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Synthesis, identification and preliminary evaluation of esters and amide derivatives of diflunisal

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Summary

This work examines the suitability of the methyl, ethyl esters and amide derivatives of diflunisal for use as prodrugs to diflunisal. Synthesis, identification and characterization of these forms is described. In vitro hydrolysis studies of these compounds in acidic and alkaline conditions were performed. The compounds were very stable in 0.1 HCl but hydrolyzed to diflunisal at pH 10 following a pseudo-first-order kinetics. The rate of hydrolysis was in the order: methyl ester > amide > ethyl ester, and the rate constants (K_{obs}) and the half-life of each prodrug was determined. Preliminary in vivo work in rabbits on the methyl ester derivative showed that diflunisal was detected in the blood after 3 h of administration and suggesting that the methyl ester was converted in vivo to the parent drug diflunisal. The very poor water solubility of these derivatives and the very low rate of hydrolysis in 0.1 N HCl may overcome both the bitter taste of the drug and its gastric irritant action, respectively.

Introduction

Diflunisal (5-[2,4-difluorophenyl]salicylic acid) is a weakly acidic drug exhibiting analgesic, antipyretic and anti-inflammatory properties (Tempero et al., 1977; Stone et al., 1977). It is more potent than aspirin (Hannah et al., 1978), but similarly has two major disadvantages, namely the very bitter taste and the gastric irritant action (after oral administration). The latter may lead to gastric ulceration and hence haemorrhage. In this work an attempt is made to minimize these drawbacks while maintaining the drugs' therapeutic activity. This was achieved by utilizing the drug latentiation or prodrug approach proposed by Harper (1959, 1962) to describe the bioreversible structural modification of a compound in such a way that the synthesized derivative, while not possessing certain undesirable properties of the original compound, is easily converted to the parent compound once administered to the patient. The ester and amide derivatives of drugs (Bundgaard et al., 1988; Sinkula, 1977) were shown to exhibit very low solubility and a low rate of hydrolysis in acidic conditions. This renders them suitable for masking the taste of the bitter tasting drug as well as overcoming their gastric irritant action. This work therefore examines the suitability of using the methyl, ethyl esters and amide derivatives of diflunisal as a possible prodrug to overcome its bitter taste and gastric irritant action. This involved the synthesis, identification, and

characterization of these derivatives. The work also included the study of the acid and alkaline hydrolyses at various pHs in order to assess the ease of their conversion to the parent drug in vitro. Preliminary in vivo studies on the methyl ester using rabbits were performed in order to determine whether this derivatives is converted to diflunisal once administered to the rabbit.

Materials and Methods

Apparatus

^{1}H-NMR spectral measurements were run on a Brucker WP-SY instrument, using acetone (d_6) as a solvent and tetramethylsilane as internal standard. Mass spectra were measured on a 7070-E VG Analytical mass spectrometer. Infrared spectra were recorded using a Pye-Unicam SP 300 instrument, as KBr disk. Melting points were determined on hot-stage Electrothermal melting point apparatus and were uncorrected. Elemental analyses were done at M-H-W Laboratories, Phoenix, AZ, U.S.A. High-performance liquid chromatography (HPLC) was generally done with a system consisting of a single piston pump (Beckman, Model 114), an injector (Beckman, Model 340) with 100 μl loop size, variable UV detector (Beckman, Model 165), an integrator-plotter (SP 4270) and a spherisorb 5 μm, RPC-8 column (250 × 4.6 mm, Phase Separation Inc., Norwalk, CT, U.S.A.).

Preparation of derivatives

Diflunisal (**1**) was obtained from Sigma Chemical Co, and used without any further purification. The ester and amide derivatives were prepared as follows.

(a) Synthesis of ethyl-5(2,4-difluorophenyl)salicylate (2). Diflunisal (3.0 g, 0.012 mole) was dissolved in absolute ethanol (50 ml). A few drops of sulfuric acid were added with stirring at room temperature. The solution was kept under reflux overnight, then poured into ice to give a white crystalline solid which was filtered by suction and washed with cold water. The product, after being absorbed in ether was washed with aqueous $NaHCO_3$, water and the ethereal solution was dried over anhydrous $MgSO_4$. The ethereal solution after being concentrated was chromatographed on silica column using 2% ethylacetate in petroleum ether eluent. Evaporation of solvent in the fast running band gave the desired ester product (2.5 g, 80% yield); m.p. 73–75 °C. I.R. spectrum showed absorptions at 3110 (br), 1670, 1600, and 1480 cm^{-1}.

(b) Synthesis of methyl-5(2,4-difluorophenyl)salicylate (3). This was prepared by a procedure similar to that adopted for the preparation of the ethyl ester (Vide-Supr), using methanol instead of absolute ethanol. M.p. of diflunisal methyl ester is 95–97 °C.

(c) Synthesis of 5-(2,4-difluorophenyl)salicylamide (4). To diflunisal ethyl or methyl ester (3.0 g) in a minimum volume of methanol was added excess concentrated ammonia with stirring. The mixture was refluxed for 2 h before being extracted with ether. Evaporation of the ether leaves behind a crystalline solid (2.3 g, 75% yield) which was recrystallized from chloroform; m.p. 190–192 °C. I.R. shows a doublet at 3480, 3420 cm^{-1} and absorptions at 3200 (br), 1670, 1600 and 1480 cm^{-1}.

All these new derivatives of diflunisal gave a microanalysis (C, H, F, N) data within ±0.4% of the calculated values.

HPLC analytical method

Methyl ester, ethyl ester of diflunisal and diflunisalamide derivatives were determined by a specific stability-indicating HPLC assay. In this method, solutions of each compound were eluted with a mobile phase composed of acetonitrile and deionized water (50 : 50 v/v) at pH 3.2. The flow rate was 1.3 ml · min^{-1} and the effluent was monitored at 270 nm and sensitivity range 0.04 AUFs. The chart speed was 0.1 cm · min^{-1}. Quantitation of each compound was achieved with reference to a suitably prepared calibration curve, using peak height ratio of the compound to flufenamic acid as internal standard. The linear regression equations were:

$y = 0.092 + 0.076$

($r = 0.9907$) for methyl ester

$y = 0.100 + 0.046$

($r = 0.9945$) for ethyl ester
$y = 0.033 + 0.067$
($r = 0.9941$) for diflunisal amide

In vitro kinetic measurements

Accurate quantities (10.0 mg) samples of methyl ester, ethyl ester and diflunisalamide were separately transferred to 10 ml volumetric flasks. The powder in each flask was dissolved in a solution of acetonitrile/water (50 : 50 v/v) preheated to 60 °C and the solution pH was adjusted to 10 using 2 M sodium hydroxide solution. The flasks were placed in thermostatically controlled water baths at 60 ± 1 °C. Aliquots (10 µl each) were withdrawn from each solution at zero time and then at 10 min intervals for a period of 1 h. Each aliquot was transferred to 1.5 ml Eppendorff tube, mixed with 10 µl of 1 mg · ml^{-1} flufenamic acid solution (internal standard) and then diluted to 1 ml with acetonitrile to quench the hydrolysis. The mixture was vortexed for 30 s and a 25 µl volume was injected onto the column, and eluted with a mobile phase composed of acetonitrile/water (48 : 52 v/v). Residual concentrations of each compound were determined from calibration curves and then plotted against the corresponding times on semilogarithmic graph paper. The observed rate constant (k_{obs}) was calculated from the slope of the linear plot by regression analysis. The half-life ($t_{1/2}$) was determined using the formula $t_{1/2} = 0.693/k_{obs}$.

In vivo studies

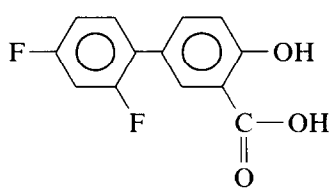
The potential of the methyl ester and amide derivatives of diflunisal as prodrugs was assessed in vivo using the rabbit as an animal model. Adult female white New Zealand rabbits weighing 3–4 kg were used. Food was not given to the rabbits for 16 h prior to and during the experiment, but water was allowed ad libitum. Diflunisal and its derivatives were separately suspended in water (50 mg in 20 ml) prior to gastric intubation. Blood samples (1.5 ml) were collected just prior to the drug administration and at 0.5, 1, 2, 3, 4 and 5 h after the drug intubation. The blood was obtained from the marginal ear veins and allowed to clot (1 h) prior to centrifugation. Serum samples obtained were freezed pending assay. A 0.5 ml aliquot serum was transferred to a 15 ml screw-capped tube and mixed with 0.5 ml of 0.2 M hydrochloric acid solution. The serum sample was extracted by shaking with 10 ml ether for 10 min. The organic and aqueous layers were separated by centrifugation at 2500 rpm for 5 min. The organic layer was separated and the ether evaporated under a stream of nitrogen gas at 45 °C. The residue was taken in 200 µl of the mobile phase (acetonitrile/water 45 : 55 v/v, pH = 3.2), transferred into 1.5 ml Eppendorff tube, and then centrifuged for 2 min in a microcentrifuge. An appropriate volume of the resulting clear solution was injected on the HPLC column.

Results and Discussion

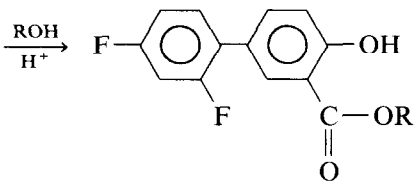
Synthesis and spectroscopic identification

Among the overwhelming number of salicylic acid derivatives (over 500 are known), diflunisal proved to be the most potent (Hannah et al., 1978). Being associated with the bitter taste and gastric irritant action, a bioreversible derivatization of diflunisal (a prodrug approach) seems feasible. We thought of examining the methyl, ethyl esters and amide derivatives for this prospect. This esterification procedure was not of good premise in the case of salicylic acid because the ester derivatives have a relatively low melting point (ethyl salicylate m.p. = 40–50 °C). However, such derivatives of diflunisal are expected to have a much higher melting point than those of salicylic acid because of the increased molecular weight effect (Bailey and Bailey, 1981).

The standard acid-catalyzed esterification procedure was adopted for the preparation of diflunisal ethyl ester (**2**) and methyl ester (**3**) derivatives, as shown below:

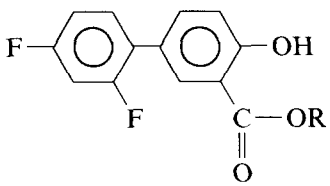


(**1**)

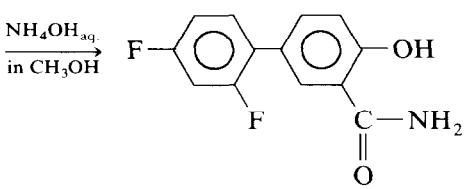


$R = C_2H_5$ (**2**)
$R = CH_3$ (**3**)

The ^{1}H-NMR spectra of the methyl and ethyl esters confirm their structures and the phenolic OH appeared in the range 9–11 ppm as a broad signal which disappears upon addition of a few drops of D_2O. The absence of carboxylic OH proton and the correct relative integrations of the methyl and ethyl protons to that of the aromatic protons assure the complete esterification. Either of these esters, upon reflux with concentrated ammonia gave a high yield of the diflunisalamide (**4**).

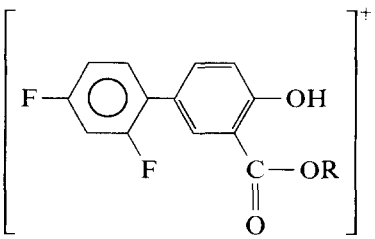


(**3**)

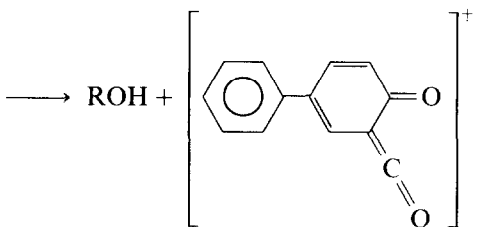


(**4**)

The ^{1}H-NMR spectrum of the amide (**4**) shows the phenolic OH proton signal at 13.0 ppm and the amide NH_2 protons at 3.0 ppm. Both of these peaks disappear upon addition of D_2O. The mass spectra of these esters and amide derivatives show a high relative abundance of the molecular ion (M^+) at m/e values corresponding to the respective molecular weight. This is usually the case in the mass spectra of aromatic compounds (Pavia, 1979a). In the mass spectra of the methyl and ethyl esters, it is worth mentioning that the base peak corresponds to the removal of neutral alcohol molecule (ROH) from the molecule ion as shown below:



M^+



$M^+ - ROH$

This behaviour is characteristics for ortho-substituted aromatic esters. However, this type of fragmentation was not observed in the mass spectrum of the amide (**4**). The base peak was due to M^+-(NH_2) rather than M^+–NH_3, i.e. in the typical fragmentation patterns of amides (Pavia, 1979b). The methyl and ethyl esters, as well as the amide derivatization procedures proved to increase the lipophilicity tremendously. The water solubility of these compounds was poor and a less polar organic solvent (alcohol or acetonitrile) had to be mixed with the water to enhance the solubility of the derivative for the HPLC and hydrolyses kinetic studies (vide infra).

Chromatographic identification

HPLC chromatograms for solutions of the methyl, ethyl esters of diflunisal, and that of diflunisalamide are reproduced in Fig. 1. The retention times of the parent acid, methyl ester, amide and ethyl ester derivatives are 6, 14, 15 and 21 min, respectively. All peaks were sharp and well-defined. The higher retention times are attributed to the lower polarity (higher lipophilic character) of these derivatives, as compared to diflunisal. This chromatographic identification procedure was

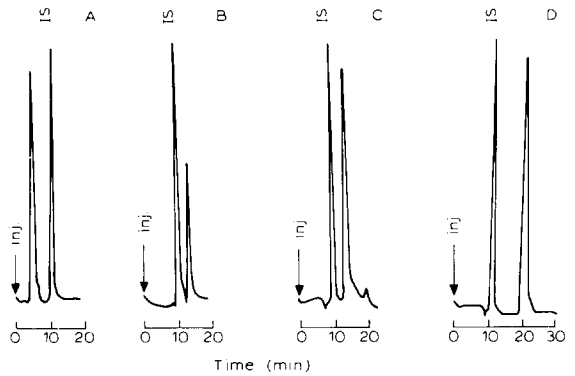


Fig. 1. HPLC chromatograms of standard solution containing diflunisal (20 $\mu g \cdot ml^{-1}$) (A), methyl ester of diflunisal (10 $\mu g \cdot ml^{-1}$) (B), diflunisalamide (20 $\mu g \cdot ml^{-1}$) (C) and ethyl ester of diflunisal (20 $\mu g \cdot ml^{-1}$) (D). Chromatographic conditions: mobile phase acetonitrile/water 50:50 v/v, pH 3.2; flow rate 1.3 $ml \cdot min^{-1}$; $\lambda = 270$ nm, sensitivity range 0.04 and chart speed 0.1 $cm \cdot min^{-1}$. Flufenamic acid (20 $\mu g \cdot ml^{-1}$) is used as an internal standard (IS).

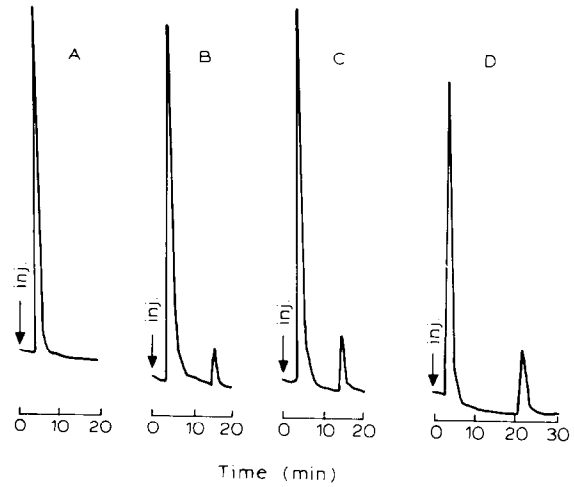


Fig. 2. HPLC chromatograms of the degraded solutions of methyl ester of diflunisal (B), diflunisalamide (C) and ethyl ester of diflunisal (D) in pH 10 at 60 °C showing the hydrolytic product, diflunisal (A) (C: each of 20 $\mu g \cdot ml^{-1}$).

adopted in the following in vivo and kinetic studies.

In vitro hydrolysis and kinetics

Due to the extremely poor water solubility of the prepared derivatives of diflunisal, the kinetic studies were conducted in 50% acetonitrile solutions. Neither the esters (methyl or ethyl) nor the amide derivatives exhibited any hydrolysis in 0.1 M HCl acetonitrile (50:50 v/v) or in phosphate buffer (pH 7.4) and acetonitrile (50:50 v/v) at 37 °C for 24 h. Similar experiments at more drastic conditions (at 60 °C for 24 h) indicated the absence of any hydrolysis product. A judicious organic chemist always expects arylcarboxylic esters to be less prone to hydrolysis than aliphatic ones. This result of resistance of acid hydrolysis indicates that the derivatives are not susceptible in the gastro-intestinal tract. Enzymatic hydrolysis, however, in the gastric and intestinal mucosa cannot be ruled out. Therefore, the compounds may be absorbed from the gut in the intact form.

To show that the prodrugs would convert to the parent compound in vitro, accelerated kinetic studies were carried out in alkaline solutions (pH 10) at 60 °C. Under these conditions of pH and temperature, the solutions of the methyl, ethyl esters and amide derivatives showed to hydrolyze to the parent compound, diflunisal. This is indicated by the HPLC analysis of the degraded solutions of compounds where two consecutive peaks corresponding to diflunisal and the ester or amide derivatives were obtained, as illustrated in the chromatograms in Fig. 2. Typical plots for the hydrolysis of the investigated compounds (percent residual concentration vs time) are illustrated in Fig. 3. It is indicated from Fig. 3 that the hydrolysis followed an overall pseudo-first-order kinetics. The rate constants (k_{obs}) were determined from the linear slopes and found to be 13.12×10^{-3}, 9.75×10^{-3} and 5.61×10^{-3} min^{-1} for the methyl ester, amide and ethyl ester of diflunisal, respectively. The respective half-lives ($t_{1/2}$) degradation was calculated to be 26, 71 and 123 min. The success of hydrolysis in basic medium but not in acidic conditions could be attributed to the immediate conversion of the acid formed (at pH 10) to the carboxylate salt which has unreactive carbonyl carbon (Lowry and Richardson, 1976). The kinetics data also indicates that the rate of the base-catalyzed hydrolysis of the methyl ester is faster than that of the amide, which in turn is faster than that of ethyl ester of diflunisal. The most reasonable mechanism for this hydrolysis is

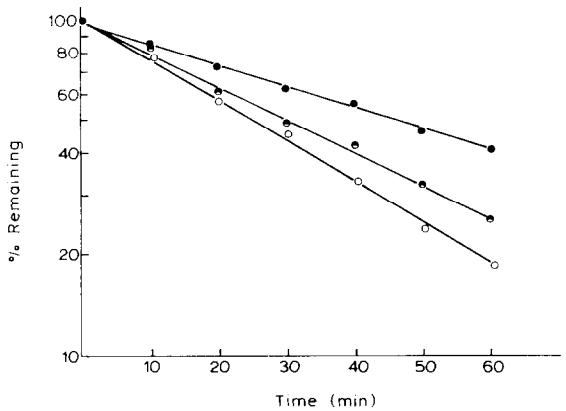
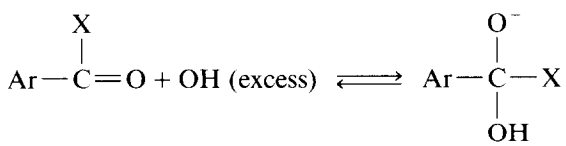
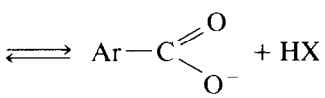


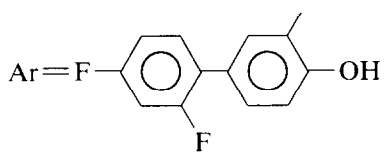
Fig. 3. First-order plots for the hydrolysis of methyl ester of diflunisal (○) diflunisalamide (◓) and ethyl ester of diflunisal (●) in pH 10 at 60 °C.

depicted below and involves a tetrahedral intermediate.





$X = OCH_3$, OC_2H_5, or NH_2



In vivo studies

The HPLC analysis of serum samples from rabbits treated with each of the methyl ester derivative, duflunisalamide, and the parent drug resulted in the chromatograms reproduced in Fig. 4. In Fig. 4, chromatogram A is for the blank serum, and chromatogram B shows the diflunisal peak at a retention time of 6 min. The latter peak was obtained for the serum sample taken from the rabbit given diflunisal, 2 h after drug administration. Moreover, it was possible to detect diflunisal

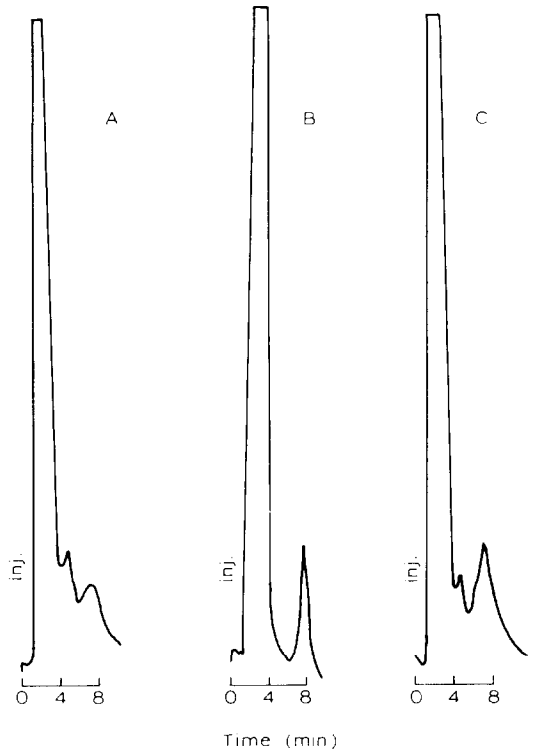


Fig. 4. HPLC chromatograms of rabbit serum. (A) blank; (B) 3 h after administration of 50 mg diflunisal; (C) 3 h after administration of 50 mg methyl ester of diflunisal. Chromatographic conditions: acetonitrile/water 45 : 55 v/v, pH 3.2; flow rate 1.3 ml·min^{-1}; sensitivity range 0.04; $\lambda = 270$ nm; and chart speed 0.25 cm·min^{-1}.

in serum samples collected after 3 h following administration of the methyl ester (Fig. 4C). However, it was not possible to detect the diflunisal peak from the serum samples collected over 5 h after administration of diflunisalamide. Similar results were reported for salicylamide (Lednicer and Mitschev, 1977) which was shown not to undergo conversion to salicylic acid. In this preliminary in vivo experiment, it was not intended to quantitate the rate of conversion of the methyl ester to the parent drug, but work regarding this aspect is in progress. Regarding the site of conversion of this prodrug to diflunisal, the liver seems to be the most likely. This speculation is based on the exclusion of the gastrointestinal lumen as a site of hydrolysis, since no hydrolysis was observed in the in vitro experiment using highly acidic conditions. The contribution of plasma esterases in the hy-

drolysis, however, cannot be excluded. On the basis of this preliminary experiment, it can be concluded that the methyl ester of diflunisal behaves as a prodrug in which the bitter taste problem of the parent drug is solved. The non-conversion to the parent drug in acidic conditions might eliminate a great deal of the gastric irritant action associated with diflunisal. Further research is in progress to evaluate the extent of gastric irritant effect and the site and rate of bioconversion.

Acknowledgement

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