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CORRELATION APPROACH OF *IN-VIVO* AND *IN-VITRO* HYDROLYTIC METABOLISM OF ESTER LINKAGE OF PRODRUG MADE OF INDOMETHACIN AND PARACETAMOL IN RP-HPLC IN ACIDIC AND ALKALINE MEDIUM

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ABSTRACT

The desired prodrug made of indomethacin and paracetamol has been synthesized in which ester and amide linkages both have been present and in-vitro and in-vivo biotransformation by hydrolysis pattern has been correlated for this prodrug. This prodrug has two amide linkages (–CONH–) and one ester linkage (–COO–). Since the ester and amide both are susceptible for hydrolysis but in amide (–CONH–) three lone pairs of electrons are present [two for oxygen and one for nitrogen] and in ester (–COO–) four lone pairs of electrons are present [four for two oxygens]. So the electron density of ester is greater than amide and electronegativity of oxygen is 3.44 and for nitrogen is 3.04. So total electronegativity of ester (–COO–) is 3.44+3.44=6.88 and for amide (–CONH–) is 3.44+3.04=6.44. Hence possibility of hydrolytic biotransformation of this prodrug having ester and amide linkages, the ester linkage hydrolyses prior to amide so the prodrug produces two fractions: indomethacin and paracetamol both in acidic as well as in alkaline medium. The release of free drug both in in-vitro and in-vivo so it will be implemented as a prodrug which can show prolong action on pain and fever after getting release into free parent drug by biotransformation. The HPLC (High Performance Liquid Chromatography) study reports the retention time (R_t) and release kinetics of prodrug by taking HPLC degradation datas of three samples of Prodrug and individual HPLC data of parent drugs separately to compare the R_t value of release of two drugs from Prodrug in both acidic and alkaline pH. logP of prodrug=3.94 releases indomethacin (logP=3.10) and paracetamol (logP=0.34) both in acidic and alkaline pH. This is a comparison study of drug release in in-vitro gastric as well as intestinal pH focusing on in-vivo biotransformation.

KEYWORDS: Prodrug, Indomethacin, Paracetamol, Molecular weight, logP, UV λ_{max}, IR, Mobile phase, TLC-R_f value, HPLC-R_t value, LOD, LOC.

INTRODUCTION

Prodrug of indomethacin and paracetamol has been synthesized by reacting 0.01mole of indomethacin (Molecular Formula=$C_{19}H_{16}ClNO_4$, Formula Weight=357.78g) with excess of thionyl chloride which converts carboxylic acid group (–COOH) of indomethacin into free acid chloride group (–COCl) which is then condensed with 0.01mole of paracetamol (Molecular Formula=$C_8H_9NO_2$, Formula Weight=151.16g) to get the desired prodrug where acid chloride reacts with phenolic group of paracetamol to get the desired prodrug (Molecular Formula=$C_{27}H_{23}ClN_2O_5$, Formula Weight=490.93g) having both ester and amide linkages.

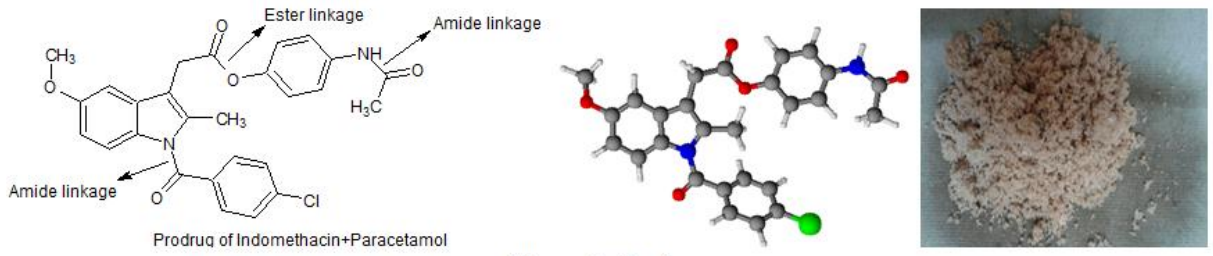
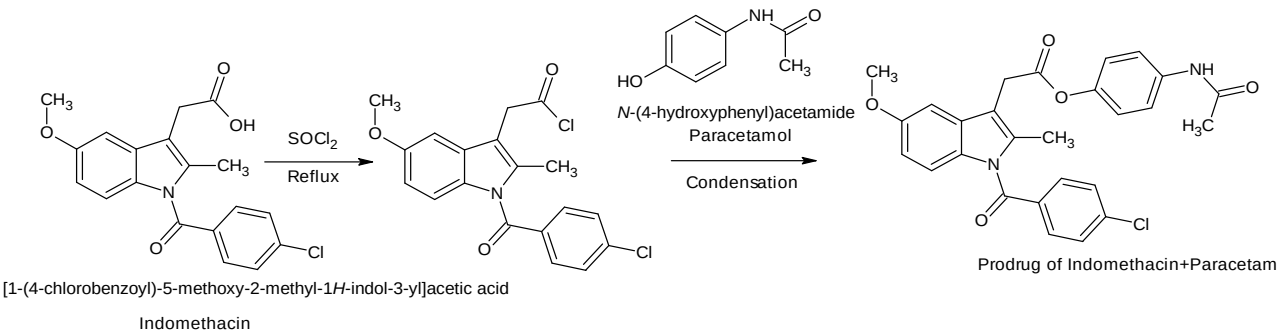


Figure-1: Prodrug

Prodrug has been successfully synthesized and has shown different melting points from individual parent drugs (Indomethacin and Paracetamol) which indicates the authenticity of fulfillment of Prodrug synthesis. [Jalpa G. Patel and Prof. Dr. Dhrubo Jyoti Sen; *Synthesis of Prodrug of ester and amide linkages of NSAID having carboxylic acid, phenolic and imino groups*: World Journal of Pharmacy and Pharmaceutical Sciences: 5(11), 897-908, 2016. (ISSN: 2278-4357, Impact Factor: 6.647, Index Copernicus Value: 52.51); Dhrubo Jyoti Sen and Jalpa G. Patel; *Logarithmic partition coefficient comparison study and molecular weight of synthesized Prodrugs of ibuprofen+paracetamol, diclofenac sodium+paracetamol and ibuprofen+diclofenac sodium*: American Journal of Advanced Drug Delivery: 4(05), 064-068, 2016. (ISSN: 2321-547X, Impact Factor: 0.786)].[1,2]



[1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid

Indomethacin

Figure-2: Synthesis

The synthesized Prodrug was characterized by m.p., logP values and IR (Infra Red) spectrum for structural identification. Their solubility parameters also found different from parent drugs. Prodrug is a substance having no medicinal importance but after biotransformation in GIT it releases the parent drug which is able to show the pharmacological activity.[3-5]

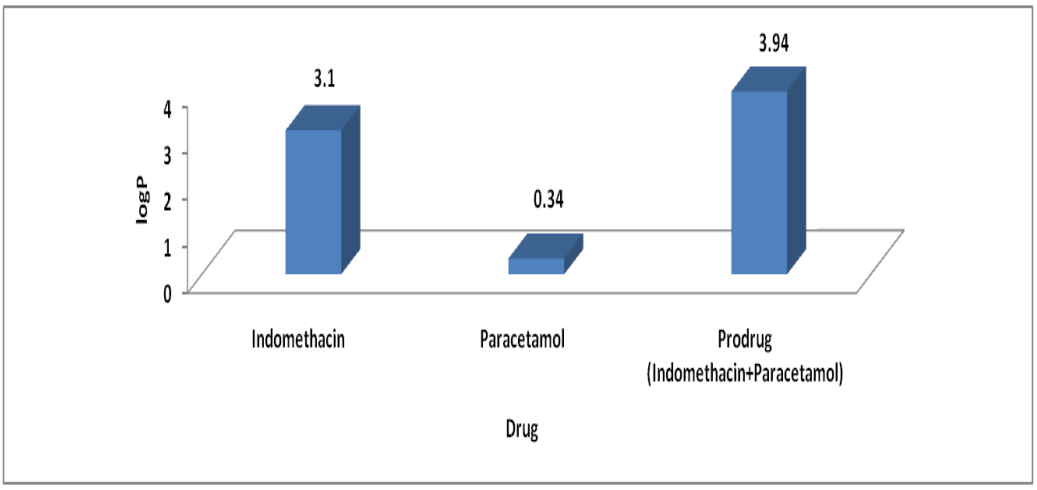


Figure-3: Histogram

Indomethacin [$C_{19}H_{16}ClNO_4$; MW=357.78g] ([1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl]acetic acid), is used as NSAID to treat pain and fever. logP=3.10 (Semipolar), pKa=4.5. Indomethacin m.p.=theoretical (**152-154°C**), practical (**152°C**).

Paracetamol [$C_8H_9NO_2$; MW=151.0g] (N–(4–hydroxyphenyl)acetamide), also known as acetaminophen or APAP, is a medication used to treat pain and fever. logP=0.34 (Polar), pKa=9.38. Paracetamol m.p.=theoretical (**169°C**), practical (**170°C**).

Prodrug [$C_{27}H_{23}ClN_2O_5$; MW=490.93g] [(1-(4-chlorobenzoyl)-4'-(acetylamino)phenyl-(5-methoxy-2-methyl-1*H*-indol-3-yl)acetate]. logP=3.94 (Nonpolar), **m.p.=125-127°C**.

Experimental part

Mobile phase of TLC values has been selected after trial and error for all individual NSAIDs (indomethacin and paracetamol) according to their logP and R_f values have been determined and after that R_f values have been obtained for Prodrug. Mobile phase solvent ratio of TLC helped to select the mobile phase selection for HPLC.[6-8] The values are as follows:

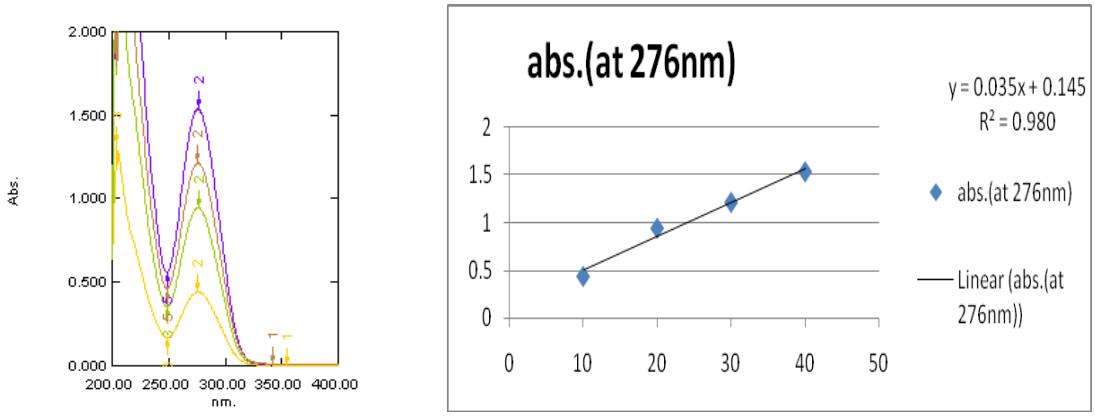


Figure-4: λ_{max} of Prodrug

Table-1: Physicochemical parameters of Prodrug

Samples	Molecular Formula	% yield	Melting Point (°C)	Molecular Weight	λ_{max} (nm)	logP
Prodrug	$C_{27}H_{23}ClN_2O_5$	98.32	125–127°C	490.93g	276	3.94

Solubility study of Prodrug

The solubility of Prodrug was practically determined by taking 100mg of Prodrug in 10ml volumetric flask, adding required quantity of solvent at room temperature and shaken for few minutes.[9-11] Solubility data for each study was observed and recorded in Table-2.

Table-2: Solubility parameters of Prodrug

Solvent	Solubility
Water	Insoluble
Methanol	Freely soluble
Acetone	Freely soluble

Instrument

A double beam UV visible spectrophotometer: Manufacturer: Shimadzu. Model: UV-1800, Shimadzu, Japan.

Preparation of Standard solution of Prodrug: Weighed accurately about 100mg of Prodrug transferred quantitatively to 100ml volumetric flask. Dissolved in about 70ml of Methanol by sonication and diluted to volume with methanol and mixed. Transferred 0.1ml of this solution to 10ml volumetric flask, diluted to volume with diluents (methanol) and mixed (10µg/ml) and absorption was observed at 276nm.[12] The values of Prodrug was given in the Table-3.

Table-3: Absorption datas of Prodrug

	Conc. (µg/ml)	Abs.
Prodrug	10	0.441
	20	0.945
	30	1.216
	40	1.536

Infra Red Spectral studies of Prodrug

IR spectras of Prodrug was measured in KBr pellets in Shimadzu FT-IR spectrophotometer and values in cm^{-1} were obtained **for** interpretation of structural framework.

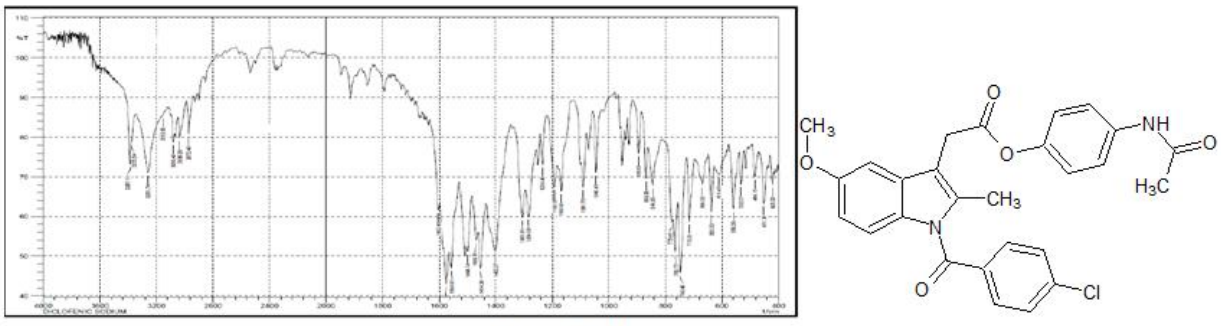


Figure-5: IR spectra

Prodrug: IR (cm^{-1}; ν): N-H stretching: (standard=3500-3100, found=3343), C-H stretching (aromatic): (standard=3150-3050, found=3136.36), C=C stretching: (standard=1615–1580, found=1621), C-N stretching: (standard=1650–1550, found=1580), >C=O stretching: (standard=1725-1705, found=1721), C-CO-C stretching: (standard=1320-1210, found=1284.63).[13]

Development and validation of Stability Indicating RP-HPLC Method for Prodrug (Indomethacin and Paracetamol)

Experimental: Reagents and Materials, Prodrug synthesized in college lab, Methanol (HPLC grade, Finar

Chemicals Ltd, Ahmedabad, India), Water (HPLC grade, Finar Chemicals Ltd, Ahmedabad, India).

Equipments and Instruments: Shimadzu HPLC instrument (LC-2010 CHT) equipped with prominence diode array detector (SPD-M20A) (Software LC Solution), Analytical balance (Acculab ALC-2014, Huntingdon Valley, PA), Ultra sonicator (EN 30 US, Enertech Fast Clean, Mumbai, India), Hot air oven (TO-90S, Thermolab, Mumbai, India), pH meter (Thermo Electron Corp., Pune, India).

Development and Optimization of RP-HPLC Method

a) Selection of Wavelength: The sensitivity of HPLC method that used UV detection depends upon proper selection of detection wavelength. An ideal wavelength is the one that gives good response for the drugs that are to be detected. The λ_{max} of Prodrug was 276nm in methanol.[14]

b) Selection of Chromatographic Conditions: Proper selection of the HPLC method depends upon the nature of the sample (ionic or ionisable or neutral molecule), its molecular weight, pKa and solubility. RP-HPLC was selected for the initial separation based on literature survey and its simplicity and suitability. To optimize the chromatographic conditions the effects of chromatographic variables such as mobile phase, pH, flow rate and solvent ratio were studied and the chromatographic parameters such as capacity factor, asymmetric factor, resolution and column efficiency were calculated. The pH of gastric acid varies from 1.5-3.5 in the human stomach lumen, the acidity being maintained by the proton pump H^+/K^+ ATPase. So the pattern for acid hydrolysis was adjusted at pH=3-3.5 by HCl. The pH of intestine varies from 5.6-6.9, so the pattern for alkaline hydrolysis was adjusted at pH=7.0-8.0 by NaOH. Finally the condition was chosen that gave the best resolution, symmetry and capacity factor was selected for estimation of Prodrug.[15]

c) Selection of Ratio of Mobile phase: The solution containing 100µg/ml of Prodrug was chromatographed with mobile phase of different ratio of methanol and water.[16]

Table-4: Selection of mobile phase for Prodrug

	Trials	Ratio	Remark
Prodrug	1	ACN:Water (80:20)	Tailing
	2	ACN:Water (70:30)	Tailing
	3	ACN:Methanol (80:20)	Tailing
	4	ACN:Methanol (70:30)	Tailing
	5	Methanol:Water (80:20)	Tailing
	6	Methanol:Water (70:30)	**Symmetrical peak**

Preparation of working solution containing Prodrug: From the above solution (1000µg/ml of Prodrug) 0.2, 0.4, 0.6, 0.8, 1.0, 1.2ml was taken into 10ml volumetric flask to get concentration 20, 40, 60, 80, 100, 120µg/ml of Prodrug respectively.[17]

Method Validation for Prodrug

1. System suitability test parameters: System suitability tests are used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were the chromatographic peak resolution, theoretical plate number and tailing factor. The repeatability of these parameters was checked by solutions of Prodrug.[18]

2. Linearity and Range: Aliquots of stock solution (0.2, 0.4, 0.6, 0.8, 1.0, 1.2ml) were transferred into series of 10ml volumetric flasks and diluted up to mark with mobile phase. This yielded solution of 20, 40, 60, 80, 100, 120µg/ml of Prodrug. An aliquot of 10µl of each solution was injected under operating chromatographic condition. Calibration curve of area versus respective concentration was plotted and correlation co-efficient and regression line equation for Prodrug was calculated. Each response was an average of three determinations.[19]

Standard Paracetamol peak at 230nm in mobile phase Phosphate Buffer:ACN (40:60v/v):-

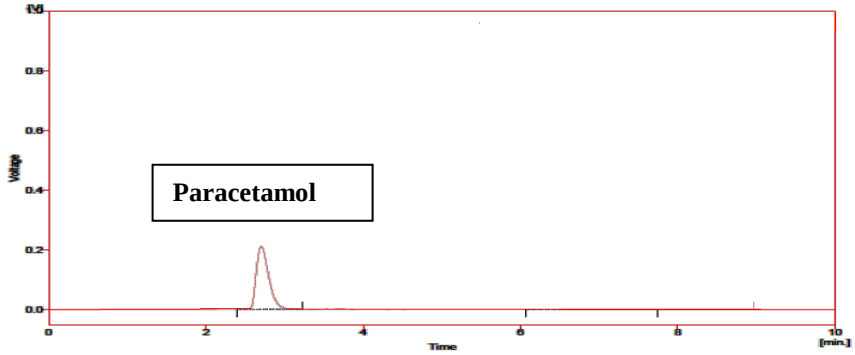


Figure-6: Chromatogram of Standard Paracetamol (R_t=2.3min)

Paracetamol releases first (R_t=2.3min) because it's logP is 0.46 (highly polar).

Standard Indomethacin peak at 276nm in mobile phase ACN:triethylamine buffer (50:50v/v):-

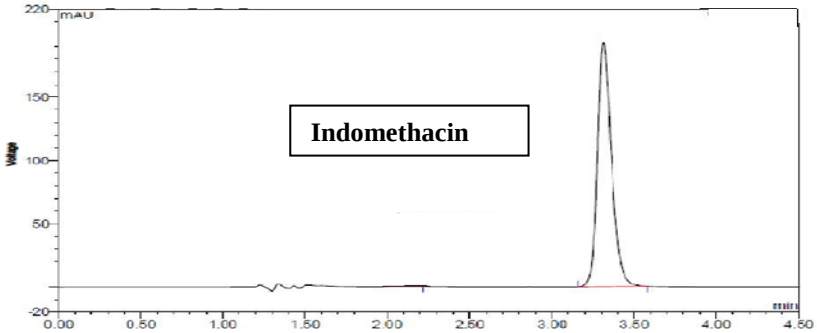


Figure-7: Chromatogram of Standard Indomethacin (R_t=3.4min)

Indomethacin releases moderate (R_t=3.4min) because it's logP is 3.94 (semipolar).

Optimized Chromatographic Conditions for Prodrug: HPLC system: LC 2010 CHT (Shimadzu), PDA detector (PDA-SPD-M10AVP, Shimadzu), Column (Stationary Phase): Kromasil C_{18} (150mm×4.6mm, 5µm particle size), Mobile phase: (Methanol: Water)(80:20 v/v), Flow Rate: 1.0 ml/min, Detection Wavelength: 276nm, Column oven Temp: 40ºC, Run time: 10 mins. Diluent: All the final dilution of sample was done with methanol.[20]

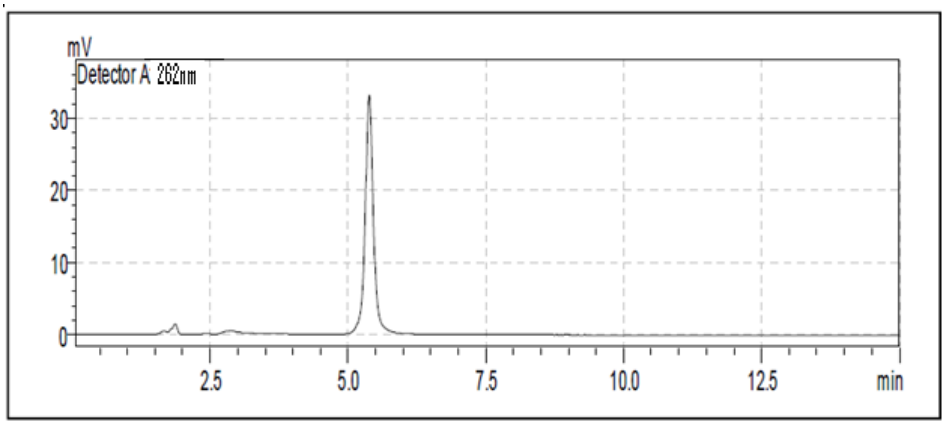


Figure-8: Chromatogram of Prodrug (10µg/ml) (R_t=5.5min)

Prodrug has logP 3.94 so it releases slowly (5.5min) due to nonpolar nature.

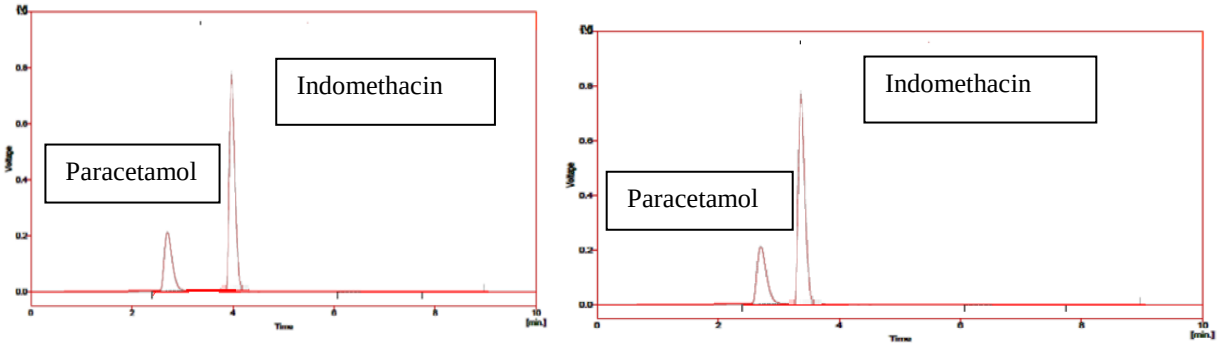


Figure-9: Chromatogram of Prodrug in Acidic medium (HCl: pH=3.0) and in Alkaline medium (NaOH: pH=7.5 at 37°C for *in-vivo* and 40°C for *in-vitro* profile)

Paracetamol has R_t=2.75min in acidic medium and 2.8min in alkaline medium; Indomethacin has R_t=4min in acidic medium and 3.8min in alkaline medium.

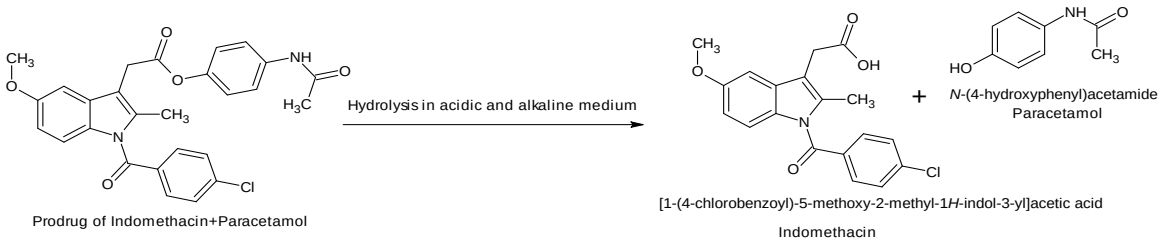


Figure-10: Biotransformation

Method Validation

Linearity and Range: Overlain chromatogram of Prodrug was shown in Figure-11. The linearity of Prodrug was found to be in the range of 1-25µg/ml with correlation coefficient 0.999 as shown in Figure-12.[21]

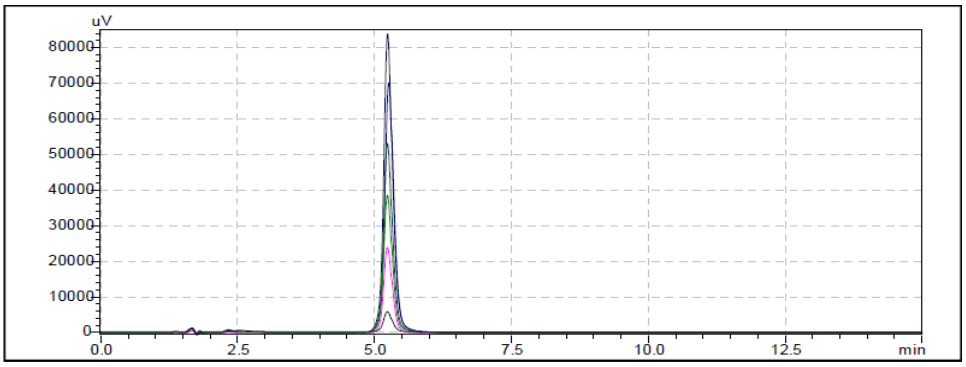


Figure-11: Overlain Linearity Chromatogram of Prodrug (1-25µg/ml)

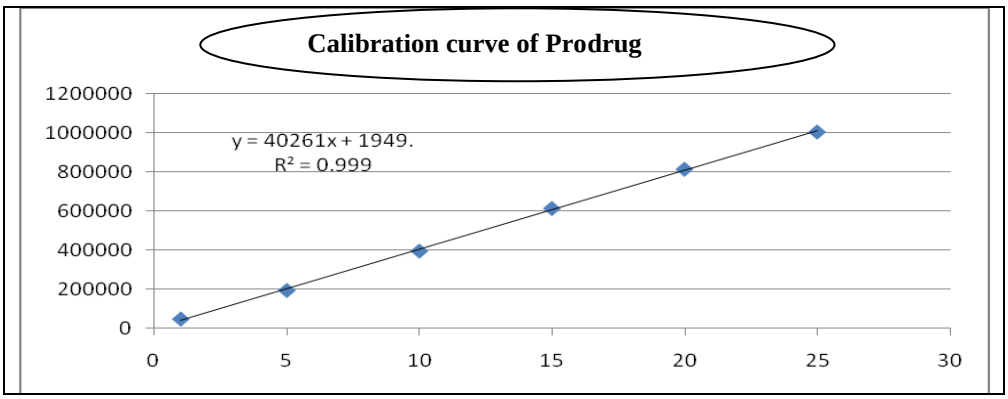


Figure-12: Linearity data of Prodrug

Precision

1. **Repeatability (Intra-day precision):** The data for Intra-day precision for Prodrug is shown in Table-5. The %RSD for Intra-day precision was found to be 0.12-0.27% for Prodrug.
2. **Inter-day Precision (different days):** The data for Inter-day Precision for Prodrug is shown in Table-5. The %RSD Inter-day Precision for was found to be 0.80-1.06% for Prodrug.[22]

Intra-day precision data and Inter-day precision data for estimation of Prodrug-:

Table-5: Intra-day precision data and Inter-day precision data for estimation of Prodrug

Intra-day precision	Conc.(µg/ml)	Mean peak Area±S.D.	%RSD	Inter-day precision	Conc.(µg/ml)	Mean peak Area±S.D.	%RSD
	10	396807±1078	0.27		10	395876±3195	0.80
	15	613783±1209	0.19		15	619236±6593	1.06
	20	814533±1005	0.12		20	819518±7767	0.94

*= Average of three determinations.

LOD and LOQ: The Limit of Detection (LOD) was found to be 0.023µg/ml; while the Limit of Quantification (LOQ) was found to be 0.071µg/ml for Prodrug.[23]

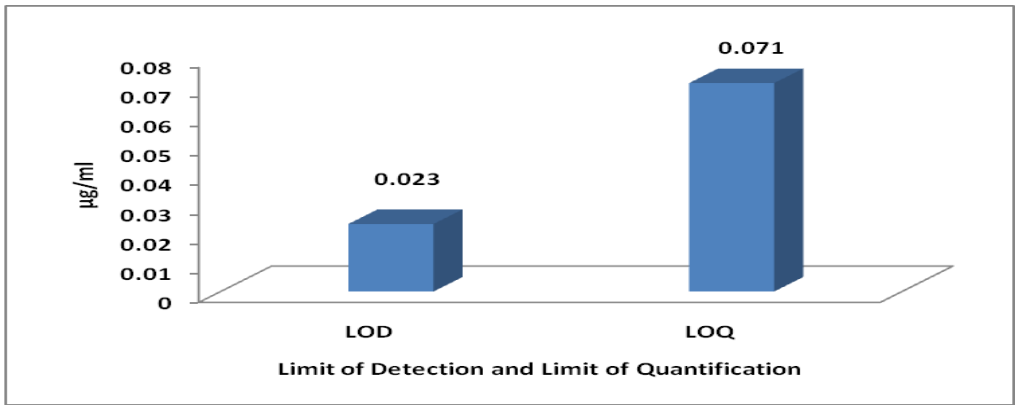


Figure-13: LOD and LOQ

Value of System Suitability Parameter of Prodrug

Table-6: Value of System Suitability Parameter of Prodrug

Sr.No.	Parameters	Prodrug
1	Retention time (min.)	5.4
2	Theoretical plates	6696
3	Tailing factor	1.04
4	Resolution	5.566
5	Capacity factor	2.237

Linearity data of Prodrug

Table-7: Linearity data of Prodrug

	Conc. (µg/ml)	Peak area mean	SD	%RSD
Prodrug	1	49515	287	0.57
	5	195469	1328	0.67
	10	395999	1062	0.26
	15	613574	877	0.14
	20	813557	1314	0.16
	25	1003451	1297	0.12

Linearity Results for Prodrug

Table-8: Linearity Results for Prodrug

Regression Analysis	Prodrug
Regression equation	Y= 40261x+1949
Correlation co-efficient	0.999
Slope	40261
Intercept	1949

Summary of Validation Parameters for HPLC method of Prodrug

Table-9: Summary of Validation Parameters for HPLC method of Prodrug

Sr.No.	Parameters		Prodrug-B
1	Linearity Range		1–25µg/mL
2	Regression equation		y=40261x+1949
3	Correlation co-efficient		0.999
4	Precision (%RSD)	Interday	0.80-1.06%
		Intraday	0.12-0.27%
5	Limit of Detection		0.023µg/ml
6	Limit of Quantification		0.071µg/ml

CONCLUSION

Paracetamol and Indomethacin have been taken as NSAID and Prodrug has been synthesized by reacting of acid chloride of indomethacin with paracetamol to get Prodrug of ester linkage. The synthesized Prodrug has two amide and one ester linkages. Prodrug is a substance which after administration is metabolized into a pharmacologically active drug. Actually Prodrug has least medicinal value in *in-vitro/in-vivo* but after biotransformation by metabolism in *in-vivo* it releases the active medicament. A drug is a substance which is a chemical entity, has definite structural skeleton, obtained by natural or synthetic or semisynthetic source, which can fit on bioreceptor platform having controlling capacity to control over the biochemical malfunction. Every drug is xenobiotic because it is coming from outer source (xeno) and active in biological unit (biotic). Prodrug is the precursor of drug which is made by derivatization of the same to enhance the bioavailability by pharmacokinetics, lipid solubility by partition coefficient and increase the physicochemical & biochemical parameters by pharmacodynamics. Prodrug showed different logP value and molecular weight according to the solubility parameters and electronegativity: logP profile: Prodrug=3.94, molecular weight profile: Prodrug=490.93g.

The main side effect of NSAID is gastric acidity due to release of free H^+ because all NSAIDs have free –COOH (carboxylic acid) group which act by competitive inhibition of cyclooxygenase enzyme (COX_1/COX_2). Here the target of this project has been designed in such a way to convert the free –COOH of API (indomethacin/paracetamol) into Prodrug of ester (–COO–) as well as amide (–CONH–) linkage which releases free API after metabolic hydrolysis in acidic pH: 1-4 and alkaline pH: 7-9 at 37°C in *in-vivo* (body temperature) and 40°C in *in-vitro* (HPLC column oven temperature). Since the Prodrug is a repository form so chances to release gastric acid have been minimized due to non availability of free –COOH group in stomach. The biotransformation of active drug from Prodrug takes such a time in stomach that all goes upto duodenum and then ileum of small intestine that chances of acidity is reduced. Finally Prodrug goes to small intestine where alkaline pH starts so gastric acidity is reduced. Since all this Prodrug is made of two NSAIDs: Prodrug (logP=3.94) releases Indomethacin & Paracetamol which shows distinct two R_t values in HPLC both in acidic an alkaline hydrolysis and these R_t values of Prodrug matches with the individual API components so the purpose of our goal has been completed successfully. The pH of gastric acid varies from 1.5-3.5 in the human stomach lumen, the acidity being maintained by the proton pump H^+/K^+ ATPase. So the pattern for acid hydrolysis was adjusted at pH=3-3.5 by HCl. The pH of intestine varies from 5.6-6.9, so the pattern for alkaline hydrolysis was adjusted at pH=7.0-8.0 by NaOH.

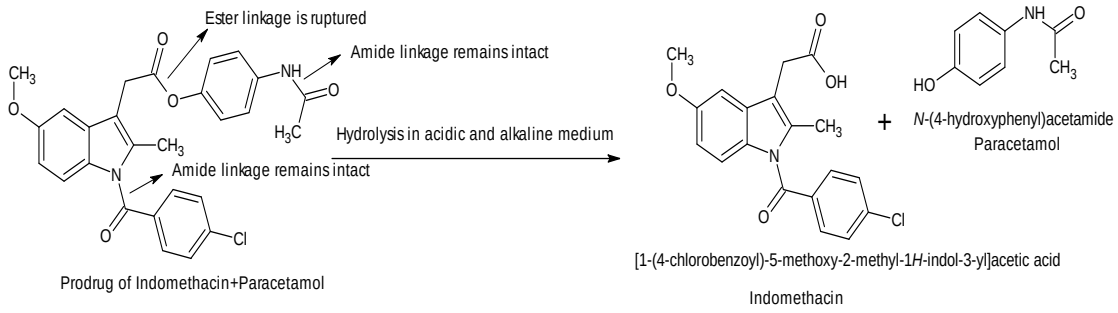
Standard R_t of NSAIDs

1. Paracetamol releases first (R_t=2.3min) because it's logP is 0.34 (highly polar). 2. Indomethacin releases slow (R_t=3.4min) because it's logP is 3.97 (semipolar). 3. Prodrug releases slowly because it has R_t=5.5min (nonpolar).

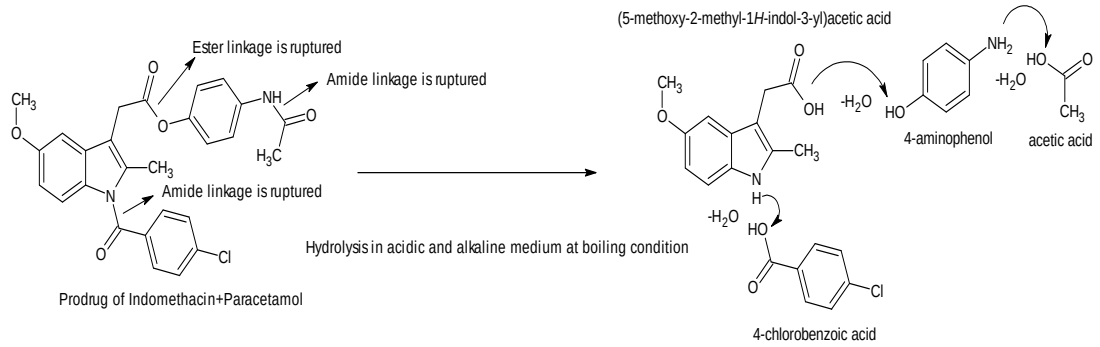
Prodrug R_t after hydrolysis

Prodrug [-COO-; ester linkage]: Paracetamol has R_t=2.75min in acidic medium and 2.8min in alkaline medium; Indomethacin has R_t=4min in acidic medium and 3.8min in alkaline medium.

R_t of paracetamol varies between 2.5-3.5min; R_t of indomethacin varies between 3.8-4.06. The focus of *in-vivo* metabolic profile of Prodrugs has been implemented into *in-vitro* hydrolytic reaction in both acidic and alkaline pH to get the satisfactory desired result.

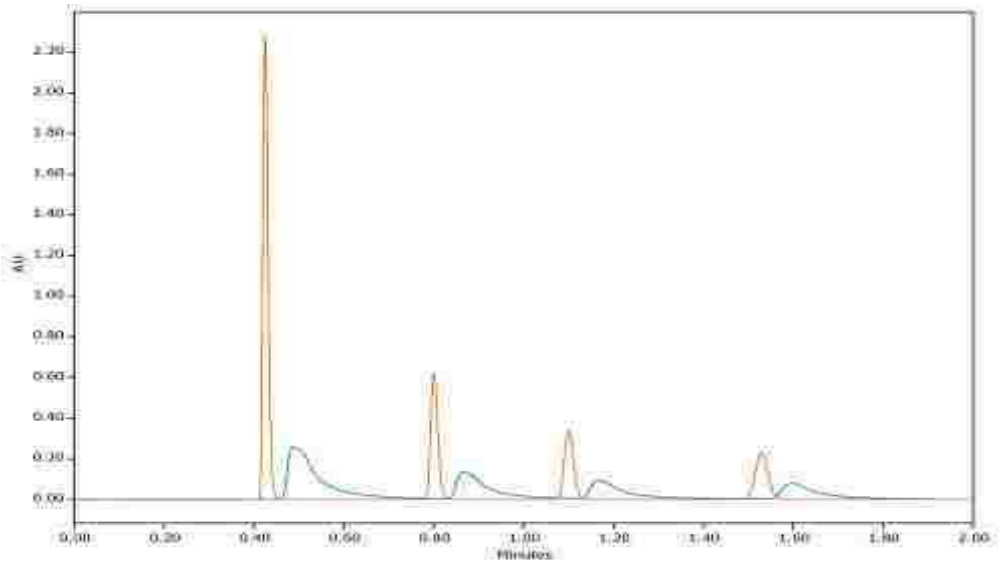


In this hydrolytic biotransformation of Prodrug in acidic and alkaline pH only ester linkage is ruptured at physiological temperature (37°C) for *in-vivo* profile and HPLC column temperature (40°C) for *in-vitro* profile due to electron density as well as electronegativity of ester is greater than amide and for this only indomethacin and paracetamol are released and nothing else.



Four fractions obtained are for (acetic acid: R_t=0.4min; polar), (4-chlorobenzoic acid: R_t=0.8min semipolar), (4-aminophenol: R_t=1.2min; polar), (5-methoxy-2-methyl-1*H*-indol-3-yl)acetic acid: R_t=1.6min; nonpolar).

If this prodrug is hydrolyzed by boiling in both acid and alkaline medium then both amide and ester linkages are ruptured to get four products (amide: -NH_2 group: 5-methoxy-2-methyl-1*H*-indol-3-yl)acetic acid/4-aminophenol + -COOH group: 4-chlorobenzoic acid/ acetic acid) and (ester: -COOH group: 5-methoxy-2-methyl-1*H*-indol-3-yl)acetic acid + -OH group: 4-aminophenol).



Methoxy (CH_3O-) group remains unaffected by acid/alkali hydrolysis because dealkylation is not possible by HCl/NaOH. Hydrolysis is only possible in (ester: –COO–) and (amide: –CONH–).

REFERENCES

1. Jalpa G. Patel and Prof. Dr. Dhrubo Jyoti Sen; Synthesis of Prodrug of ester and amide linkages of NSAID having carboxylic acid, phenolic and imino groups: World Journal of Pharmacy and Pharmaceutical Sciences; 2016; 5(11): 897-908.
2. Dhrubo Jyoti Sen and Jalpa G. Patel; Logarithmic partition coefficient comparison study and molecular weight of synthesized Prodrugs of ibuprofen+paracetamol, diclofenac sodium+paracetamol and ibuprofen+diclofenac sodium: American Journal of Advanced Drug Delivery: 2016; 4(05): 064-068.
3. J.E. Slemmer, B.R. Martin and M.I. Damaj. Bupropion is a nicotinic antagonist. J. Pharm. Exp. Ther., 2000; 295: 321–327.
4. C. Wu, J. Quan, J. Xie, C Branford-White, L. Zhu, Y. Yu and Y. Wang. Preparation and controlled release of degradable polymeric ketoprofen-saccharide conjugates. Polym Bull., 2011; 67: 593–608.
5. M, Babazadeh and T. Mosanejhad, Vinyl ester type polymers containing ibuprofen pendents: synthesis, characterization and evaluation. Iran Polym J., 2009; 18: 179–186.
6. L. Shargel, Susanna Wu-Pong and B.C. Andrew, Applied Biopharmaceutics & Pharmacokinetics, 6th Ed. McGraw-Hill Medical Publishing Division, US., 2012; 47-66: 129-154.
7. S. Shirke, S. Shewale and M. Satpute, Prodrug design: an overview. International Journal of Pharmaceutical, Chemical and Biological Sciences., 2015; 5(1): 232-241.
8. A.V. Bhosale, G.P. Agrawal and P.Mishra: Preparation and Evaluation of Directly Compressible Forms of Mutual Prodrugs of Ibuprofen Forms of Mutual Prodrugs of Ibuprofen. Indian Journal of Pharmaceutical Sciences, 2006; 68(4): 425-431.
9. Beckett AH and Stenlake JB, Practice pharmaceutical chemistry; 4th Edn; CBS Publishers and Distributors, New Delhi, 1997; 293-304.
10. Ewing GW, Instrumental Methods of Chemical Analysis; 5th Edn; McGraw- Hill Book Company, New York, 1985; 1-7.
11. Kasture AV, Mahadik KR, Wadodkar SG and More HN, Instrumental methods of pharmaceutical analysis; 14th Edn; Nirali Prakashan, Pune, 2006; 1-30.
12. Wikipedia: The free Encyclopedia, "Ultraviolet-visible spectroscopy", 2013, http://en.wikipedia.org/wiki/Ultraviolet-visible spectroscopy.
13. Skoog DA, Holler FJ and Nieman TA, Principle of Instrumentation Analysis; 5th Edn; Thomas Asia Pvt Ltd, 2005; 580.
14. Beckett AH and Stenlake JB, Practical Pharmaceutical Chemistry; 4th Edn; CBC Publication and distribution, 1977; 1-26.
15. Snyder LR., Kirkland JL and Glajch JL., Practical HPLC Method Development.,2nd Edition, Wiley Interscience, 1977; 1- 26.
16. Halmilton RJ and Sewell PA., Introduction to HPLC., 2nd Edition; Chapman and Hall, 1982; 189.
17. Cartensen JT and Rhodes CT., Drug stability: Principles and practices New York – Marcel Dekker; 3rd Edition, 329 – 384.
18. American Society of Health-System Pharmacists 2013; Drug Information 2013. Bethesda, MD. 2013; 2212.
19. O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. 13th Edition, Whitehouse Station, NJ: Merck and Co., Inc., 2001; 876.
20. O'Neil, M.J. (ed.). The Merck Index - An Encyclopedia of Chemicals, Drugs, and Biologicals. Whitehouse Station, NJ: Merck and Co., Inc., 2006; 522.
21. Hosale AV, Agrawal GP and Mishra P: Preparation and characterization of mutual Prodrugs of ibuprofen. IJPSR 2004; Vol. 2, Issue 4 ISSN: 0975-8232.
22. Koren, K., Mann, A.R., Colosimo, A.L., Diaz, Z., Nuclelman, A., Levovich, I., Jing, Y., Waxman, S. and Miller, W.H. Jr., Mol. Cancer Res., 2003; 1: 903.
23. Slemmer J.E., Martin B.R., Damaj M.I. Bupropion is a nicotinic antagonist. J. Pharm. Exp. Ther. 2000; 295: 321–327.