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SYNTHESIS OF PHOSPHORAMIDATE DERIVATIVES OF 5-FLUROURACIL AS A POSSIBLE PRODRUGS FOR TARGETING CANCER TISSUE

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Abstract

5-Fluorouracil (5-FU) is used widely as an anticancer drug to treat solid cancers, such as colon, breast, rectal, and pancreatic cancers; although it's clinical application is limited because 5-FU has gastrointestinal and hematological toxicity. An approach to improve the cancer cell selective properties of 5-fluorouracil is the chemical transformation into reversible derivatives (prodrugs) which are converted to the parent drug by virtue of enzymatic and / or chemical hydrolysis within the cancer tissue. In the present study, three derivatives of 5-fluorouracil has been designed to be synthesized as 5-fluorouracil phosphoramidate prodrugs, compounds (I, II and III) to selectively deliver the 5-fluorouracil into the cancer cells.

The generation of the target compounds I, II and III were accomplished following one-pot reaction procedures. The reaction and purity of the products were checked by TLC, the structure of the final compounds was confirmed by their melting points, infra red spectroscopy and elemental microanalysis. The hydrolysis of compounds I, II and III in aqueous buffer solution of pH 6, pH 7.4 and in serum were studied.

Compounds I, II and III had acceptable rate of hydrolysis at pH 6 ($t_{½}$ = 45.05 min, $t_{½}$ = 41.22min and $t_{½}$ = 38.80min respectively) and enough stability at pH 7.4 ($t_{½}$ = 348.72 min, $t_{½}$ = 395.31min and $t_{½}$ = 345.38min respectively); and enough stability at serum; therefore these three compounds can selectively deliver 5-fluorouracil into the tumor cells which have pH approximate to (6).

According to the results mentioned above, compounds I, II and III can be good candidates as 5-fluorouracil prodrugs that can selectively deliver the parent drug into the cancer cells by the effect of pH and /or enzyme.

Keyword: 5-Fluorouracil, Phosphoramidate prodrug, Cancer targeting.

Introduction

5-fluorouracil (5-FU) is being widely used in oncology for treatment of various cancers including head and neck, colorectal, breast, and pancreatic tumors [1].The use of 5-fluorouracil accompanied by several disadvantages including sever adverse effects [2,3] drug resistance[4], limitation of uses [5,6], and variable bioavailability [7]. The cytotoxic effect of 5-FU in most systems is attributed primarily to its anabolism to 5-fluoro-2′-deoxyuridine monophosphate (FdUMP), a potent inhibitor of thymidylate synthase [8], a pivotal enzyme in pyrimidine biosynthesis [9].

Prodrugs are defined as per se therapeutically inactive agents but that can be predictably transformed into active metabolites. In other words, prodrugs act as precursors of parent drugs, with no intrinsic activity, and must undergo, by enzymatic and/or chemical, transformation into active agents in vivo. Simple prodrugs contain a covalent link between the drug and the strategically selected chemical/transport moiety or promoiety. [10]. Lowering extracellular pH (pHe) is one of the few well documented physiological differences between solid tumor and normal tissues, with an absolute value as low as 5.8[11]. prodrugs that could be selectively activated at the lower than normal pHe (occurring in tumor tissue) could have some theoretical advantage as drug for cancer therapy[12]. The prodrug strategy for a site-specific or tumor-targeting delivery has been employed, and much effort has been expended in searching for prodrugs that might improve the clinical utility of 5-FU as an important cancer chemotherapeutic agent. Examples include 1-prodrug forms of

5-FU such as tegafur (Ftorafur) [1-(2-tetra-hydrofuranyl)-5-fluorouracil] derivatives [13], 1-alkylcarbamoyl-5-fluorouracils [14], 5-fluoro-2′-deoxyuridine (5-FdUrd) derivatives [15], and polymeric matrix systems for the controlled release of 5-FU [16]; 2- the recently advanced tumor-specific targeting of 5-FU prodrugs using tumor specific gene expression such as antibody-directed enzyme prodrug therapy [17] and targeting carcinoembryonic antigen-promoted activity [18]; and 3- intratumoral prodrug activation in which a nontoxic drug is converted into 5-FU by intratumorally expressed enzymes [19].

Materials and Methods

5-Fluorouracil was purchased from EBEWE pharma (Austria); Benzyl alcohol and Benzyl amine were purchased from Fluka (Germany); Phosphoroxychloride was purchased from Fluka (Switzerland). All chemicals were reagent grade and obtained from standard commercial sources. Elemental micro analysis were performed using Carlo Euro-vector EA 3000A(Italy); Melting points were measured on Thomas Hoover Electronic melting point apparatus; and are uncorrected; Infra red spectra were recorded as KBr disks on Back IR spectrophotometer (College of Pharmacy, University of Baghdad); and UV spectrophotometer (College of Pharmacy, University of Baghdad).

Synthesis of (Compound I):

To a stirred solution of Phosphor-oxychloride (0.93 ml, 10 mmol) in dry chloroform (50ml) at –20 ^{o}C, a solution of benzyl alcohol (1.04 ml 10 mmol) and dry triethylamine (1.53 ml, 11 mmol) in dry chloroform (10ml) was added. After 30 min at –20 ^{o}C a solution of benzyl amine (1.09 ml, 10 mmol) in dry chloroform (10 ml) was added into the reaction mixture. Then, dry triethylamine (1.53 ml, 11 mmol) was added. After 30 min at –20 ^{o}C a solution of 5-FU(1.31gm,10 mmol) in dry tetrahydrofuran (200ml) was added in three portion into the reaction mixture Then, dry triethyl amine (1.53ml,11mmol) was added again and the mixture was kept at room temperature for 1 hour. The obtained suspension was filtered and the filterate was washed with distilled water (3×20 ml),dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained compound was crystallized from ethyl ether to give White crystal of compound I. Percent yield (38%), melting point (169-170 ^{o}C decomp.).And elemental microanalysis calculated/found: C55.53/54.71, H4.40/4.22, N10.79/10.49. The infrared characteristic bands (cm^{-1}): 3060: N-H (str.vib.) of pyrimidine and N–H (str.vib.) of 2° amine, 1958, 1892 (str.vib.) of mono substituted benzene, 1724, 1521and1346 NH(C=O)–C= C–amide I, II, and III (str.vib.) of uracil, 1663C=O (str.vib.) of Pyrimidine, 1246 C–F(str.vib.), 1111 P=O (str.vib.), 1600, 1550 and 1467 C=C of benzene ring, 1058 P–O–C (str.vib.), 746 and 694 C–H out-of-plane bending vib. of mono substituted benzene.

Synthesis of (Compound II):

To a stirred solution of Phosphor-oxychloride (0.93 ml, 10 mmol) in dry chloroform (50 ml) at –20 ^{o}C a solution of benzyl alcohol (2.07 ml, 20 mmol) and dry triethylamine (3.06 ml, 22 mmol) in dry chloroform (20ml) was added. After 30 min at –20 ^{o}C a solution of 5-FU (1.31 gm, 10 mmol) in dry tetrahydrofuran (200 ml) was added in three portion into the reaction mixture. Then, dry triethylamine (1.53 ml, 11 mmol) was added again and the mixture was kept at room temperature for 1 hour. The obtained suspension was filtered and the filterate washed with distilled water (3×20 ml), dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained compound was crystallized from ethyl ether to give off white powder of compound II. Percent yield (38%), melting point (144-146 ^{o}C decomp.) and elemental microanalysis calculated/found: C55.39/54.59, H4.13/4.01, N7.18/7.01. The infrared characteristic bands (cm^{-1}): 3174: N-H (str.vib.)Of pyrimidine, 1892, 1780(str.vib.) of mono substituted benzene, 1722, 1521and1350 NH(C=O)–C= C–amide I, II, and III (str.vib.) of uracil and C=C of benzene ring, 1654 C=O (str.vib.), 1275 P=O (str.vib.), 1248 C–F (str.vib.), 995 P–O–C (str.vib.).

Synthesis of (Compound III):

To a stirred solution of Phosphor-oxychloride (0.93ml, 10mmol) in dry chloroform (50ml) at –20 ^{o}C a solution of benzyl alcohol (1.04 ml, 10 mmol) and dry triethylamine (1.53 ml, 11 mmol) in dry chloroform (10 ml) was added. After 30 min at –20 ^{o}C a solution of benzyl amine (1.09 ml, 10mmol) in dry chloroform (10ml) was added into the reaction mixture. Then, dry triethylamine (1.53 ml, 11 mmol) was added.After 30 min at room temperature a suspension of 5-fluorouracil sodium salt (1.53 g, 10 mmol) in freshly distilled acetonitrile (10ml) was added and stirred mixture for 10 hr at room temperature. The obtained suspension was filtered and the filterate washed with distilled water (3×20 ml), dried with anhydrous sodium sulphate and the solvent was evaporated. The obtained compound was crystallized from ethyl ether-petroleum ether to give Pale yellow powder of compound III. Percent yield (60.5%), melting point (90-91 ^{o}C) and elemental microanalysis: C55.53/54.62, H4.40/4.23,N10.79/10.47. The infrared characteristic bands (cm^{-1}): 3316: N–H (str.vib.) of 2° amine, 3160: N-H (str.vib.)of pyrimidine, 1953,1894 (str.vib.) of mono substituted benzene, 1723, 1500 and 1344 NH(C=O)–C=C– amide I, II, and III (str.vib.) of uracil and C=C of benzene ring, 1670C=O (str.vib.), 1250 C–F (str.vib.), 1201 P=O (str.vib.), 1028 P–O–C (str.vib.), 744 and 696 C–H out-of-plane bending vib. of mono substituted benzene.

Hydrolysis of compounds I, II and III at pH 6, pH 7.4 and serum:

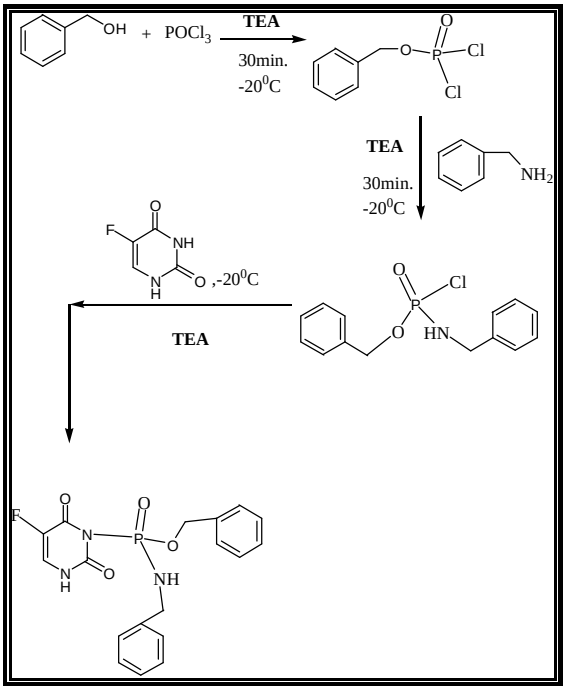
The hydrolysis of compounds I, II and III were carried out for the equivalent of (0.01mg/ml) in aqueous phosphate buffer solution of pH 6, pH 7.4 and serum at 37 ^{o}C. The total buffer concentration was 0.1M and the ionic strength (μ) of 1 was maintained by adding calculated amount of NaCl. Different sample were taken for analysis at specific time interval (15, 30, 60, 120, 240 min) and the rate of hydrolysis was followed spectro-photometrically by recording 5-fluorouracil absorbance increase accompanying the hydrolysis at 266nm pH6, pH7.4 and serum. The observed pseudo-first order rate constant was determined from the slope of the linear plot of log concentration of compound vs. time.

Results and Discussion

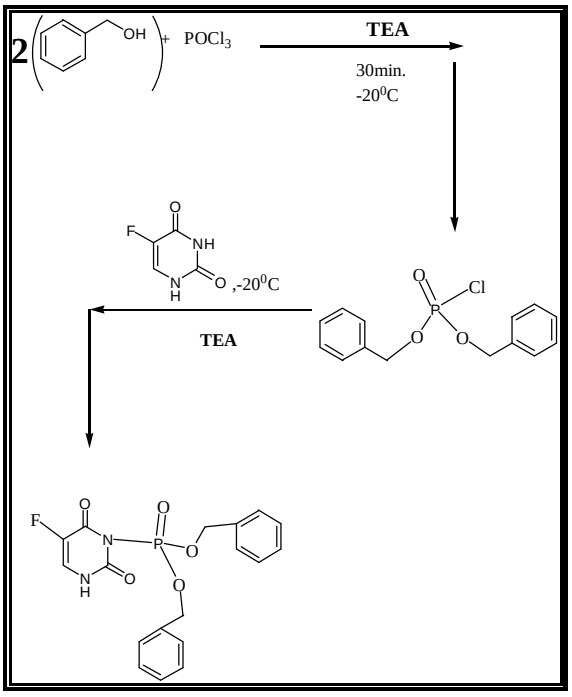
The synthetic procedures for the designed target compounds I, II and III are illustrated in [schemes 1, 2 and 3]. Benzyl Compounds I was obtained in one pot-reaction of benzyl alcohol with phosphorusoxychloride in the presence of triethylamine. The obtained dichlorophosphate intermediate was reacted, without isolation, with benzyl amine in the presence of triethylamine, then the obtained monochlorophosphate intermediate was reacted, without isolation, with 5-flourouracil.

The Benzyl Compound II was synthesized from reaction in one pot-reaction of benzyl alcohol with phosphorus oxychloride in the presence of triethylamine. The obtained monochlorophosphate intermediate was reacted, without isolation, with 5-flourouracil. This method was previously established by us for the synthesis of isotopically labeled IPAM [20].

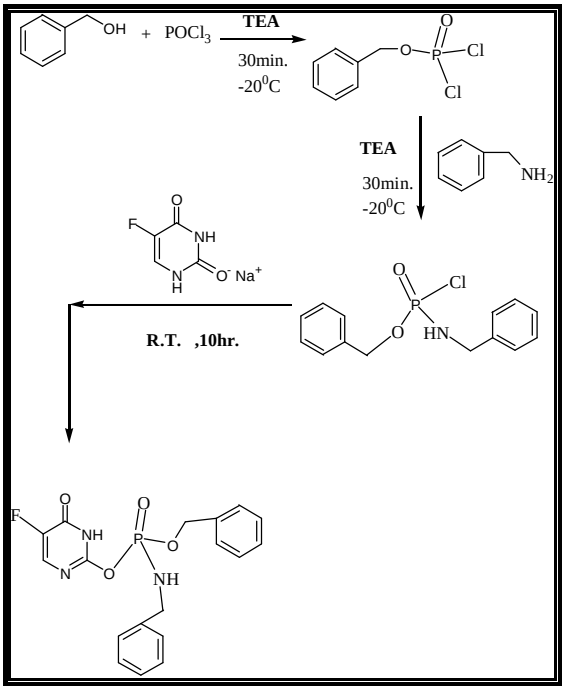
Compound III was obtained in one pot-reaction of benzyl alcohol with phosphorus oxychloride in the presence of triethylamine. The obtained dichlorophosphate intermediate was reacted, without isolation, with benzyl amine in the presence of triethylamine [21], then the obtained monochlorophosphate intermediate was reacted, without isolation, with an acetonitrile solution of 5-flourouracil sodium salt [22].



Scheme (1): Synthesis of compound I.

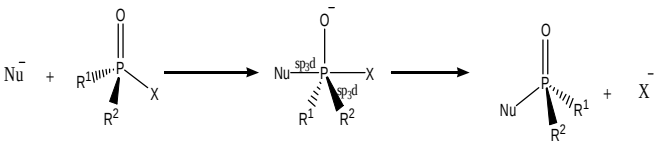


Scheme (2): Synthesis of compound II.



Scheme (3): Synthesis of compound III.

The reactions were proceeded through addition-elimination (AE) mechanism involves the formation of a true penta-coordinate intermediate, generally thought to have trigonal bipyramidal geometry, and constructed by apical approach of the attacking nucleophile with departure of the displaced group at the opposite apex (Scheme 4), [33].



Scheme (4): Mechanism of phosphoramidate reaction.

Under experimental conditions used the hydrolysis of compounds I, II and III followed pseudo first order kinetic, since plot of log concentration vs. time resulted in straight line. From it slope, the observed rate constant of hydrolysis was calculated.

The N-phosphates is highly sensitive to acidic conditions, whereas it was considerably resistant to hydrolysis under alkaline conditions [23]; the O-phosphates groups are generally resistant to both acidic and alkaline

conditions [24] and sensitive to phosphatase enzyme [25].

Figs. (1), (2) are representive graphs for hydrolysis of compounds I, II while Figs. (3),(4) are representive graphs for hydrolysis of compound III respectively, while Table (1) shows the pH values, the corresponding Kobs and half-lives of the hydrolysis of 5-FU derivatives. The half-life was calculated using the following equation, which derives from the first order kinetic law.

$$t_{1/2} = \frac{0.693}{Kobs}$$

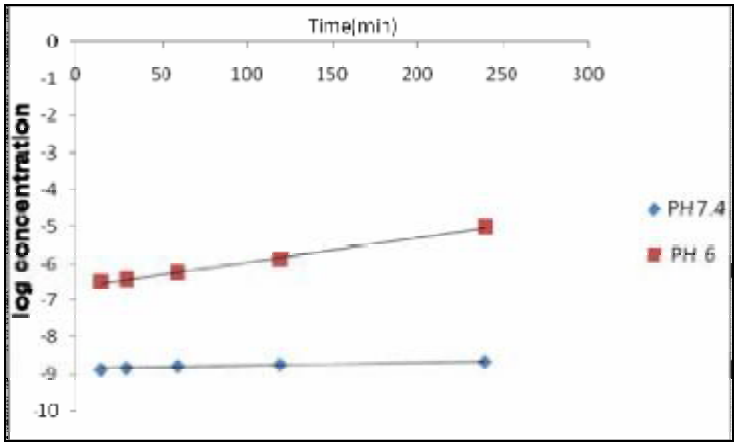


Fig. (1): First order plot for the hydrolysis of compound I in 0.1M Phosphate buffer of pH 6 and 7.4 at 37℃ (μ = 1), at pH 6 and pH 7.4, (r = 0.998 and 0.975 respectively).

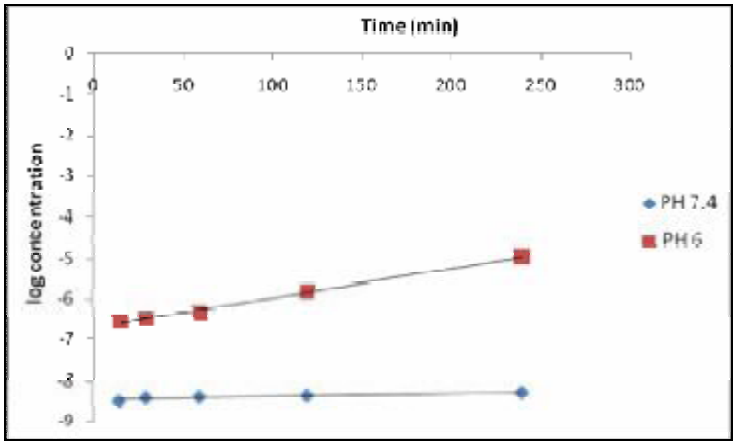


Fig. (2): First order plot for the hydrolysis of compound II in 0.1M Phosphate buffer of pH 6 and 7, 4 at 37℃ (μ = 1), at pH 6 and pH 7.4,(r = 0.997 and 0.928 respectively).

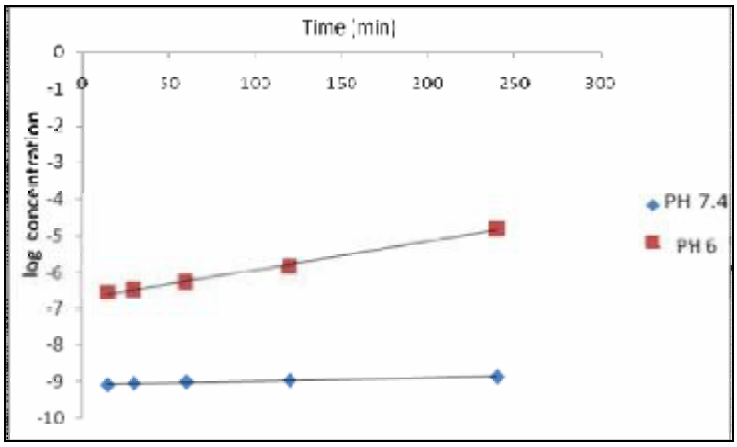


Fig.(3): First order plot for the hydrolysis of compound III in 0.1M Phosphate buffer of pH 6 and 7, 4 at 37℃ (μ=1), at pH 6 and pH 7.4, (r = 0.998 and 0.979 respectively){we determine the release of benzyl amine}.

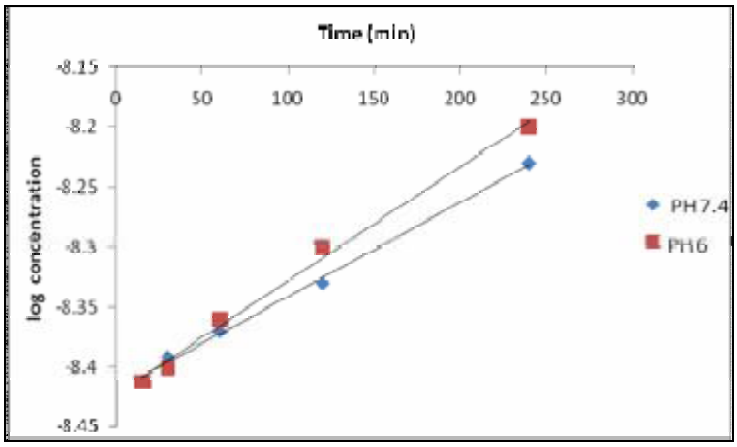


Fig.(4): First order plot for the hydrolysis of compound III in 0.1M Phosphate buffer of pH 6 and 7, 4 at 37℃ (μ = 1), at pH 6 and pH 7.4, (r = 0.993and 0.995 respectively){we determine the release of 5-fu}.

Table (1)
The rate constant of hydrolysis of compounds I, II and III at pH 6 and pH 7.4 at 37℃, and μ = 1.

Compound	pH	Kobs(min^{-1})	t½(min)
I	6 7.4	0.1538 1.98726×10^{-3}	45.05 348.72
II	6 7.4	0.01681 1.75304×10^{-3}	41.22 395.31
III[a]	6 7.4	0.01786 2.00645×10^{-3}	38.80 345.38
III[b]	6 7.4	2.15895×10^{-3} 1.78358×10^{-3}	320.98 min 388.54 min

a; we determine the release of benzyl amine.
b; we determine the release of 5-fu.

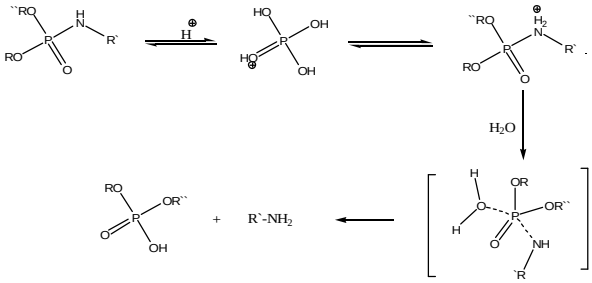
One of the crucial reguirements for a prodrug to be used in directed prodrug therapy is good stability under physiological conditions providing enough time to reach the site of action [26].Incubation of compounds (I, II and III) in serum shows insignificant digestion of these compounds and enough stability at serum; as shows in Table (2).

Table (2)
Hydrolysis (%) in serum of compounds I, II and III in serum.

Compound	Hydrolysis (%) in serum				
	15 min.	30 min.	60 min.	120 min.	240 min.
I	2.8	3.4	3.998	4.764	6.36
II	3.01	4.03	5.55	6.12	7.53
III	4.04	4.6	5.79	7.29	9.18

Compounds I, II and III are susceptible to general base catalyze hydrolysis and this was attributed to the protonation of phosphoryl nitrogen [27]. On the other hand the relative stability of compounds I, II and III at pH 7.4 and in serum could be attributed to the lower degree of ionization of compounds I, II and III at these media.

The phosphoramidate linkage of compounds I, II and III is cleaved at mildly acidic condition predominantly via the acid-catalyzed cleavage of the P-N linkage (Scheme 5), as observed previously with both ribonucleoside[28]and deoxyribonucleoside [29] phosphoramidates. Under these condition the phosphoramidate may in principle be protonated either at the phosphoryl oxygen or at the amide nitrogen [Scheme 5]. No conclusive evidence for the preferred site of protonation exists, and indirect evidence in favor of both the oxygen [30] and nitrogen [31] protonation has been reported. The rate-limiting step is the nucleophilic attack of a water molecule on the phosphorus atom [27]. The involvement of water as a nucleophile in the transition state receives support from previously published studies on the hydrolysis of simple phosphoramidates [32, 33].



Scheme (5): Hydrolysis of phosphoramidate in mildly acidic medium.

The preliminary kinetic studies showed that compounds I and II was librated 5-FU at pH 6 faster than at pH 7.4 as shown in Table (1) and that in serum Table (2). On the other hand compound III was liberated benzyl amine and O-diphosphate 5-FU derivative at PH6 and has enough stability at pH 7.4 Table (1). The diphosphate ester may be susbtable to the hydrolysis by the acid phosphatase enzyme in the cancer tissues ; will make these compounds good candidates as prodrugs for targeting cancer cells since the pH of there is approximate 6 and the tumors are known to overexpress acid phosphatases [34] ; while these compounds are relatively stable at pH 7.4 which is the pH of normal cells.

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الخلاصة

5- فلورويوراسيل وهو من الأدوية المضادة للأورام و يستخدم لعلاج سرطان القولون وسرطان البنكرياس، سرطان الثدي، و سرطان القولون، و سرطان المستقيم، على الرغم من إن استعمالاته حددت لان 5-فلورويوراسيل لديه سمّية للجهاز الهضمي و النظام الوعائي الدموي، ا ن من أهم المعادلات المستخدمة لدعم خاصية انتقاء الخلايا السرطانية لـ (5- فلورويوراسيل) هي تحضير مشتقات ذات خواص محسنة و قابلة لتحرر الدواء فيها بالتحلل الإنزيمي أو الكيماوي الموجودة في الأنسجة السرطانية. تم في هذه الدراسة تحضير ثلاثة مركبات فوسفور آمايديت مشتقة من لـ(5-فلوروبوراسيل) (I, II and III) وهي لكي يتم تخليقها كمشتقات محسنة لإيصال الدواء و تحريره بانتقائية في الخلايا السرطانية.

تم تحضير هذه المركبات بإتباع طريقة التفاعل ذات الخطوة الواحدة. و تم مراقبة جميع التفاعلات و التأكد من نقاوة المركب بواسطة كروماتوغرافيا الطبقة الرقيقة (Thin layer chromatography)، كما تم متابعة المركبات النهائية و التأكد من تحضيرها من خلال قياس درجات الانصهار والتحليل الطيفي للأشعة تحت الحمراء، و التحليل الدقيق للعناصر.

وقد تم دراسة تحلل المركبات (I, II and III) في المحاليل الدارئة ذات الأس الهيدروجيني PH(6) و (7,4) و المصل. وقد أوضحت النتائج المستخلصة من هذه الدراسة إن المركبات (I, II and III) يملكون سرعة تحلل مقبولة في المحاليل الدارئة ذات الأس الهيدروجيني (عمر النصف=45.05 دقيقة و41.22 دقيقة و38.80 دقيقة على التوالي) و بثباتية كافية في المحاليل الدارئة ذات الأس

الهيـــدروجيني (7.4) (عمـــر النـــصف=348.72 دقيقـــة و395.31 دقيقة و345.38 دقيقة على التـــوالي) و بثباتيـــة كافية في المحاليل الدارئة المصلية لذلك فان هذه المركبـــات تستطيع إيـــصال الـــــ (5-Fluorouracil) إلـــى الخلايـــا السرطانية ذات الأس الهيدروجيني المقارب (6).

يتضح بـــان المركبـــات (I, II and III) مرشـــحات كمشتقات محسنات لـــ(5- فلورويوراسيل) لهم القدرة على إيصال الدواء بانتقائية للخلايا السرطانية و باليـــة تحريـــر تتضمن تأثير الأس الهيدروجيني و/ أو ألإنزيمي.

BATCH AND FLOW INJECTION SPECTROPHOTOMETRIC METHODS FOR DETERMINATION OF PARACETAMOL IN PHARMACEUTICAL PREPARATIONS VIA OXIDATIVE COUPLING WITH 4-AMINOANTIPYRINE

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Abstract

A simple, rapid and sensitive batch and flow injection spectrophotometric methods have been developed for the determination of paracetamol in pure form and pharmaceutical preparations. The proposed methods are based on the oxidative coupling reaction of paracetamol with 4-aminoantipyrine in presence of ammonium persulfate in alkaline medium to produce an orange-reddish product that having absorptivity maximum at 461 nm. The optimum reaction conditions and other analytical parameters have been evaluated. Linearity was observed from 2-16 and 100-700 μg mL^{-1} paracetamol by batch and flow injection procedures, respectively. Statistical analysis of the results and comparison with results by the British Pharmacopoeia method are also reported.

Keywords: Paracetamol; Spectrophotometric; Flow Injection; 4-Aminoantipyrine; Oxidative Coupling Reaction.

Introduction

Paracetamol (N-acetyl-p-aminophenol) is well known as analgesic anti-pyretic drug. It is the active metabolite of phenacetine responsible for its analgesic effect. It is well tolerated, lacks many of the side effects of aspirin, So it's commonly used for the relief of fever, head ache and other minor aches and pains[1-3].

Various methods for paracetamol determination have been described, including chromatography[4,5], spectrophotometry[6-9], fluorimetry[10,11] and chemiluminescence[12].

The British Pharmacopoeia (BP) method describes a titrimetric procedure for paracetamol determination in pharmaceutical formulations using Ce (IV) in acidic media and 1, 10-phenanthroline-iron(II) complex (ferroin) to determine the end point. The titration is performed in ice[13].

Flow injection (FI) system are adequate procedures to use in routine analysis in pharmaceutical laboratories control due to their simplicity, high analytical frequency and capacity to reduce reagent consumption when compared with batch procedure[14,15].

Oxidative coupling organic reactions are recently used for spectrophotometric determination of several drugs such as phenylephrine hydrochloride[16], folic acid[17], salbutamol[18], amoxicillin[19] and catecholamine drugs[20].

In this paper, two batch and FI methods using spectrophotometric detection at 461 nm are described for the determination of paracetamol via oxidative coupling reaction. The method are depends on the formation of orange-reddish product between this drug and 4-aminoantipyrine in presence of ammonium persulfate in alkaline medium. The proposed methods have been successfully applied to the determination of paracetamol in pharmaceutical preparations.

Experimental

Apparatus

All spectral and absorbance measurements were performed on a Shimadzu UV - VIS 260 (Tokyo, Japan) digital double-beam recording spectrophotometer using 1 cm quartz cells.

The FI system comprised a peristaltic pump (Ismatec, Labortechink-Analytic, CH-8152, glatbrugg-zurich, Switzerland, six channels) with poly vinyl chloride flow tubes