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SPECTROPHOTOMETRIC DETERMINATION OF PYRIMIDINE NUCLEOSIDES WITH PHLOROGLUCINOL

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ABSTRACT

A new absorptiometric procedure for the determination of pyrimidine nucleosides in pharmaceutical preparations is presented, based on the reaction of pyrimidine nucleosides with a mineral acid-containing phloroglucinol solution. Absorbance readings at 435-450 nm obey Beer's law within the concentration range from 40 to 140 $\mu g \cdot mL^{-1}$ (2'-deoxythymidine). The method is operationally simple, and precise. A chemical mechanism is proposed.

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INTRODUCTION

Most of the known chemical methods for the absorptiometric determination of pyrimidine nucleosides are based on chromogenic reactions which depend on the chemical properties of the carbohydrate fragment of the molecule (1-3).

In this publication, a new absorptiometric method for the determination of pyrimidine nucleosides is presented. The procedure, which is sensitive and operationally simple since it does not require any chemical treatment prior to the chromogenic reaction, is based on heating the sample compound with a solution of phloroglucinol in mineral acid at 100 °C. The furfuraldehyde produced by the acid hydrolisis of the pyrimidine nucleosides condenses directly with phloroglucinol, yielding a polycyclic dibenzopiran chromophore.

EXPERIMENTAL

Reagents

All reagents (analytical grade) used as solvents and in the preparation of the solutions were obtained from E. Merck A.G. (Darmstad, West Germany). The pyrimidine nucleosides used (uridine, cytidine, and 2'-deoxythymidine) were also from E. Merck.

Reagent Solutions

Two reagent solutions (A, and B) were used. The reagent solution A contains 0.50 % phloroglucinol in aproximately 5.25 M hydrochloric acid, and is prepared by mixing

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concentrated hydrochloric acid (35 mL), and an ethanolic 5 % phloroglucinol dihydrate solution (10 mL). The reagent solution B contains 0.30 % phloroglucinol in ca. 1.85 M hydrochloric acid, and is prepared by mixing concentrated hydrochloric acid (20 mL), glacial acetic acid (100 mL), and an ethanolic 5 % phloroglucinol dihydrate solution (10 mL). These solutions (A, and B) should only be used when freshly prepared.

Equipment

Beckman (ACTA III) double-beam spectrophotometer with 1.0 cm fused silica cells.

Electrically heated water bath, thermostated to ± 0.1 °C.

Method

An aqueous solution (2.0 mL) of the pyrimidine nucleoside is transferred to a 10 mL volumetric flask; reagent solution A (5.0 mL) is added and the contents are well mixed. The flask, well stoppered, is maintained in a boiling water bath for exactly the recommended time (Table I). It is then inmersed for one minute in an ice-water bath to stop the reaction and to bring the contents to room temperature. Finally it is made up to volume with unheated reagent solution A. The absorbance of this solution is measured at the wavelength of maximum absorption (Table I) in a 1.0 cm fused silica cell, against a reference sample obtained by subjecting distilled water (2.0 mL) to the same procedure.

A constant heating time of 70 minutes is the only difference in the method when reagent solution B is used.

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RESULTS AND DISCUSSION

The chromogenic reaction with phloroglucinol is only possible with compounds containing a furan-like group, since this is critical for the condensation step of the procedure. The oxygen heteroatom of these compounds is linked to carbon atoms which, in most cases, are bonded to an hydroxy group in order to allow the dehydration step.

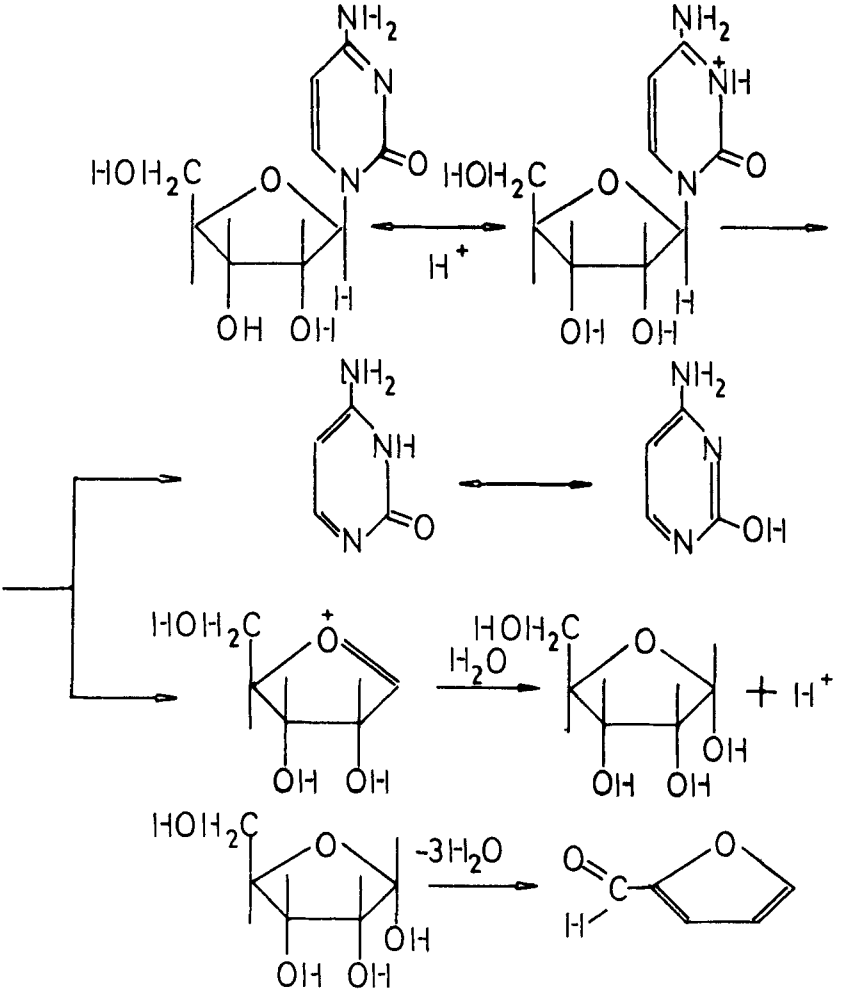

Fig. 1

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The mechanism for this reaction with the pyrimidine nucleosides follows a complex path that includes an initial acid hydrolisis of the structure with a subsequent multistep dehydration of the resultant ribose, or deoxyribose, giving rise to furfuraldehyde (Fig. 1), and terminates with the condensation of this compound with phloroglucinol to yield a dibenzopyrane derivative, as has been described (4). This last step is facilitated by the absolute symmetry in the phloroglucinol molecule, and the resultant high activating effect of its non-substituted carbon atoms.

Analysis of Uridine, Cytidine, and 2′-Deoxythymidine

Aqueous uridine, cytidine, and 2′-deoxythymidine solutions processed in this method develop an intense colour, which is strongly dependent on the duration of the heating period (Fig. 2). The corresponding spectra show two absorption bands, with maxima at the wavelengths recorded in Table I for each nucleoside.

TABLE I

ANALYTICAL CONDITIONS AND APPLICABILITY RANGE

Nucleoside	Heating time (min)	Wave length (nm)	Beer's interval ($M \cdot 10^{-5}$)	Ringbom's interval ($\mu g \cdot mL^{-1}$)
Uridine	40	450	8.9–82.2	36.2–100.4
Uridine	20	558	8.9–82.2	74.2–176.0
Cytidine	50	435	8.5–116.9	122.4–252.0
2′-Deoxythymid.	50	445	1.2–15.3	7.8–27.7
2′-Deoxythymid.	50	558	32.9–970.1	251.6–1815.0

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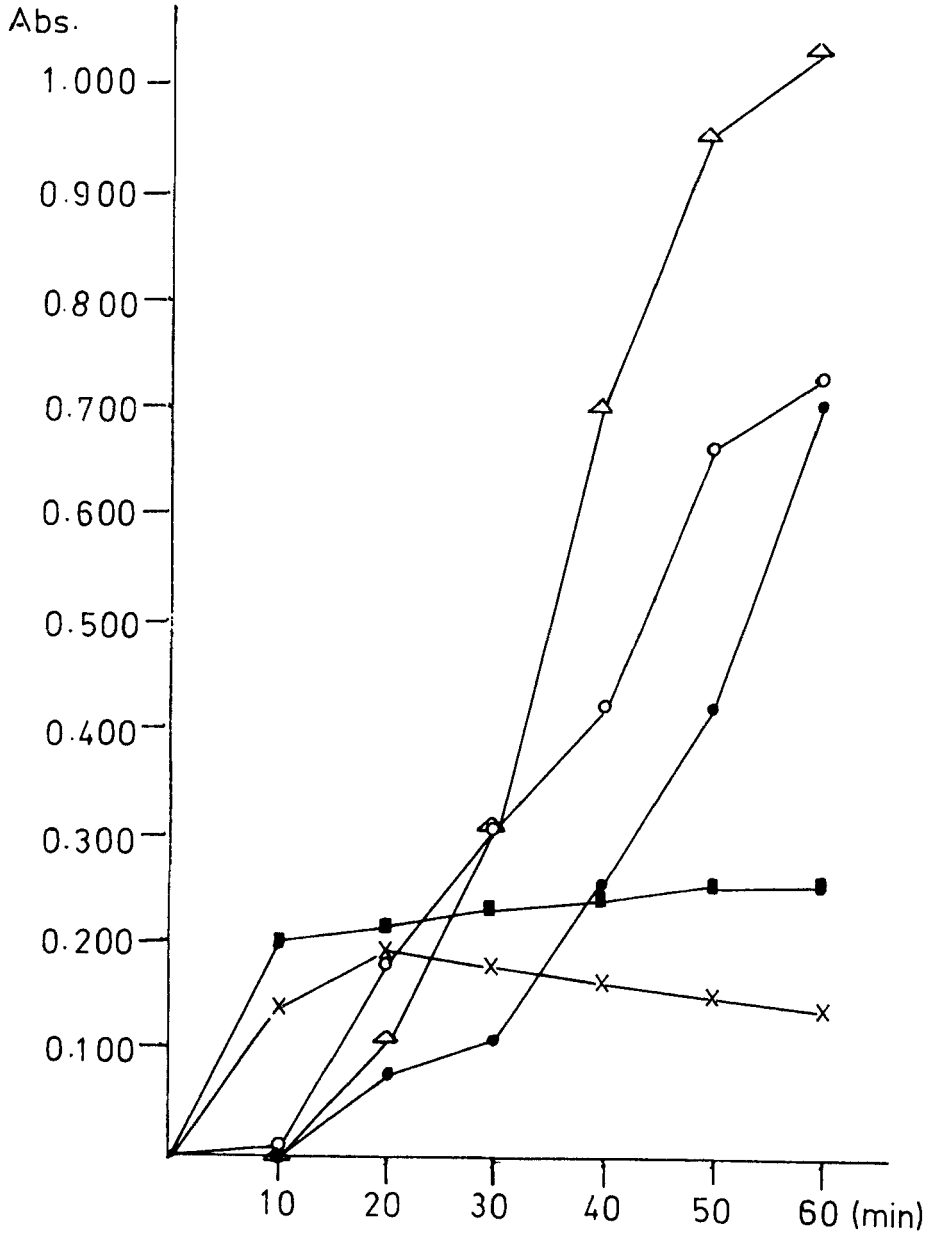

Fig. 2

Effect of heating time

Uridine at 445 nm (△), uridine at 558 nm (×), cytidine at 435 nm (●), 2′-deoxythymidine at 445 nm (○), and 2′-deoxythymidine at 558 nm (▮).

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Several series of standard solutions containing uridine, cytidine, or 2'-deoxythymidine within the concentration ranges shown in Table I, were used to check adherence to Beer's law, at the wavelengths of maximum absorption. The concentration ranges of maximum accuracy were obtained from Ringbom's graphs (5) in accordance with standard practice.

Good agreement was found in all cases over the studied ranges. When analyzed by a least-squares procedure, the data fitted straight lines whose parameters are given in Table II, with very good correlation coefficients (signifiant to a $P = 0.001$ level [6]).

TABLE II

CALIBRATION DATA

Nucleoside	Wave length (nm)	Number of results	Correl. Coef.	Slope ($\Delta A/\Delta M$)	Intercept (A)
Uridine	450	10	0.998	1920	−0.107
Uridine	558	10	0.993	399	0.070
Cytidine	435	9	0.996	375	0.286
2'-Deoxythy.	445	11	0.995	763	−0.023
2'-Deoxythy.	558	14	0.997	790	0.140

The coefficient of variation of the analytical results was in all cases less than 1.75 % (Table III) and shows the suitability of the method for routine replicate analysis.

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TABLE III

REPLICATE ANALYSIS DATA

Nucleoside	Wave length (nm)	Number of results	Nominal ($mg \cdot mL^{-1}$)	Found ($mg \cdot mL^{-1}$)	Standard Deviation ($mg \cdot mL^{-1}$)	Coef. of Variation (%)
Uridine	450	9	0.190	0.186-0.190	0.0016	0.830
Uridine	558	8	0.190	0.182-0.190	0.0030	1.600
Cytidine	435	7	0.400	0.394-0.410	0.0031	0.770
2'-Deoxythy.	445	10	0.090	0.089-0.090	0.0003	0.349
2'-Deoxythy.	558	12	0.090	0.087-0.093	0.0015	1.730

Selectivity of the reaction

In a previous study (4), the pyrimidinic nucleosides were shown to be very resistant to the colour-forming reaction with the low acidity phloroglucinol reagent solution, that enabled the quantitative reaction with purinic nucleosides. This different behaviour was explained in terms of the conformation of the nucleic base relative to the carbohydrate, that can be described by the torsion angle C_5-N-C'_1-O'_1 (lover for pyridimide nucleosides [7-11]), and assuming that the freedom of rotation about the glycosidic bond is closely related to the puckering in the furanose ring (12).

The lack of reaction of cytidine (lovest angle C_5-N-C'_1-O'_1) even with the moderately acidic phloroglucinol reagent solution B, with which uridine normally reacts, confirms the previous statement. This behaviour shows the greater

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strength of the cytosine-ribose bond versus the uracil-ribose one, where the electronic desplacement towards the nucleic base is higher due to the mesomeric effect of its two carbonyl groups.

At the proposed high acidity level for the reagent solution A, purinic nucleosides strongly interfere with the colorimetric reaction.

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