflavin is known to sensitize the photodegradation of folic acid resulting in the deactivation of the vitamin [10,11]. The details of the method, its validation and applications are being reported.

2. Experimental

2.1. Materials

Potassium dihydrogen phosphate, methanol and tetrabutyl ammonium hydroxide (20%, aqueous) were obtained from Merck. Reference standards were obtained from Sigma.

2.2. HPLC method

The chromatograph used in this study consisted of a LC-10AS pump (Shimadzu, Japan), an SIL-9A injector, a SPD-10A detector and an C-R4A integrator. μ-Bonda Pak C_{18} 300 mm × 3.9 mm i.d. columns, protected by an ODS (octadecyl silane) pre-column were used.

A degassed and filtered mixture of 0.017 M potassium dihydrogen phosphate, tetrabutyl ammonium hydroxide (20%, aqueous) and methanol (870:15:250, v/v) was used as eluent. The flow rate was maintained at 1.0 ml min^{-1}. Detection was performed at 254 nm. Chromatographic time for each analysis was 30 min. All separations were carried out at ambient temperature.

2.3. Calibration curves

Calibration standards were prepared by dissolving the reference standards in citrate-phosphate buffer, pH 7.0, in the range: *p*-aminobenzoic acid, $0.01–1.25 \times 10^{-5}$ M; pterine-6-carboxylic acid, $0.01–2.0 \times 10^{-5}$ M; *p*-aminobenzoyl-L-glutamic acid, $0.02–2.0 \times 10^{-5}$ M; pteroic acid, $0.02–2.5 \times 10^{-5}$ M; folic acid, $1.0–5.0 \times 10^{-5}$ M and riboflavin, $1.0–5.0 \times 10^{-5}$ M. Three replicates were used for each concentration employed.

Calibration curves were constructed by plotting the peak height against concentration of each compound using linear regression analysis. Unknown concentrations of each compound were quantified by relating the respective peak heights to the regression line.

2.4. Observed retention times

The observed retention times of the reference compounds were found to be 4.90, 5.60, 9.00, 11.80, 15.10 and 27.50 min for *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid, pteroic acid, riboflavin and folic acid, respectively.

Table 2
Simultaneous determination of *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid, pteroic acid, riboflavin and folic acid by the proposed HPLC method

Compound	Amount added ($\times 10^{-5}$ M)	Amount found ($\times 10^{-5}$ M)	Recovery (%)
p-Aminobenzoic acid	0.0102	0.0098	96.07
	0.0204	0.0206	100.98
	0.0408	0.0410	100.49
	0.2040	0.2051	100.54
	0.4005	0.4065	101.50
	0.8010	0.7650	95.51
	1.0012	0.9540	95.28
Mean (± R.S.D.)			98.62 (± 2.839)
Pterine-6-carboxylic acid	0.0095	0.0098	103.15
	0.0190	0.0195	102.63
	0.0380	0.0392	103.15
	0.1900	0.1961	103.21
	0.4828	0.4806	99.54
	0.9656	0.9926	102.80
	1.9312	1.9860	102.83
Mean (± R.S.D.)			102.47 (± 1.311)
p-Amino-benzoyl-L-glutamic acid	0.0204	0.0210	102.94
	0.0408	0.0411	100.73
	0.2040	0.2035	99.75
	0.4693	0.4679	99.70
	0.9386	0.9508	101.29
	1.8772	1.9002	101.22
Mean (± R.S.D.)			100.93 (± 1.199)
Pteroic acid	0.0200	0.0209	104.50
	0.0400	0.0391	97.75
	0.2000	0.2011	100.55
	0.4828	0.4754	98.47
	0.9614	0.9216	95.86
	1.9228	1.9740	102.66
Mean (± R.S.D.)			99.96 (± 3.23)
Riboflavin	0.9829	0.9983	101.57
	1.9658	2.0270	103.15
	4.9000	4.8640	99.26
Mean (± R.S.D.)			101.32 (± 1.956)
Folic acid	0.9968	1.0250	102.83
	1.9936	2.0108	100.86
	4.9840	5.0578	101.48
Mean(± R.S.D.)			101.72 (± 1.0072)

3. Results and discussion

Individual standard solutions of each compound including folic acid, riboflavin, *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid and pteroic acid were examined by HPLC method to assess the UV response. In each set of standard solutions, a linear relationship between peak height and concentration was observed in the specified range.

Regression analysis of the data ($n = 3$) for each compound are reported in Table 1. The validity of the listed regression data was confirmed by the assay of an authentic mixture containing known

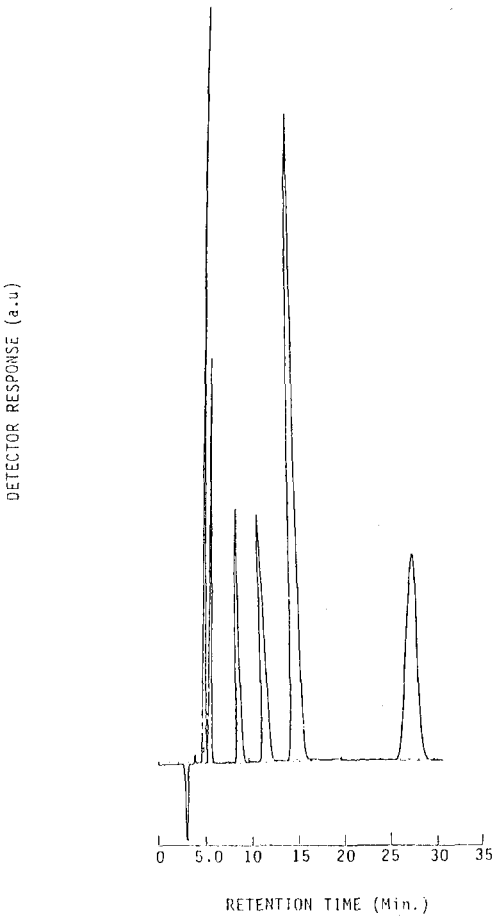

Fig. 1. Chromatogram of a mixture of authentic standard solution containing 2.5×10^{-5} M, each of *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid and pteroic acid and 5×10^{-5} M each of riboflavin and folic acid.

quantities of the individual compounds. The results showed good accuracy, as revealed by the percentage recovery (Table 2).

Under the proposed HPLC conditions, chromatographic time for each analysis is 30 min. A typical chromatogram of a mixture of standard solution containing 2.5×10^{-5} M *p*-aminobenzoic acid, pterine-6-carboxylic acid, *p*-aminobenzoyl-L-glutamic acid and pteroic acid and 5×10^{-5} M folic acid and riboflavin is shown in Fig. 1.

The use of tetrabutyl ammonium hydroxide as a pairing ion has been found to provide the desirable separation and acceptable chromatographic times. Some variation of methanol content or mobile phase pH may be required to improve resolution due to slight deterioration of the chromatographic column being used. However, chromatographic conditions described above provide the best resolution for all the six compounds in a single analysis. The minimum column efficiency required to carry out this method has been found to be 6000 plates. After use, the column is washed with 30 ml each of water, water:methanol (50%, v/v) and methanol to avoid any significant deterioration in column performance.

In order to test the validity of the method in the assay of the components of a photolysed solution of folic acid in the presence and absence of riboflavin, the method has been applied to a reaction carried out at pH 6.5 and 9.0. The results of the assay of folic acid and its photoproducts

Table 3
Assay of folic acid and photoproducts ($M \times 10^5$) at pH 9

Time (h)	Folic acid	*p*-Aminobenzoyl-L-glutamic acid	Pterine-6-carboxylic acid	Total[a]
0	4.984	—	—	4.984
1	4.961	0.053	0.017	5.030
2	4.920	0.068	0.021	5.008
3	4.878	0.083	0.023	4.984
4	4.820	0.108	0.028	4.955
5	4.734	0.135	0.036	4.905
6	4.664	0.162	0.042	4.868
7	4.581	0.196	0.052	4.829
8	4.467	0.237	0.062	4.766

[a]Total moles equivalent to folic acid.

Table 4
assay of folic acid and photoproducts ($M \times 10^5$) in presence of riboflavin (2×10^{-5}) at pH 6.5

Time (mins)	Folic acid	*p*-Aminobenzoyl-L-glutamic acid	Pterine-6-carboxylic acid	Total[a]
0	4.500	—	—	4.500
2	0.429	0.195	0.042	4.530
4	4.132	0.299	0.071	4.502
6	3.935	0.416	0.010	4.452
8	3.713	0.555	0.143	4.410
10	3.488	0.685	0.183	4.356

[a]Total moles equivalent to folic acid.

determined at various time intervals are given in Table 3 and Table 4. The uniformly increasing values of *p*-aminobenzoyl-L-glutamic acid and pterine-6-carboxylic acid and an almost constant molar balance, with time, indicate that the method has a good reproduciblity and can be conveniently applied to the assay of photolysed solutions. The values of the molar balance are in good agreement with the initial concentration of folic acid. However, a slight decrease in molar balance at 5 h (folic acid solution) and at 6 min (folic acid and riboflavin solution) may be due to the presence of some minor products (~ 5%) which could not be accounted for in the assay. The other two products of folic acid, i.e., *p*-aminobenzoic acid (a minor hydrolytic degradation products) [12] and pteroic acid (a bacterial degradation product) [13] may also be analysed by the proposed method.

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