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RESEARCH

SYNTHESIS, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF PALMITIC ACID DERIVATIVES OF SALICYLIC ACID AND ANTHRANILIC ACID

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ABSTRACT

The use of high dose of NSAID is accompanied by a number of side effects in disease conditions such as jaundice, loss of consciousness, liver damage and death. Non-steroidal anti-inflammatory drugs (NSAID) like aspirin, paracetamol and diclofenac are known for their widely valued pharmacological properties; however, they are toxic to the bio systems. One can overcome this limitation by decreasing the solubility of NSAID in aqueous phase. In the present study, salicylic acid and anthranilic acid have been modified as lipids PSA and *N*-PAA by a simple esterification / amidation reaction of the respective acid with palmitoyl chloride. On screening for the pharmacological activity, PSA has been found to exhibit potent and persistent analgesic and anti-inflammatory effects, while N-PAA, demonstrated novel analgesic and antipyretic effects.

INTRODUCTION

The pharmacological action of non-steroidal anti-inflammatory drugs (NSAID) like acetylsalicylic acid, paracetamol, indomethacin, diclofenac, ibuprofen, phenylbutazone, sulfasalazine is related to their inhibitory action upon cyclooxygenase and lipoxygenase enzyme by inhibiting prostaglandins and leukotriene synthesis(1,2). However, the use of high dose of NSAID is accompanied by a number of side effects in disease conditions such as jaundice, loss of consciousness, liver damage and death.

Aspirin based prodrugs produce compounds with lower gastric toxicity, greater stability or enhanced percutaneous absorption relative to aspirin (3). Published data offers numerous examples of lipophilic modification of various high and low molecular mass compounds like lipid derivatives of antitumor preparations such as 5-flourouracil conjugated with cholesterol (4), 5-fluoro-2'-deoxyuridine conjugated with palmitic acid (5), 1,2-dipalmitoyl phosphatidic acid (6) and phospholipids with ether and thioester bond (7,8) to increase their efficacy. Also, a much lower ulcerogenic potential is inherent in anti-inflammatory preparations that are poorly soluble in acidic medium of stomach (5).

In this connection a series of triglyceride derivatives of acetylsalicylic acid, indomethacin, 2-(4-isobutylphenyl) and 2-(4-benzoylphenyl)-propionic acid have been synthesized (9, 10, 11).However, such modifications lead to decreased anti-inflammatory activities. The conjugates of acetylsalicylic acid with 1, 3-dipalmitoylglycerol, 1, 3-didodecanoylglycerol and 1, 3-dioctanoylglycerol also showed lower activities than the parent compound. The pharmacokinetics of acetylsalicylic acid bound to 1, 3-diglycerides also differs from that of the initial compound (11).

The lipid-linked acetylsalicylic acid is known to stay in the stomach for at least 8 h and do not produce damage to the stomach mucous membrane. Investigations have shown that lipid bound acetylsalicylic acid is not altered in the stomach and is readily absorbed in the small intestine. Thus the attachment of lipid moiety to acetyl salicylic acid allows the drug to pass through the gastrointestinal tract without significant damage (10).

A considerable decrease in the solubility of drugs in the aqueous phase, including acid medium can be achieved through chemical modification of these drugs by conjugating with lipid molecules. This communication is aimed at designing the palmitoyl derived NSAID molecules like palmitoyl salicylic acid (PSA) and palmitoyl anthranilic acid (*N*-PAA). We also report that conversion into a lipid bound derivatives, resulted in enhanced anti-inflammatory, analgesic and anti-pyretic activities.

EXPERIMENTAL

Synthesis

Palmitoyl chloride

Palmitoyl chloride (Scheme.1) was prepared by gently refluxing a mixture of palmitic acid (25.6g, 100 *mmole*) and thionyl chloride (23.7g, 14.3 mL, 200 *mmole*)

on a steam bath for about 30 min. The excess of thionyl chloride was removed by washing successively with water and the residue was dried over anhydrous Na_2SO_4 to get palmitoyl chloride (20.4g, 74.5%).

Palmitoyl salicylic acid

To a mixture of palmitoyl chloride (2.74g, 10*mmole*) and salicylic acid (1.38g, 10*mole*), pyridine (0.79g, 9.7mL, 10 *mmole*) was added slowly and stirred at 25°C for 10 min, after the completion of the reaction, neutralized with ice cold diluted HCl. The solid thus separated was filtered, washed with water, dried and recrystallised from ethanol to get pure palmitoyl salicylic acid (PSA) (3.0g, 79.8 %).

Brown solid, Melting point- 250^{0}C - 251.5^{0}C

IR (KBr): υ 1265, 1560, 1750, 3320, 3580.

^{1}H NMR ($CDCl_3$): δ 0.96(t, 3H, CH_3), 1.29(qn, 22H, $(CH_2)_{11}$), 1.56(qn, 2H, CH_2), 1.7(m, 2H, CH_2), 2.23(t, 2H, CH_2), 7.28-8.10(m, 4H, aromatic CH), 11.0 (s, 1H, OH).

^{13}C NMR ($CDCl_3$): δ 13.8 (aliphatic CH_3), 23.1(aliphatic CH_2), 23.6(aliphatic CH_2), 24.8 (-C (=O)-C), 29.8(aliphatic CH_2), 30.0(two aliphatic CH_2), 30.3 (seven aliphatic CH_2), 32.5(aliphatic CH_2) 43.3(aliphatic CH_2), 115.6- 127.6(four aromatic CH) 122.1(aromatic C), 155(aromatic C), 163.5(carbonyl C carboxyl group), 207.1(carbonyl C amide bond).

N-Palmitoyl anthranilic acid

Palmitoyl chloride (2.8g, 10*mmole*), anthranilic acid (1.37g, 10*mmole*) were taken in a two necked flask, pyridine (0.79g 9.7mL, 10*mmole*) was added slowly to this mixture and stirred at 25ºC for 10 min, neutralized with ice-cold dilute HCl to get a solid. Filtration followed by washings with water, and recrystallization from hot ethanol, afforded pure *N*-palmitoyl anthranilic acid (*N*-PAA) (3.2 g, 85.4 %).

Colourless solid, melting point- 250.5^{0}C- 252.2^{0}C

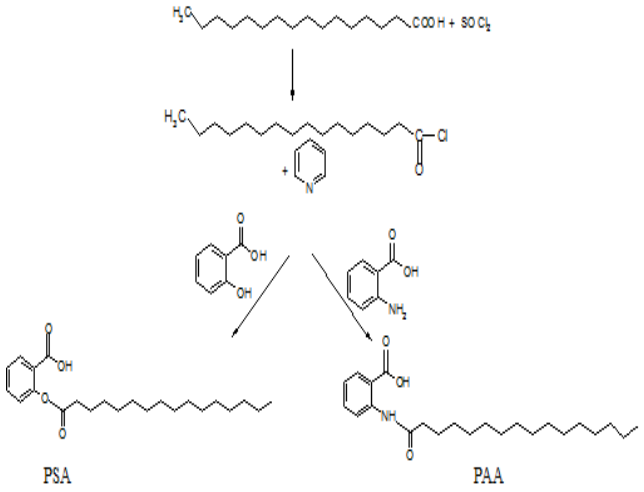
IR (KBr) : υ 1265, 1560, 1600, 2800, 3320, 3335, 3580

^{1}H NMR ($CDCl_3$): δ 0.96(t, 3H, CH_3), 1.29(qn, 22H, $(CH_2)_{11}$), 1.56(qn, 2H, CH_2), 1.72(m, 2H, CH_2), 2.23(t, 2H, CH_2), 7.28-8.10(m, 4H, aromatic CH), 8.0(s, 1H, OH), 11.0 (s, 1H, OH).

^{13}C NMR ($CDCl_3$) : δ13.6 (aliphatic CH_3), 23.1(aliphatic CH_2), 23.6(aliphatic CH_2), 29.8(aliphatic CH_2), 30.0(two aliphatic CH_2), 30.3 (six aliphatic CH_2), 32.5(aliphatic CH_2), 43.3(aliphatic CH_2), 115.0- 128.5(four aromatic CH), 121.5 (aromatic C), 144.4(-C (=O)-C), 163.5(carbonyl C for carboxyl group), 207.1(carbonyl C for amide bond).

Scheme 1. Chemical synthesis of palmitoyl derivatives; palmitoyl salicylic acid (PSA) and

palmitoyl anthranilic acid (N-PAA)



MATERIALS AND METHODS

The synthetic pathway for PSA and *N*-PAA is given in scheme.1. Melting points of the reaction products were determined with a Bu¨chi melting point apparatus. IR,^{1}H NMR,^{13}C NMR mass spectra were recorded on Nicolet 400D FT-IR spectrophotometer, 400 MHz Brucker spectrometer respectively. Purity of the compounds was ascertained by thin Layer Chromatography on silica gel plates using iodine as visualizing agent. All reagents were of analytical grade commercial products and used without further purification.

Pharmacological analysis

Wistar rats (180-230g) and albino mice of either sex chosen as experimental models were maintained at Government Pharmacy College, Bangalore. The animals were maintained in an animal house under standard environmental conditions. Ethical approval was obtained from Animal Ethical Committee, Government Pharmacy College, Bangalore, India.

All the compounds (200 mg kg^{-1} body mass) and reference NSAID, acetyl salicylic acid, paracetamol (100 mg kg^{-1} body mass) were suspended in 1ml soya oil and administered orally using an animal feeding needle. The control group received appropriate volume of vehicle (1ml soya oil, oral).

Anti-inflammatory activity

Animals (rats) were injected with 0.1 mL of freshly prepared suspension of carageenan (1.0% w/v) in the plantar region of right hind paw. Test animal groups were pretreated with test drug suspended in soya oil one hour before carrageenan treatment. Control group animals received only soya oil. The quantity of edema formed was measured for three hours after every hour using plethysmograph (12).

Antipyretic activity

Rats were given (*s. c.*) 20 ml/kg of 20% aqueous suspension of sterilized bacterial LPS. After 18 h animals showing an increase of rectal temperature >0.5ºC were selected. Control group received 1mL soya oil; treated groups received PSA and *N*-PAA respectively. Rectal temperature was determined by digital thermometer before (pretreatment) 0.5 h and at 0.5, 1, 2, 3 and 4 h after administration of drug (13).

Analgesic activity

Tests were carried by hot plate method, tail immersion method or acetic acid induced writhing test.

Hot plate test

Male and female albino mice showing reaction time of 10 sec to 55 ± 1ºC thermal stimulus were selected. The time for hind paw licking or jumping on heated plate of analgesiometer was taken as reaction time for every 30, 60 and 90 min intervals. Acetyl salicylic acid was used as standard.

Tail immersion test

Mice were placed in a restrainer cage after oral dose of drug. Animal was placed in such a way that its tail hangs out. In this position the tail (5 cm) dipped into the water bath maintained at 55 ± 1ºC. The reaction times (time to withdraw tail) were recorded after every hour.

Acetic acid induced writhing test

The mice were divided into four groups. 1st group received 0.3mL of carrier soya oil and they served as control The next two groups of 16 hours fasted mice were administered with PSA and PAA orally. Mice from 4th group received aspirin (200 mg kg^{-1} body mass). One hour after introduction of the drug all the mice received 0.1 mL of 3%

acetic acid to induce characteristic writhing response. The numbers of writhing occurring in 30 min were recorded. Analgesic activity was calculated as the percentage inhibition of abdominal constrictions (14).

Analgesic activity (%) = $(n - n^1 / n) \times 100$

Where n = mean numbers of writhes of control group and n^1 = mean no of writhes of test group.

Maximum tolerance studies

Acute toxicity studies were performed with rats of either sex by administration of single oral dose of 2000 mg / kg body weight for three different groups of animals (n = 6). The 1st group (control) was treated with soya oil while other two groups received drug to be tested and observed for 48 h. All the test animals survived without any side effect; hence dosage till 200 mg / kg body weight is safe. Hence, 1/10th of maximum dose was considered safe for further studies (15).

Statistics analysis

The statistical significance of treatments between groups was determined by one way ANOVA followed by Student's unpaired *t*-test to find correlation between the control group and groups of animals treated with the test compound. Results shown are mean ± S.E mean, n = 6,*** p<0.01

RESULTS AND DISCUSSION

Chemistry

The palmitic acid derivatives of salicylic acid and anthranilic acid were prepared by esterification and amide bond formation reactions of SA and AA with palmitoyl chloride in the presence of pyridine as a base. The reaction of salicylic acid and anthranilic acid in basic medium afforded PSA and *N*-palmitoyl anthranilic acid (*N*-PAA). The IR spectra of the these compounds revealed presence of absorption band at 1750 cm^{-1} and 1600 cm^{-1} for ester and amide bond respectively; also ^1H NMR of PSA is in agreement with its structure, displayed multiplet at δ7.28 - 8.10 for four aromatic protons and triplet at δ 0.96 indicating terminal two protons of long chain fatty acid .whereas to fatty acid ^{13}C NMR spectra show signal δ 207.1 for carbonyl carbon of ester bond confirming the formation of long chain ester. *N*-PAA ^1H NMR showed singlet at δ 8.0 for NH proton and triplet at δ 0.96, while ^{13}C NMR displayed signal at δ 207.1 for carbonyl carbon confirming the formation of amide bond.

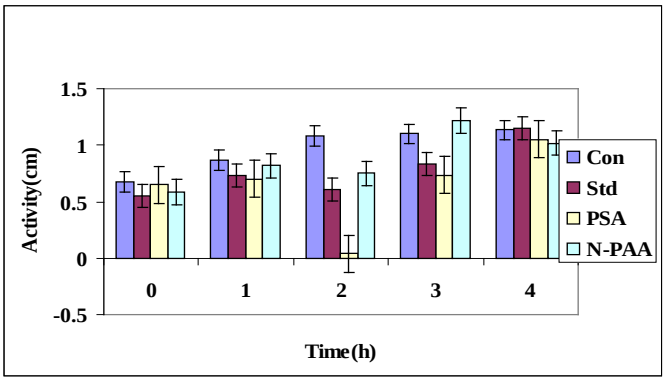
Pharmacology

Anti-inflammatory studies

The anti-inflammatory potential of PSA and *N*-PAA against various animal models exhibited significant (P< 0.05) results. The effect of PSA, *N*-PAA and acetyl salicylic acid on inflammation induced by carrageenan is summarized in figure.1.

As shown in figure.1, pretreatment with PSA showed maximum inhibition of 33. 4 % after 3 h of treatment and aspirin 24.3%. Whereas, *N*-PAA reduced edema only by 10.7%. These results suggest that anti-inflammatory effect of PSA and *N*-PAA on carrageenan induce edema. PSA is more potent than acetyl salicylic acid; the anti-inflammatory effect was started from 1st h and retained till 3rd h. whereas for *N*-PAA it was persistent till 4th h may be due to inhibition of inflammation mediators.

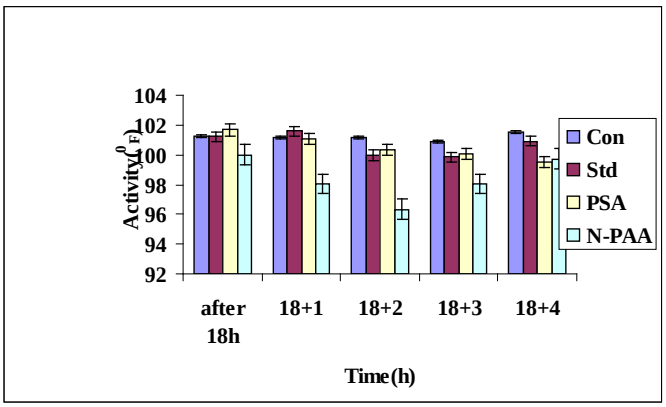
Figure 1. The anti-inflammatory effect of palmitoyl salicylic acid and palmitoyl anthranilic acid in animal models by the carrageenan induced paw edema method.



Antipyretic activity

The subcutaneous injection of yeast suspension markedly elevated the rectal temperature after 24 h of administration. Treatment with PSA and *N*-PAA decreased the rectal temperature. The antipyretic effect of *N*-PAA started from 1st h and maintained till 4th h.Whereas acetyl salicylic acid (aspirin) showed significant activity only in 1st h (figure. 2)

Figure 2. Anti-pyretic activity of palmitoyl salicylic acid and palmitoyl anthranilic acid in rats.



Analgesic activity

As shown in table.1 acetic acid induced writhing response in mice was carried out to examine analgesic action of PSA and *N*-PAA. Writhing test is not only simple and reliable but also affords rapid evaluation of peripheral type of analgesic action. In this test animal reciprocated with characteristic stretching behaviour called writhing. PSA and *N*-PAA inhibited writhing response by 59.23 % and 79.44% respectively.

The abdominal constriction is related to sensitization of nociceptive receptors to prostaglandins. It is therefore possible that PSA and *N*-PAA produce analgesic effect due to inhibition of prostaglandins synthesis

The mechanical hyperalgesia by hot plate method was found to be suitable for evaluation of central but not peripheral acting analgesics (15).The validity of this test has been shown even in presence of substantial impairment of motor performance (16). The results showed that PSA and *N*-PAA confer significantly enhanced and persistent analgesic activity for 30 - 90 min.

The thermal hyperalgesia as measured by tail immersion test (17) showed persistent analgesic activity from 1st -3rd h. The results showed that PSA is more efficient than acetyl salicylic acid. *N*-PAA also showed persistent analgesic activity. Therefore, these compounds

produce their effect both peripherally (writhing tests) and centrally (tail immersion and hot plate tests) (18).

Table 1. Comparison of analgesic activities of PSA and PAA.

Groups	Treatment	Dose mg/kg body weight	Writhing test (% inhibition)	Hot plate method			Tail immersion test			
				Time in minutes			Time in hour			
				30	60	90	0	1	2	3
Group-1 (Control)	Soya oil	1ml	-	14.667 ± 1.229	16.00 ± 1.528	12.667 ± 1.202	3.3 ± 0.2108	3.66 ± 0.5578	3.33 ± 0.6146	2.33 ± 0.2108
Group-II (Standard)	Aspirin	700	59.92	Ns 20.167 ± 2.994	*** 66.667 ± 4.595	*** 232.33 ± 1.081	Ns 3.667 ± .7149	* 5.33 ± 0.5578	Ns 5.0 ± 0.3651	Ns 3.0 ± 0.6325
Group-iii (PSA)	Palmitoyl Salicylic acid	500	59.23	* 27.333 ± 4.302	*** 106.67 ± 11.377	*** 90.833 ± 18.573	Ns 3.833 ± .3073	*** 6.833 ± .7032	*** 6.833 ± 18.573	*** 4.667 ± 0.2108
Group-IV (PAA)	Palmitoyl nthranilic acid	500	79.44	Ns 14.33 ± 1.687	** 37.500 ± 6.292	** 29.500 ± 3.948	Ns 3.500 ± 0.2236	** 5.750 ± .2582	* 5.000 ± 0.2582	Ns 2.000 ± 0.1291

CONCLUSION

At physiological pH the gastric tolerability of NSAIDs may be markedly improved by decreasing their solubility. Hence conversion of salicylic acid and antranillic acid into palmitoyl derivatives through an ester bond or amide bond to the OH or NH_2 residue of the latter may reduce their acidic nature. Thus the results of this study suggest that PSA possesses promising anti-inflammatory, anti-pyretic and analgesic properties compared to acetyl salicylic acid, and *N*-PAA exhibited potent pharmacological properties. Thus, the conjugation of palmitic acid to NSAID leads to new compounds that possess improved and efficient pharmacological properties. Hence, conjugation reaction products with lipids can attribute to eliminate side effects inherent in the parent compounds. The mechanisms involved in the cell membrane penetration of these compounds are underway. Such a study may identify the interactions of modified drugs with cell receptors and their metabolic roles.

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