Synthesis and in vitro kinetic study of new mutual prodrug for colon cancer associated with constipation

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Abstract

Objective: a new mutual prodrug was synthesized for colon targeting in the treatment of colon cancer associated with constipation. ***Methods:*** a new mutual prodrug was synthesized through several steps included amide hydrolysis in a strong acidic medium, amide synthesis, diazotization and coupling reactions. The stability of this prodrug in HCl buffer, in phosphate buffer and in rat fecal matter was monitored. The chemical structure of mutual prodrug was characterized by physical and spectroscopic techniques as FTIR, UV-Visible and ^{13}C NMR spectra. In colon, the mutual prodrug was proposed to split by the action of bacterial azoreductase into two N-substituted benzamides, metoclopramide and declopramide, that constituted two apoptotic agents, also the local application of metoclopramide on colonic smooth muscles was proposed to enhance their contractions affords relief of constipation. In vitro kinetic studies in a hydrochloric acid buffer showed an insignificant release of metoclopramide and declopramide while in a phosphate buffer, only (9.12٪) release was observed over six hours. In order to confirm the hydrolysis of mutual prodrug in colon, the release study in a rat fecal matter was monitored over six hours and showed that the hydrolysis was almost complete (90.88٪) with a half-life of (166.19 min) followed first order kinetics. The prodrug approach that is based on enzymes specification may offer a new method to improve drug efficacy and reduce side effects.

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الملخص

الهدف: تصنيع بادئ دواء تبادلي جديد لغرض استهداف القولون لعلا ج سرطان القولون المترافق مع الإمساك . طرق العمل: تم تصنيع بادئ دواء تبادلي جديد من خلال عدد من الخطوات ضمت تحلل أصرة الامايد في وسط حامضي قوي، تصنيع أصرة الامايد، تفاعلا ت الدايزونيم والاقتران. كما تم ت دراسة استقرارية بادئ الدواء التبادلي في محلول حامض الهايدروكلوريك البفري، في محلول الفوسفيت البفري وكذلك في مادة براز الجرذان . النتائج: تم تشخيص الشكل الكيميائي لبادئ الدواء التبادلي باستخدام الوسائل الفيزيائية والطيفية كطيفي الأشعة تحت الحمراء وفوق البنفسجية /المرئية والرنين النووي المغناطيسي للكربون . في القو لون وتحت تأثير إنزيم الازوريدكتيز البكتيري يفترض أن ينشطر بادئ الدواء التبادلي ليحرر عقاري الميتوكلوبرامايد والديكلوبرامايد اللذان ينشطان الموت المبرمج للخلايا كما أن التماس الموضعي لعقار الميتوكلوبرامايد مع عضلات القولون الملساء يفترض أن يزيد من تقلصها وبالتالي يساعد في علاج الإمساك . لقد أظهرت الدراسات الحركية خارج جسم الكائن الحي استقرارية بادئ الدواء التبادلي في محلول حامض الهايدروكلوريك البفري وتحرير فقط (٩,١٢٪) في محلول الفوسفيت البفري خلال فترة ٦ساعات. لغرض التأكد من تحلل بادئ الدواء التبادلي في القولون، تمت دراسة تحر ره في مادة براز الجرذان والذي كان شبه كامل (٩٠,٨٨٪) خلال فترة ٦ساعات وبعـــمر نصف قدره (١٦٦,١٩دقيقة) متبعا حركيات الرتبة الأولى. الاستنتاج: إن نهج بادئ الدواء الذي يعتمد على خصوصية الأنزيمات قد يشكل طريقا جديدا لتحسين الأداء العلاجي للدواء وتقليل أثاره الجانبية.

Introduction

Colon cancer is the second cause of cancer related deaths in the world. Although improvements have been made in surgical techniques and in chemotherapies, the survival rate is still low.[1] Variability in the signs and symptoms of the colon cancer poses a significant challenge to the physician.[2, 3] The patients are more likely to notice a change in the bowel habits, as constipation which can occur owing to a constricting lumen due to tumor mass in addition to the psychological and diet factors.[4, 5] Apoptosis, or programmed cell death, plays an important role in the development and maintenance of tissue homeostasis.[6] Multicellular organisms also use apoptosis to eliminate potentially dangerous cells, such as genetically damaged cells, including tumor cells.[7] Abnormalities in apoptotic function have been identified as contributing events in the pathogenesis of colon cancer. Furthermore, resistance to apoptosis induction by chemotherapeutic drugs or radiotherapy frequently hampers their efficacy in the treatment of established tumors.[8] During apoptosis, a complex death program is initiated that ultimately leads to the fragmentation of the cell.[9] The death program can be induced by two major apoptosis signaling pathways namely the 'extrinsic' and the 'intrinsic' pathway. The extrinsic pathway is initiated by triggering cell death receptors on the cell surface, leading to activation of the intracellular apoptotic machinery. The intrinsic pathway of apoptosis is initiated via the mitochondria by cellular stress, for example following DNA damage caused by chemotherapeutic drugs and radiation.[10-12] The nuclear factor kappa B (NF-κB) signaling pathway has also been the subject of intense study as a drug target.[13] From its initial description as a transcription factor for the immunoglobulin κ locus,[14] this multimember family of transcriptional activators has been implicated in a large number of biological mechanisms in both health and disease.[15] Recently, it was also shown that NF-κB was involved in the regulation of apoptotic cell death.[16] Hence, if NF-κB activation was blocked, cells that had received an apoptotic stimulus showed a more pronounced response than a corresponding cell population where the NF-κB activation pathway was intact.[17, 18] This salvage pathway from apoptotic cell death can thus be seen as a potential mechanism for the survival of tumor cells and the inhibition of NF-κB activation may thus be a mean for the treatment of colon cancer.[19, 20] N-substituted benzamides have been exploited in the clinic as anti-emetics, anti-psychotics, anti-arrhythmics, local anesthetics, anti-inflammatory, anti-tumor agents, radio- and chemo-sensitizers.[21-23] These compounds as metoclopramide and declopramide have been shown to have several targets like dopamine and hydroxytryptamine receptors and apoptosis through the inhibition of NF-κB signaling pathway.[24, 25] The aim of study was the synthesis of a new mutual azo prodrug which was proposed to split in colon by the action of bacterial azoreductase leading to liberate two apoptotic agents, metoclopramide and declopramide, these agents were proposed to treat colon cancer through the inhibition of NF-κB signaling pathway and also the local application of metoclopramide on colonic smooth muscles affords relief of constipation. In vitro kinetic studies of this mutual prodrug were monitored in a hydrochloric acid buffer (pH 1.2) and in a phosphate

buffer (pH 7.4) while its hydrolysis was established in a rat fecal matter.

Materials and Instruments

Materials

Metoclopramide was supplied from Al-Hokamaa Drug Industry (Iraq) as purified hydrochloride salt while declopramide was purchased from Lanospharma Laboratories Co. Ltd (China). All chemicals used in preparations were supplied from Fluka Company and the solvents were purified prior to use.

Instruments

The melting points were determined in open capillaries on electrothermal CIA 9300 melting point apparatus and are uncorrected. The ultraviolet-visible spectra were obtained via Carrywinn U.V. Varian U.V. -visible spectrophotometer while the infrared spectra were recorded by Buck 500 scientific I.R. spectrophotometer. The ^{13}C NMR spectra were recorded by Bruker AM 300 instrument (Sweden). Thin-layer chromatography (TLC) was carried out on TLC plastic sheets silica gel 60 F5 precoated, 20 ×20 cm, layer thickness 0.2 mm, the spots on the chromatograms were localized by U.V. light (at 366 nm) and iodine vapor, the solvent system employed for separation composed from methanol: strong ammonia solution (98.5:1.5).

Experimental methods

Preparation of N,N-diethylethylenediamine: [26]

In a water bath, metoclopramide (30 gm, 100 mmol) and (60 ml) of 6N HCl were refluxed at (100°C) for (5 hr). After cooling, the reaction mass was extracted three times with ether and the aqueous layer was collected and evaporated by rotary evaporator under reduced pressure. The crude product was recrystallized from ethanol-water mixture (7:3) and the compound purity was established by TLC. The melting point was (211-213°C) with (48٪) yield; the R_f value was (0.42) while the λ_{max} (ethanol) was (274 nm). The obtained hydrochloride salt of amine was dissolved in (30 ml) of distilled water and a sufficient amount of diluted NaOH was added dropwise until the solution was alkaline to litmus. The miscible solution was concentrated and the pure amine was obtained by distillation at (146°C), the distilled light yellow liquid was used immediately.

Synthesis of 3-chloro-N-(2-diethylaminoethyl)benzamide: [27]

In a water bath, 3-chlorobenzoic acid (15.6 gm, 100 mmol) and an excess of thionyl chloride were refluxed for (2 hr). The excess amount of thionyl chloride was removed under vacuum to give 3-chlorobenzoyl chloride. From a dropping funnel, toluene (20 ml) was added dropwise to a mixture of 3-chlorobenzoyl chloride (9.5 gm, 50 mmol) and N,N-diethylethylene diamine (7 ml, 50 mmol). The reaction mixture was stirred at room temperature for (30 min), refluxed for (2 hr) and then neutralized with ice cold diluted HCl. The precipitate was filtered off, washed with cold water, dried, recrystallized from ethanol and the compound purity was established by TLC. The melting point was (118-120°C) with (68٪) yield; the R_f value was (0.49) while the λ_{max} (ethanol) was (326 nm).

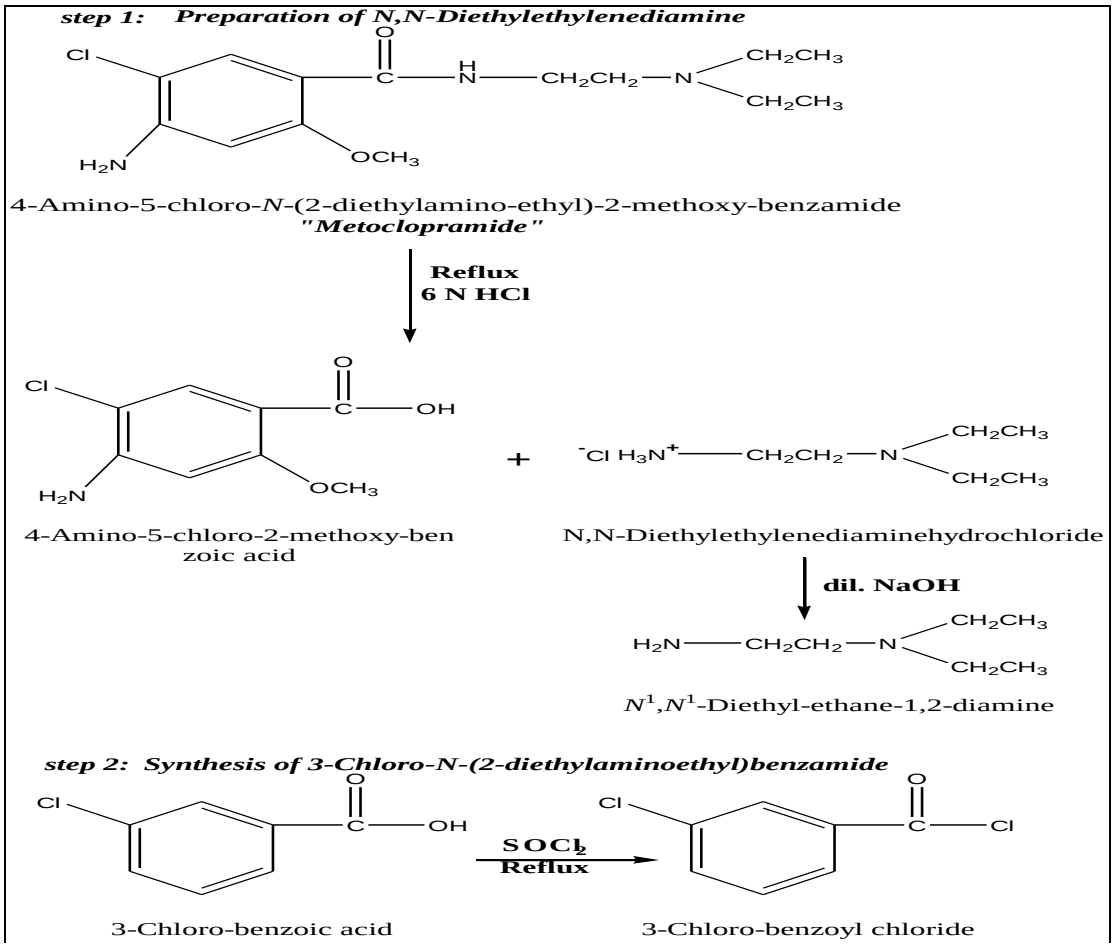
Synthesis of the diazonium salt of metoclopramide: [28]

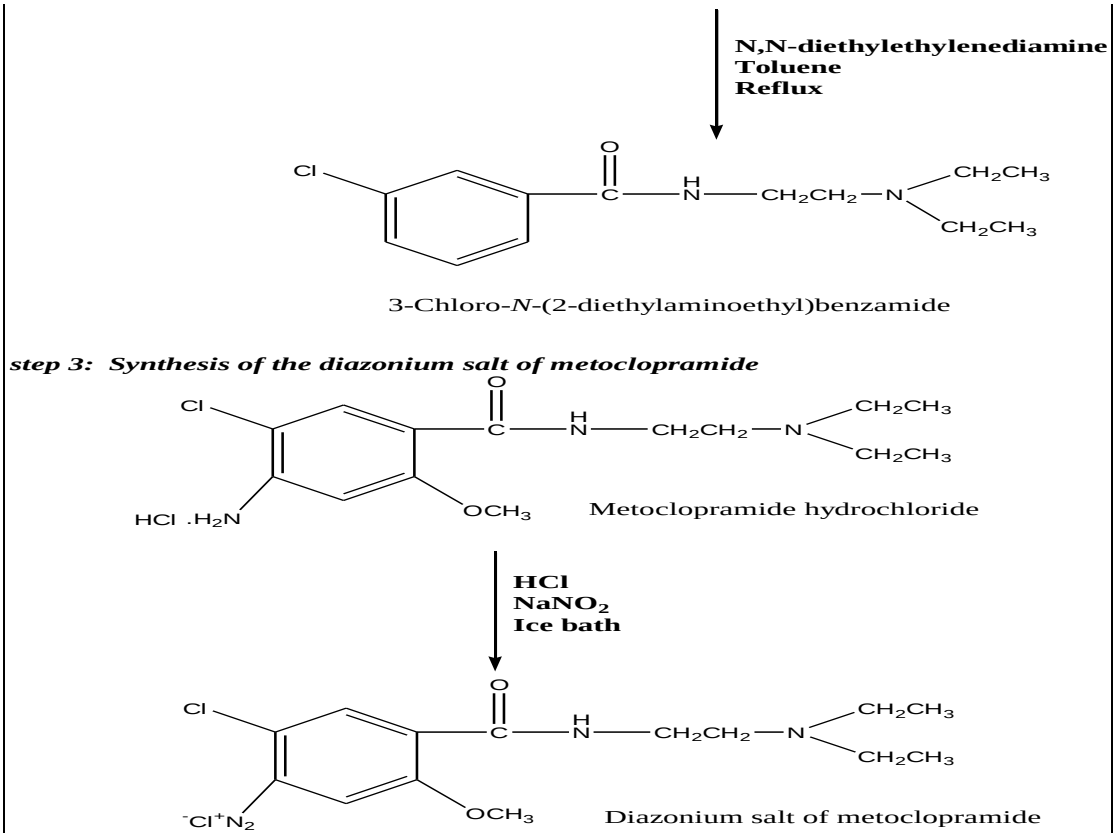
In a beaker, metoclopramide hydrochloride (3.54 g, 10 mmol) was dissolved in a mixture of (25 ml) water and (2 ml) concentrated HCl; the resulting solution was cooled by immersing in a bath of crushed ice. The cold solution of sodium nitrite (0.83 g, 12 mmol) in (12 ml) water was placed in a dropping funnel and added

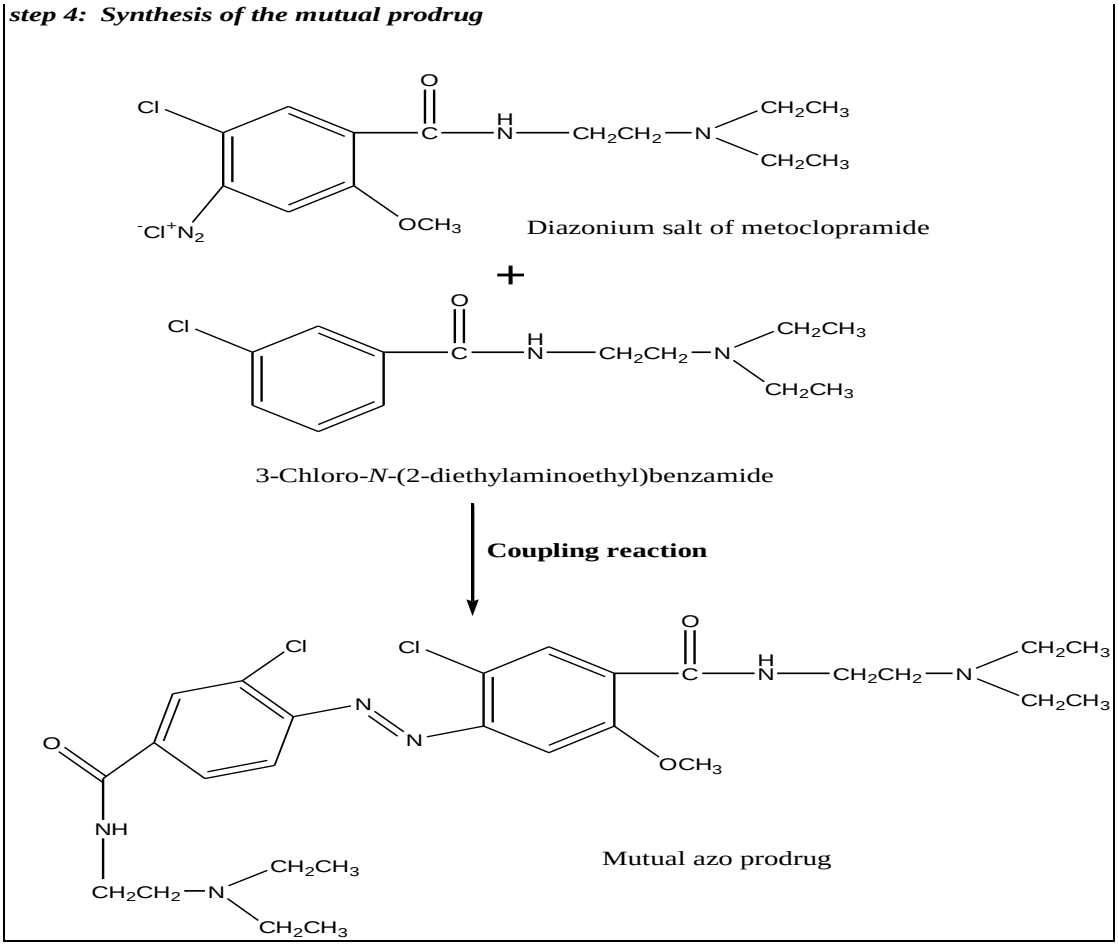
dropwise to the stirred solution of metoclopramide hydrochloride in an ice bath; the reaction temperature was kept below (5°C) by adding few grams of crushed ice when necessary. After the last addition, the resulting solution was stirred for (5 min) in an ice bath and the solution was tested with potassium iodide-starch paper until an immediate blue color was obtained at the point of contact. The diazonium salt solution of metoclopramide was kept in an ice bath until used.

Synthesis of the mutual prodrug: [29]

In an ice bath, 3-chloro-N-(2-diethylaminoethyl)benzamide (2.55 gm, 10 mmol) was dissolved in (10 ml) of glacial acetic acid and the resulting solution was stirred for (20 min). From a dropping funnel, the cold diazonium salt solution of metoclopramide was added dropwise to the previous solution and the reaction mixture was stirred for one hour at below (10°C). Then the pH of solution was maintained between 4.5 and 5 by adding dropwise an aqueous solution of (5٪) sodium acetate. Stirring was continued for (3 hr) at less than (10°C) and the colored crystals soon separated, filtered off and washed three times with cold water. The crude product was purified by recrystallization from chloroform and the compound purity was established by TLC. The melting point of mutual prodrug was (239-241°C) with (41٪) yield; the R_f value was (0.46) while the λ_{max} (chloroform) was (586 nm). The steps for the synthesis of mutual prodrug are illustrated in Scheme 1.







Scheme 1. Steps for the synthesis of mutual prodrug.

In vitro stability studies in hydrochloric acid and phosphate buffers: [30]

The stability of mutual prodrug in a (0.05 M) hydrochloric acid buffer (pH 1.2) was examined by dissolving (2.83 gm, 5 mmol) of compound in (1000 ml) of HCl buffer; the resulting solution was stirred and kept in a water bath at a constant temperature (37 ± 1°C). The absorbance at zero time and the absorbance coefficient (ε) were determined by using (2 cm) quartz U. V. cell. The decrease in prodrug concentration with the time was monitored every (30 min) for three hours. In order to examine the stability of mutual prodrug in a phosphate buffer, the same procedure was followed except that the phosphate buffer replaced the HCl buffer and the spectral data were taken every (30 min) for six hours. Table 1 shows the kinetic data obtained from in vitro stability studies in HCl and in phosphate buffers.

Table (1):- Kinetic data obtained from the stability studies.

Type of buffer	A	λ_{max} (nm)	a (mol×10 ^{6})	ε
HCl (pH 1.2)	0.087	564	132	330.68
Phosphate (pH 7.4)	0.109	602	132	412.80

A = absorbance, a = concentration at zero time and ε = absorbance coefficient.

In vitro release study in a rat fecal matter: [31]

The release study of mutual prodrug in a rat fecal material was examined by dissolving (0.068 g, 120×10^{-6} mol) of compound in a sufficient volume of phosphate buffer (pH 7.4) to achieve a final concentration of (300 µg /ml). In a water bath, a set of test tubes was incubated at a constant temperature (37 ± 1°C); each test tube contains (1 ml) of the above solution diluted to (5 ml) with phosphate buffer to achieve a final concentration of (60 µg /ml) and (1 g) of fresh rat fecal material. Every (30 min) for six hours, a test tube was removed from a water bath and estimated on UV-visible spectrophotometer to determine the remaining amount of mutual prodrug. The kinetic studies (stability studies in a HCl buffer; in a phosphate buffer and the release study in a rat fecal matter) were carried out in triplicate and monitored by the decrease in a prodrug concentration with the time.

Results and Discussion

Colon has recently received a great attention as a potential site for the delivery of pharmaceutical moieties; the factor usually exploited for this is the pH gradient between the stomach and the colon.[32-34] Colon-specific drug delivery was developed for treating many local conditions including colon cancer where it is necessary to attain a high concentration level of drug.[35-37] The successful delivery of drug to colon requires the protection of a drug from being released in the stomach and small intestine; this can be achieved by the use of a special drug delivery system that can protect the drug during its transfer to the colon.[38, 39] Colon is known to be a reductive medium in which azo group is reduced to the corresponding amines.[40] The azo compounds could be used for colon targeting since reduction and subsequent splitting of the azo bond occurs only in the large intestine and therefore they are highly site-specific. [41,

[42] This opportunity for reductive degradation of azo compounds by colonic bacteria was exploited in this study to prepare a new mutual azo prodrug for colon targeting in the treatment of colon cancer associated with constipation.

Preparation of N,N-diethylethylenediamine

The heating of amide in a concentrated aqueous acid leads to its hydrolysis into carboxylic acid derivative and amine. [43] The most acceptable mechanism of this hydrolysis is that the amide carbonyl accepts a proton from the concentrated acid, then a water molecule attacks the protonated carbonyl to form a tetrahedral intermediate which split to give carboxylic acid derivative and amine. [44, 45]

Synthesis of 3-chloro-N-(2-diethylaminoethyl)benzamide

3-Chlorobenzoyl chloride was prepared by treating 3-chlorobenzoic acid with thionyl chloride. This acid chloride has a very electrophilic carbonyl and its chloride was readily displaced by nucleophile (e.g. amine).[46] Atmospheric moisture must be carefully avoided because the acid chloride reacts vigorously with H_2O.[47] To minimize the contact with air, the 3-chlorobenzoyl chloride is not isolated or identified, but reacted directly with N,N-diethylethylenediamine. [48]

Synthesis of the diazonium salt of metoclopramide

Metoclopramide hydrochloride dissolved in a mixture of distilled water and HCl was treated with sodium nitrite to form a diazonium salt, this process called a diazotization of primary amine.[49, 50] Two important points must be taken in consideration in the preparation of diazonium salt; first, the amine is comparatively a weak base, so that, a certain amount of amine may be produced by salt hydrolysis unless an excess amount of acid is present.[51-53] Second, the reaction mixture must be kept very cold during the process; otherwise, the diazonium salt may be partially converted into the corresponding hydroxyl compound.[54-55]

Synthesis of the mutual prodrug

The prepared mutual prodrug offers several advantages; among them the prodrug was proposed to split by bacterial azoreductase in colon to give two N-substituted benzamides, metoclopramide and declopramide, which are well known antitumor compounds.[56, 57] Declopramide was used for many years ago in a combination with cis-platin or 5-flourouracil in the treatment of patients with advanced stage of colon cancer.[58, 59] The principal mechanism of action of these N-substituted benzamides is the inhibition of NF-κB that results in apoptosis and subsequently local treatment of colon cancer.[60-62] The second advantage is the local application of metoclopramide on colonic smooth muscles may enhance their contractions affords relief of constipation; this prokinetic effect of metoclopramide on colonic smooth muscles has been studied extensively for in vitro clinical models.[63-68] The third advantage is the mutual prodrug approach may minimize the administrated dose and reduce the side effects of the involved drugs.[69-74] The last advantage is the presence of azo bond may enhance the water solubility of slightly water soluble N-substituted benzamides. [75, 76] The application of the computerized program (Cambridge Internet Chemistry Software leader) which depends on the calculation of partial charge, heat of formation and steric hindrance was recommended that the mutual prodrug is more

common to be formed comparing with other possible products. Then, the structure of the mutual prodrug was established by FTIR, UV-Visible and ^{13}C NMR spectra.

The FTIR spectrum of mutual prodrug

The FTIR spectrum (KBr, ν) of mutual prodrug shows the following absorbance bands; (N-H) stretching vibrations of amide groups as medium bands at (3349, 3321, 3180, 3189) cm^{-1}, (C=O) stretching vibrations of amide groups as strong bands at (1597, 1612) cm^{-1}, asymmetrical (C-O-C) stretching vibration of aryl alkyl ether as a medium band at 1269 cm^{-1} and the strong absorbance bands of (C-Cl) groups appear at (1081, 1092) cm^{-1}. The disappearance of medium absorbance bands of primary aromatic amine groups at (3396, 3386) cm^{-1} of metoclopramide and declopramide respectively, and the appearance of a weak absorbance band of azo group at 1502 cm^{-1} were confirmed the formation of mutual prodrug.

The ^{13}C-NMR spectrum of mutual prodrug

The ^{13}C-NMR (300 MHz, $CDCl_3$) spectrum of mutual prodrug reports that the carbons of side chain methyl groups resonated at δ (31.51, 32.98, 34.87 and 37.12) ppm while the carbon atoms of methylene groups resonated at δ (53.62, 54.01, 56.11, 56.67, 58.88, 58.98, 59.23 and 59.77) ppm. The aryl carbon atoms attached to chloride atoms resonated at δ (126.56 and 131.14) ppm while the aryl carbon atom attached to ether resonated at δ 151.92 ppm and the methyl carbon atom of ether group resonated at δ 54.26 ppm. The carbonyl carbon atoms of benzamides resonated at δ (173.16 and 176.62) ppm. The disappearance of the peaks at δ (141.21 and 149.12) ppm that attributed to aryl carbon atoms attached to primary amine groups of metoclopramide and declopramide respectively; and the appearance of peaks at δ (160.22 and 164.34) ppm that attributed to aryl carbon atoms attached to azo group were confirmed the formation of mutual prodrug.

In vitro kinetic studies

The mutual prodrug in a hydrochloric acid buffer showed an insignificant release of metoclopramide and declopramide; while in a phosphate buffer, only (9.12٪) cumulative release was observed over six hours; thus, the objective of bypassing the upper gastrointestinal tract with minimum prodrug release was achieved. Further study in a rat fecal matter was carried out to confirm the reduction of mutual prodrug by colonic bacteria; the mutual prodrug gave (90.88٪) cumulative release of metoclopramide and declopramide over six hours. Table 2 shows the kinetic data obtained from the release study of mutual prodrug in a rat fecal matter at 37°C and λ_{max} (602 nm).

Table (2):- Kinetic data of the release study of mutual prodrug in a rat fecal matter.

Absorbance	Time (min.)	x (mole×10 ^{6})	(a-x) (mole×10 ^{6})	a\(a-x)	ln (a\a-x)
0.0763	30	92.41	27.59	4.35	1.47
0.0737	60	89.23	30.77	3.90	1.36
0.0701	90	84.91	35.09	3.42	1.23
0.0660	120	80.00	40.00	3	1.10
0.0618	150	74.89	45.11	2.66	0.98
0.0567	180	68.72	51.88	2.34	0.85
0.0514	210	62.31	57.69	2.08	0.73
0.0446	240	54.07	65.93	1.82	0.60
0.0383	270	46.38	73.62	1.63	0.49
0.0307	300	37.24	82.76	1.45	0.37
0.0211	330	25.51	94.49	1.27	0.24
0.0114	360	13.81	106.19	1.13	0.12
0	390	0	120.00	1	0

(a)= conc. of mutual prodrug at zero time and equal to (120×10^{-6} mole), (x) = conc. of mutual prodrug remaining for any time.

The release study of mutual prodrug in a rat fecal matter followed first order kinetics (Figures 1and 2); the $t_{1/2}$ of mutual prodrug (average of three trials) was (166.19 min) while the rate constant (*k*) was (4.17×10^{-3} ± 0.0001 min^{-1}).

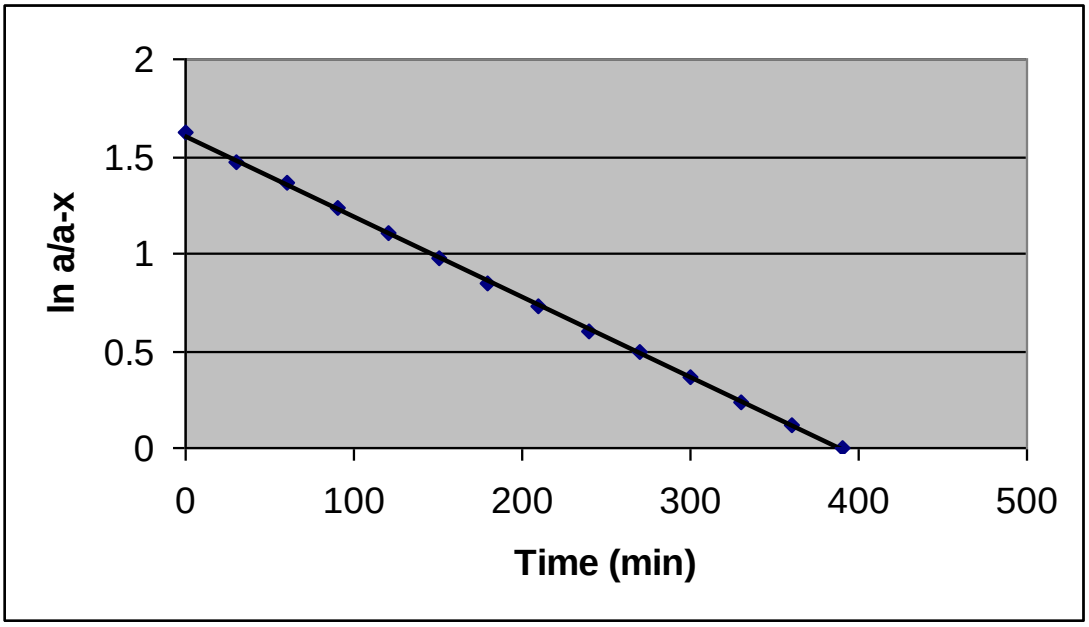


Figure (1):- The slope of the release study of mutual prodrug in a rat fecal matter.

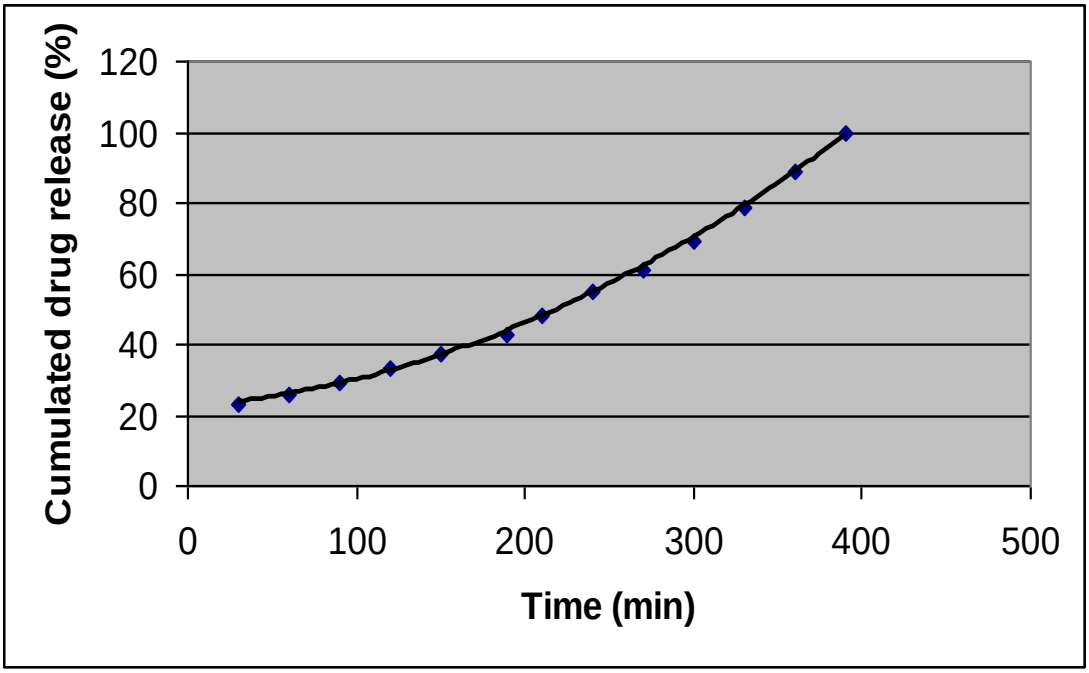


Figure (2):- The release profile of metoclopramide and declopramide from the mutual prodrug in a rat fecal matter.

Conclusion

This study reports the synthesis and in vitro kinetic studies of a new mutual azo prodrug. This mutual prodrug was synthesized through several steps and its chemical structure was characterized by physical and spectroscopic techniques as FTIR, UV-Visible and ^{13}C-NMR spectra. Properties of this mutual prodrug acting as a colon-specific compound was evaluated depending on in vitro kinetic studies in a hydrochloride buffer, in a phosphate buffer and in a rat fecal matter. The study reports that only (9.12٪) of mutual prodrug was hydrolyzed in the upper gastrointestinal tract while (90.88٪) of mutual prodrug was delivered to colon and split by bacterial azoreductase to liberate metoclopramide and declopramide, these compounds were proposed to inhibit NF-κB and induce apoptosis resulting in the treatment of colon cancer; as well as the local application of metoclopramide on colonic smooth muscles may afford relief of constipation. Therefore, this prodrug is a promising colon specific prodrug for colon cancer associated with constipation and worthy of further study.

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