

Cephalothin as a Carrier of 6-Mercaptopurine for Targeting Cancer Tissues

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Abstract

A lower extracellular pH is one of the few well-documented physiological differences between tumour and normal tissues. On the other hand, elevated glutathione (GSH) level has been detected in many tumours compared with healthy surrounding tissues. The compound II: 3-(9H-purin-6-yl-thio) carbonothionyl methyl-8-oxo-7-(2-thiophen-2-yl) acetamido-5-thia-1-azabicyclo-4-octo-ene-carboxylic acid was a cephalothin derivative contain 6-mercaptapurine (6-MP). Compound II react with general base catalysis in slightly acidic pH or with sulfhydryl nucleophiles to release the chemotherapeutic drug 6-MP. The generation of compound II was accomplished following multistep reaction procedures. The structure of compound II and its intermediate was confirmed by their melting point, infrared spectroscopy, CHN and NMR analysis. The hydrolysis of compound II in aqueous buffer solution of pH 6 and in the presence of GSH at pH 7.4 was studied. The partition coefficient (PC) of compound II was also determined. Compound II has acceptable rate of hydrolysis at slightly acidic medium pH 6 ($t_{1/2} = 56.34$ min.) and 80% of compound II had been converted to 6-MP within 30 min in the presence of GSH. And the compound has acceptable stability at pH 7.4 ($t_{0.5} = 639.65$ min.) and the rate of hydrolysis was effected by change the buffer concentration. This compound can selectively deliver 6-MP into the tumour tissues which have acidic pH or elevated GSH level. Compound II had an improved PC value of 12.23 compared to 1.22 for free drug 6-MP confirming higher lipophilicity.

Key words: 6-mercaptapurine, cancer, prodrug, targeting

الخلاصة

ان انخفاض الالاس الهيدروجيني (pH) خارج الخلية هو احد العلامات الفسلجية الفارقة الموثوقه بين الانسجة السرطانية والانسجة الطبيعية. ومن جهة اخرى فقد لوحظ ارتفاع في مستوى الغلوتاثيون (GSH) في عدد من الاورام مقارنة بالانسجة الطبيعية المحيطة. ان المركب II

3-(9H-purin-6-yl-thio) carbonothionyl methyl-8-oxo-7-(2-thiophen-2-yl) acetamido-5-thia-1-azabicyclo-4-octo-ene-carboxylic acid

وهو مشتق للسيفالوثين (Cephalothin) يحتوي على مركب الـ 6-مركبتوبورين (6-MP) ان المركب II يتفاعل مع القاعدة العامة في المحيط الحامضي الضعيف او مع سلفهايدريل (sulfhydryl) الباحث عن النواة لتحرير الـ 6-مركبتوبورين. تم تحضير المركب II باتباع طريقة التفاعل متعدد الخطوات وقد تم اثبات الصيغ الكيميائية للمركب النهائي والوسطي والتأكد منها بواسطة قياس درجات الانصهار والتحليل الطيفي للأشعة تحت الحمراء والتحليل الدقيق للعناصر. وتم دراسة تحلل المركب II في المحلول الدائري ذي الالاس الهيدروجيني (pH) 6 وكذلك تم دراسة معامل التآين للمركب II partition coefficient. اوضحت النتائج المستحصلة من هذه الدراسة ان المركب II يمتلك سرعة تحلل مقبولة في المحلول الدائري ذي الالاس الهيدروجيني (pH) 6 بعمر نصف = 56.34 دقيقة وثباتية مقبولة في المحلول الدائري ذي الالاس الهيدروجيني (pH-74)، كما تبين ان سرعة تحلل المركب تتأثر بتغير تركيز المحلول الدائري. لذلك من المتوقع ان المركب II يستطيع اوصول الـ 6-مركبتوبورين (6-MP) الى الخلايا السرطانية ذات الالاس الهيدروجيني المقارب للـ 6). وقد اتضح ايضا ان الغلوتاثيون يزيد من سرعة تحلل المركب II، حيث ان 80% من المركب قد تحول الى الـ 6 مركبتوبورين خلال 30 دقيقة. كما تم ملاحظة ان معامل التآين قد تحسن مما يدل على تحسن نفاذية المركب داخل الجسم، حيث ان معامل التآين للمركب II هو 12.23 في حين ان معامل التآين للـ 6-مركبتوبورين هو 1.22

Introduction

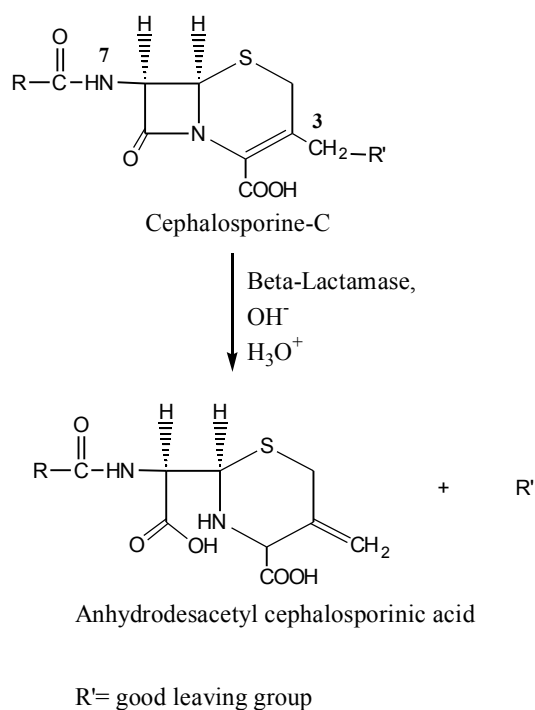
The use of antineoplastic agent 6-MP accompany by several disadvantages including sever adverse effects ⁽¹⁾, poor absorption, low bioavailability ⁽²⁾, limitation of uses ⁽³⁾, thiopurine associated leukemia ^(4,5) and drug resistance ^(6,7,8). Cephalosporins are β -lactam

antibiotics; their degradation depends on the side chain at C-7 and the substitution on C-3 ⁽⁹⁾. The presence of good leaving group at C-3 facilitate spontaneous expulsion of the 3-substituent by any general nucleophile, β -lactamase or pH change ^(10,11), scheme (1).

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Scheme (1): Possible degradation of cephalosporin C⁽¹¹⁾.

Lowering extracellular pH (pHe) is one of the few well-documented physiological differences between solid tumour and normal tissues. The main pHe in tumour tissues is 0.6-0.8 unit lower than normal tissues, with an absolute as low as 5.8^(12,13). Changes in GSH content and level of glutathione S-transferase have been detected in tumours⁽¹⁴⁾. Moreover, increased levels of GSH have been linked with drug resistance⁽¹⁵⁾ and poor patient prognosis⁽¹⁶⁾. Thus, an excellent opportunity exists for the design of prodrugs that specifically target tumours with low pHe or with abnormal GSH level. Doxorubicin armed antibody was a prodrug designed to target tumours with low pHe⁽¹⁷⁾. The 6-MP prodrug S-(9H-purin-6-yl) glutathione (figure 1a) which can be further metabolized *in vivo* to yield 6-MP⁽¹⁸⁾. Cis 6-(2-acetyl vinyl) thiopurine (AVTP). (figure 1a), a potential 6-MP prodrug targeting tumours with up-regulated GSH level. Structurally AVTP is butanone conjugate of 6-MP and a Michael acceptor that undergoes addition-elimination reaction with nucleophiles to yield 6-MP, the AVTP metabolism to 6-MP was GSH dependent⁽¹⁹⁾.

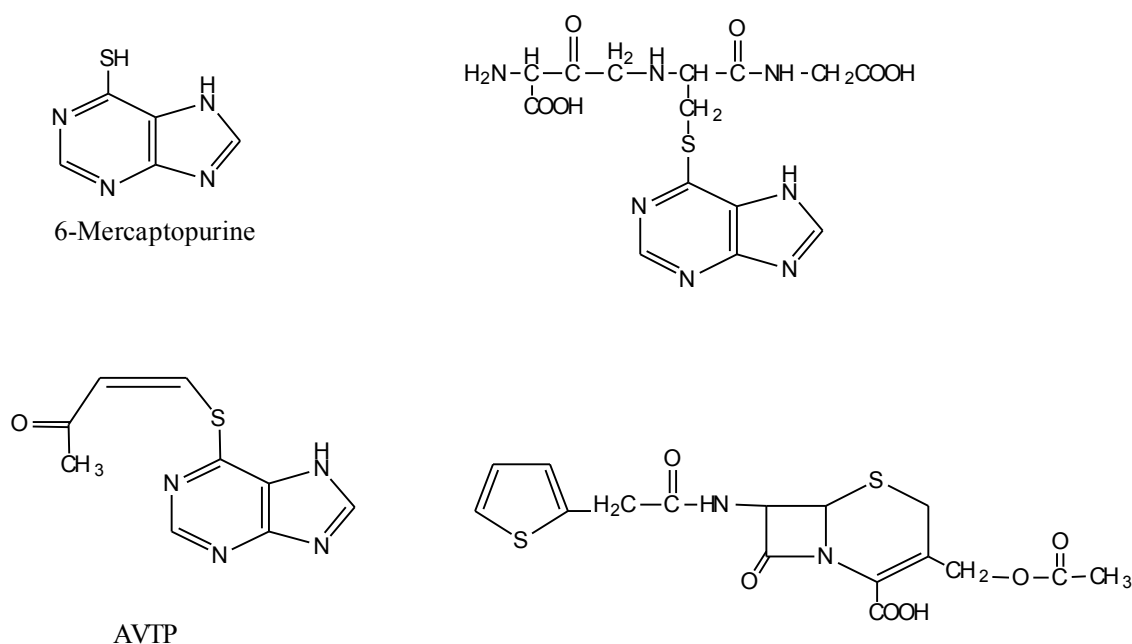


Figure (1a): Chemical structure of 6-mercaptapurine, S-(9H-purin-6-yl) glutathione, Cis-AVTP and cephalothin.

Materials and Methods

Chemicals

6-mercaptopurine and HCl were purchased from Fluka (Germany); Cephalothin sodium was purchased from Laboratories TORLAN, S.A. (Spain); GSH was purchased from Sigma (USA); and Carbon disulfide was purchased from Riedel-Dehen (Germany). All chemicals were reagent grade and obtained from standard commercial sources. Elemental micro analysis were performed using Carlo Erba elemental analyzer 1106 (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 H-NMR spectrum was carried out on Mercury MHZ-NMR spectrometer (ppm) at MDIT center in the University of Toronto.

Synthesis of potassium 7H-purinylcarbonotrithionate (compound I)

To a stirred solution of potassium hydroxide (0.313gm, 5.58 mmol) in absolute ethanol (10ml) at 15-20 °C, a 6-MP (0.96gm, 5.58 mmol) was added over (0.5 min). After 0.5 hr carbon disulfide (0.425gm, 5.58mmol) was then added and the reaction mixture was stirred for 3 hr at 25 °C. Then the precipitate of the desired compound was obtained by the addition of diethyl ether. The precipitate was filtered and re-crystallized from ethanol to give white crystalline powder of compound I, percent yield (63%), melting point (240-243 °C) and infrared yield absorption band, (cm⁻¹): 3450 of NH stretching vibration (str. Vib.) of purine, 1610 and 1556 of C=N str. Vib., 1598 of NH bending vibration (bend. Vib.) of purine, 1497 and 1423 of C=C str. Vib., 1410 of CH bend. Vib. of purine 1205 of C=S str. Vib. of trithiocarbonate and 870 of out plane CH bend. Vib. of purine.

Synthesis of 3-(9H-purin-yl thio) carbonothionyl-methyl-8-oxo-7(2-thiophen-2-yl) acetamido-5-thia-1-azabicyclo-4-octa-2-ene-carboxylic acid (compound II)

A mixture of cephalothin sodium (0.73gm, 3.4mmol) and of compound I (0.5gm, 3.4mmol) in (30ml) of 0.1M phosphate buffer (pH 7) was heated for 3hr at 60-70°C. The mixture was cooled to 5 °C and acidified with HCl (1N) to reach pH 4.0, a precipitate was obtained and collected by filtration, dried and recrystallized from ethanol to give a yellow crystalline powder of compound II. Percent yield (60%), melting point (196-199 °C), elemental microanalysis, calculated/found: (C 42.539/42.996, H 2.855/3.011, N 14.882/15.189, O 11.339/11.637, S 28.390/28.427, the infrared absorption band (cm⁻¹): 3450 of NH str. Vib., 3000-2500 group of small band due to OH str. vib. of OH of COOH, 2682 of CH₂-S str. vib. of cephalothin, 1773 of C=O str. vib. of β-Lactam, 1610 a mide str. vib., 1617 and 1570 of C=N str. vib. 61598 of NH bend-vib. of purine, 1500 and 1430 of C+C str. vib., 1408 of CH bend. vib. of purine, 1228 of C=S str. vib. of trithiocarbonate ester, 875 out plane CH bend vib. of purine, 690 str. vib. of thiophen: and HNMR in CDCl₃ 3.06, 3.16 (q, 2H of methylene α to C=C and α to S-C-), 3.44 (s, 2H of methylene α to C-R and α to (C=O)-N), 4.07 (s, 2H of methylene α to -C=C and α to -S-C-R), 5.1 (d, 1H of propiolactam α to -S R, β- to -N-C=O), 5.45 (d, 1H of propiolactam α to N-C=O, β- to -S-R), 6.83 (d, 1H of 2-thiophen), 6.93 (t, 1H of 2-thiophen), 7.4 (d, 1H of 2-thiophen), 8.32 (s, 1H of sec. amide), 8.57 (s, 1H of purine), 13.65 (s, 1H, NH of purine) as shown in (Figure 1b).

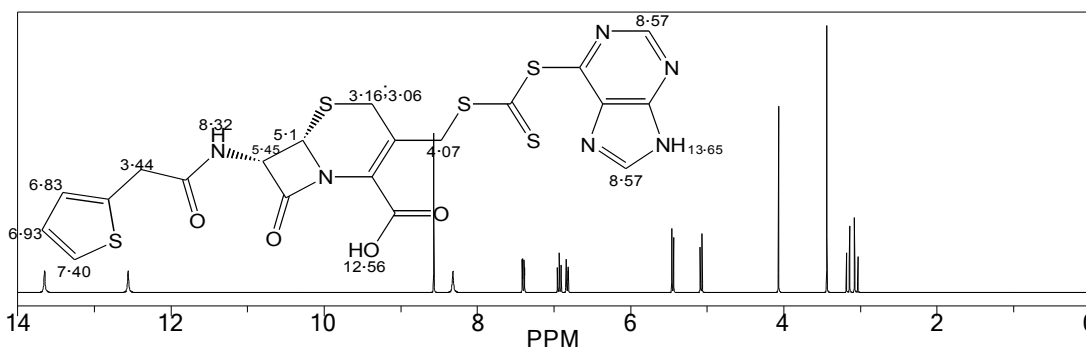


Figure (1b) : ^1H NMR spectrum of compound II .

Hydrolysis of compound II at pH 6 and pH 7-4

The hydrolysis of compound II was carried out for the equivalent of (0.01mg/ml) in aqueous phosphate buffer solution of pH 6 and pH 7.4 at 37 °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 (16, 30, 60, 120, 240 min) and the rate of hydrolysis was followed spectrophotometrically by recording 6-MP absorbance increase accompanying the hydrolysis at 324nm and 316 for pH6 and pH7-4 respectively. The observed pseudo-first order rate constant was determined from the slope of the linear plot of log concentration of 6-MP vs time.

The effect of the buffer concentration at the rate of hydrolysis

The same procedure mentioned above was followed for studying the hydrolysis of compound II in different buffer concentration (0.2, 0.5 and 0.8) and the ionic strength ($\mu=1$) to determine the effect of buffer concentration at the rate of hydrolysis.

Hydrolysis of compound II in the presence of glutathione

Compound II (0.96gm, 0.17mmol) was incubated with glutathione (0.52gm, 0.17mmol) in phosphate buffer (0.1M and $\mu=1$) at pH 7.4 in shaking water bath 37°C. A 1.0 ml sample was removed and added to 4ml of phosphate buffer. The concentration of 6-MP was determined spectrophotometrically at 314nm.

Partition coefficient estimation

Partition coefficient of compound II at pH 7-4 was estimated by using shake flask method. The amount of compound II in both phase were measured spectrophotometrically at 298 nm, and the partition coefficient value was calculated by the following equation ⁽²⁰⁾.

$$PC = \frac{\text{Conc. in organic layer}}{\text{Conc. in inorganic layer}}$$

Results and Discussion

Target compound II was obtained following procedure outlined in scheme (2), compound I was obtained from reaction of 6-MP and carbon disulfide in the presence of potassium hydroxide. The reaction was a nucleophilic addition reaction in which the thiolate anion was added to the carbon atom of the disulfide ⁽²¹⁾. Compound II have been synthesized using the method previously established for the synthesis of cephalosporin derivative ⁽²²⁾. The reaction followed a nucleophilic substitution in which the thiolate moiety attached the methylene moiety at position 3 of cephalothin leading the formation of trithiocarbonate ester with liberation of acetic acid. Under the experimental conditions used the hydrolysis of the compound II followed pseudofirst order kinetic, since the plot of log conc. of 6-MP vs time resulted in straight line from it slope, the observed rate constant of hydrolysis (K_{obs}) was calculated from figure (2a) and (2b) which representative graph for hydrolysis of compound II. The K_{obs} were 0.0123 min^{-1} and 1.083×10^{-3} at pH 6 and pH 7.4 respectively and the half-life of hydrolysis of compound II were 56.34 min and 639.65 min at pH6 and pH 7.4 respectively. The half life was calculated using the equation:

$$t_{0.5} = \frac{0.693}{K_{obs}}$$

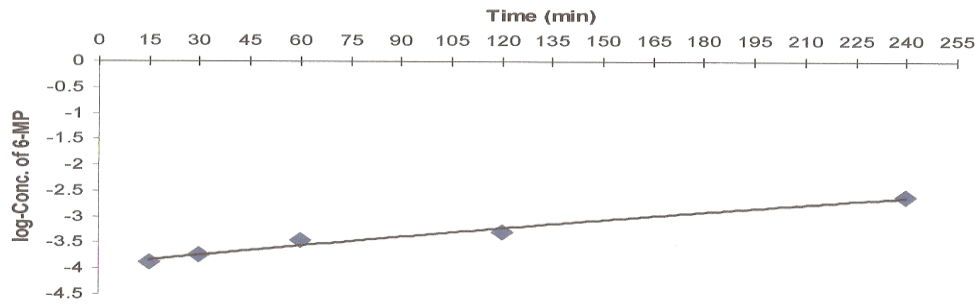


Figure (2a): First order plot for the hydrolysis of compound II in 0.1 M phosphate buffer of pH 6.0 at 37 °C ($\mu =1$).

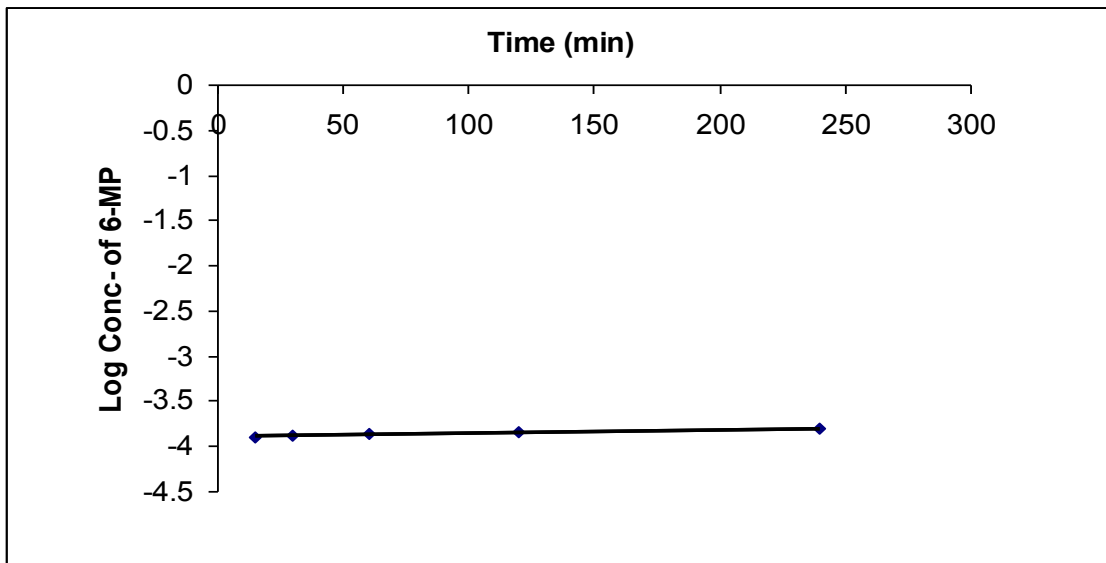
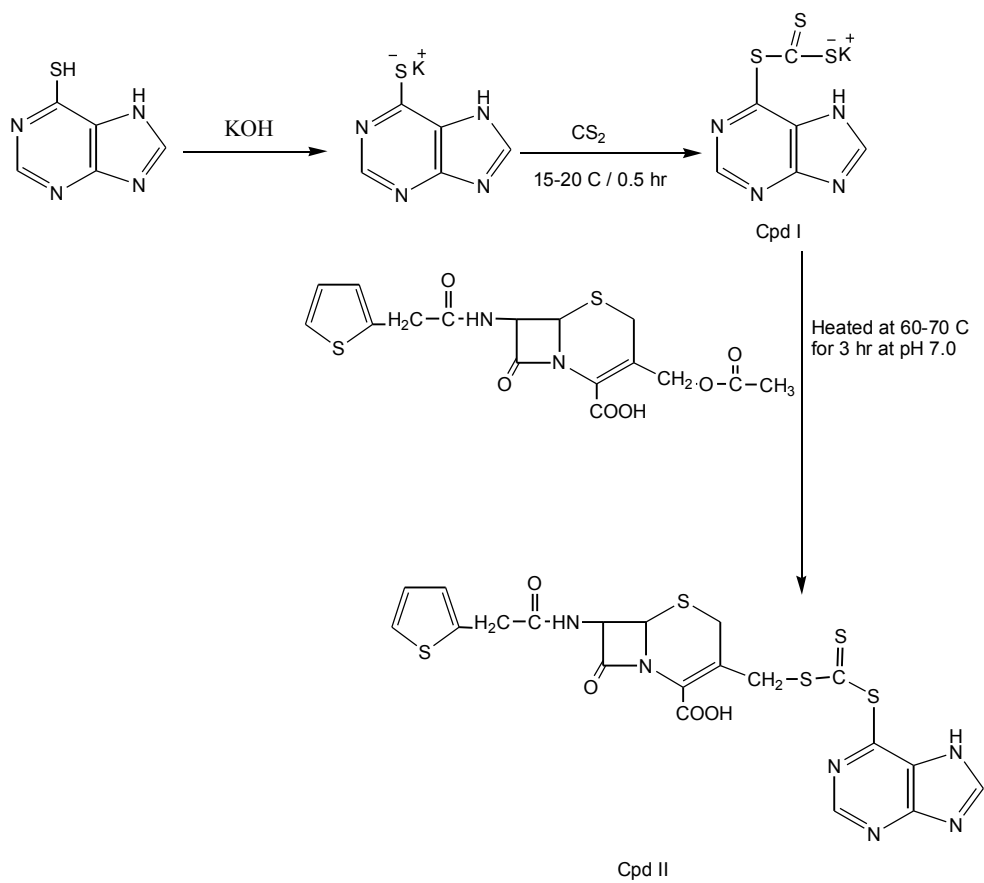
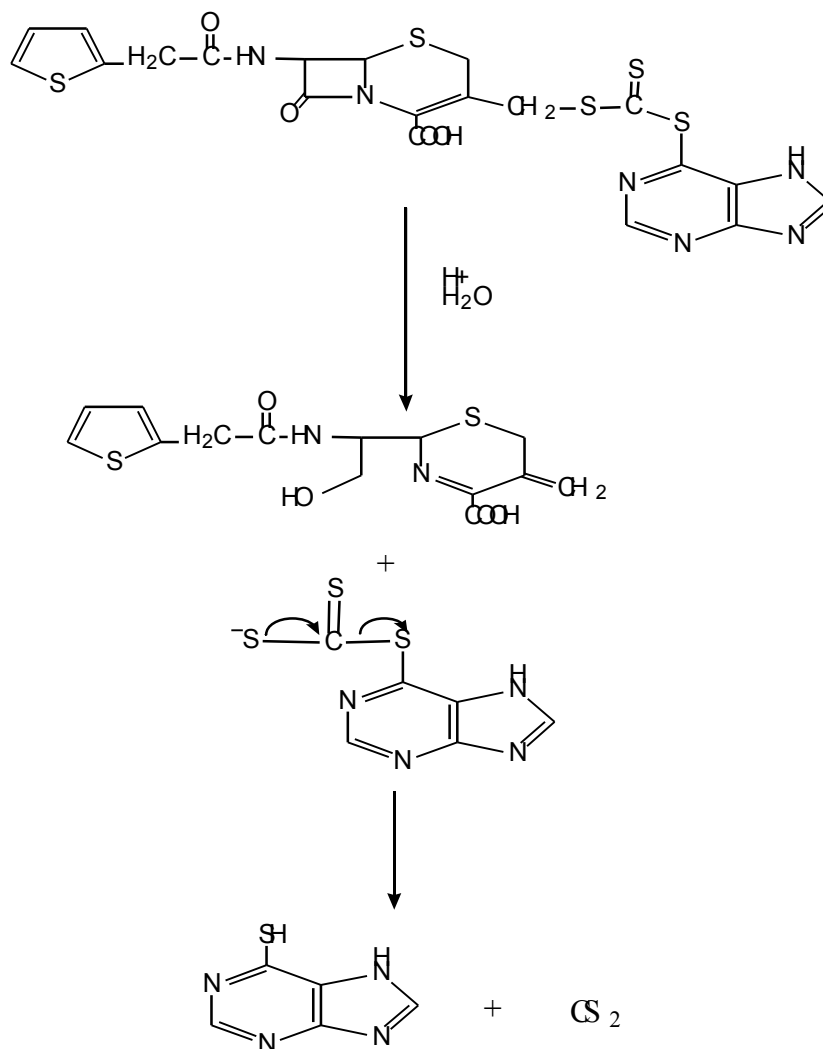


Figure (2b): First order plot for the hydrolysis of compound II in 0.1 M phosphate buffer of pH 7.4 at 37 °C ($\mu =1$).

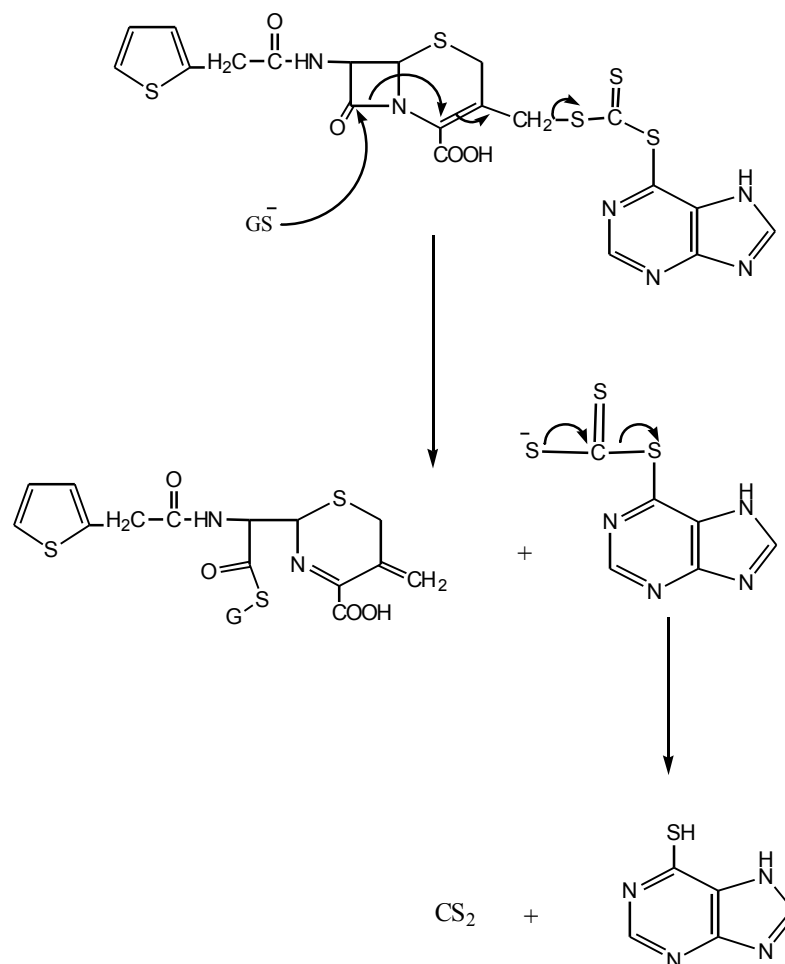
**Scheme (2): Synthesis of compound II.**

The release of 6-MP from compound II depend on the opening of β -lactam ring which in turn depend on the pH of the media and the ease with which the substitution at position 3 of cephalosporin will leave the molecule^(10,11). The thiocarbonate is better leaving group than acetoxy group due to it soft base⁽²³⁾, and this will cause the hydrolysis of compound II at pH 6 is faster than hydrolysis of cephalothin at the same pH (Scheme 3). The rate of hydrolysis of compound II at pH 6 in the presence of different buffer concentration was calculated from figure (4) which represent the hydrolysis of compound II at different buffer concentration. The K_{obs} . Were 0.0133min^{-1} , 0.0154min^{-1} and 0.0166min^{-1} at buffer concentration 0.2, 0.5 and 0.8M respectively. The half-lives were 52.13min, 45.00min. and 41.1 min. for buffer concentration 0.2, 0.5 and 0.8M respectively. Under experimental

condition used the hydrolysis of compound II followed second order kinetic, since plot of log conc. of 6-MP vs time result in curve line. The formation of 6-MP from compound II was linear for at least 30 min. (figure 3). 80% of compound II had been converted to 6-MP within 30 min. Compound II reacted rapidly with thiolate nuceophile of GSH to yield the 6-MP. The thiolate was attack the β -lactam ring of cephalosporin resulted in the opening of this ring with the release of trithiocarbonate derivative from position 3⁽²⁴⁾ (scheme 4). The partition coefficient has become the most common physicochemical property⁽²⁵⁾. The partition coefficient of compound II equal to 12.23; so this compound had an improved partition coefficient value compared to 1.2 for 6-Mp, confirming higher lipophilicity and improvement of the drug bioavailability.



Scheme (3): The liberation of 6-mercaptapurine from Compound II at pH 6.0⁽¹⁰⁾.



Scheme (4): Liberation of 6-mercaptopurine from compound II in the presence of glutathione.

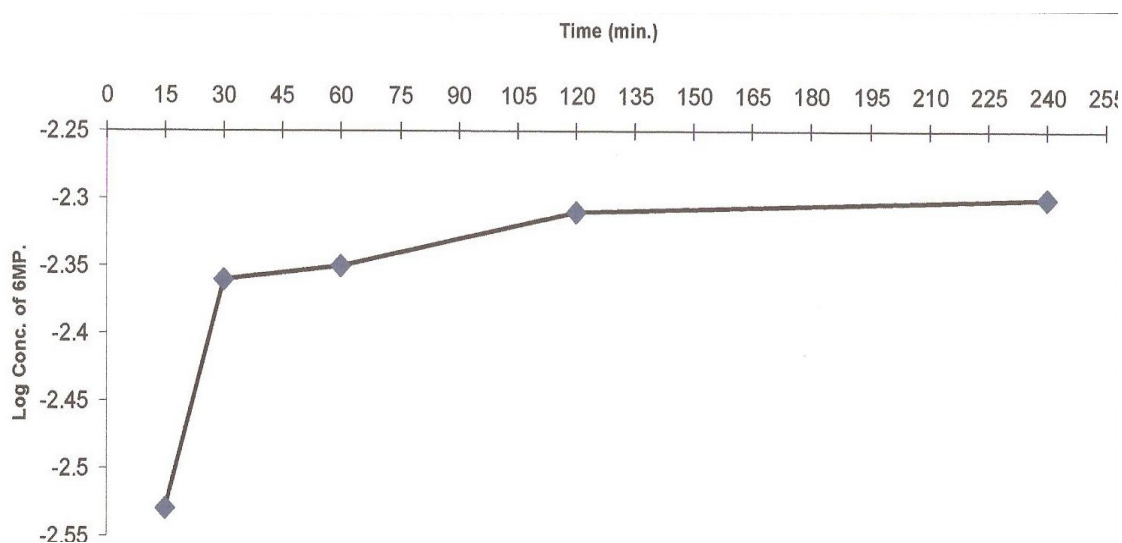


Figure (3): Plot of the hydrolysis of compound II in the presence of glutathione (0.17 mmole) in phosphate buffer of pH 7.4 at 37 °C ($\mu=1$).

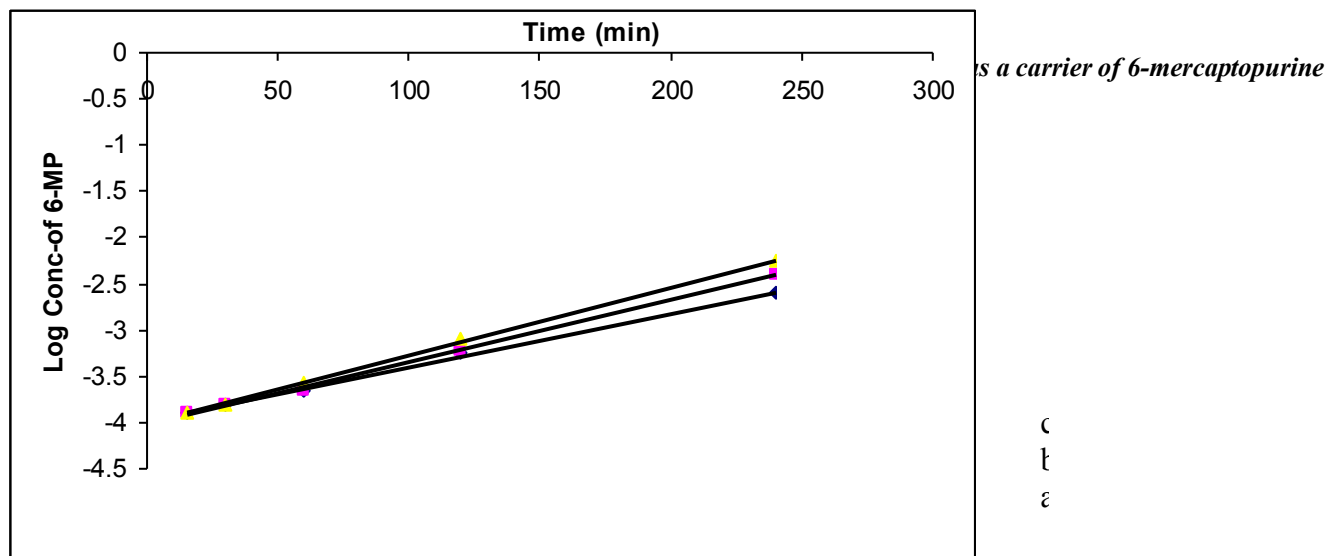


Figure (4): The effect of buffer concentration on the rate of hydrolysis of compound II at Ph 6 at 37 °C ($\mu=1$).

* buffer concentration:

a= 0.2 M , b= 0.5 M , c= 0.8 M

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