Section - I

INTRA-ARTICULAR DRUG DELIVERY

Introduction

Local drug action within the joint cavity is essential for the treatment of arthritic disorders and for relief from the associated pain and inflammation.[1] Intra-articular (IA) injection is one of the methods to deliver drugs within the joint cavity and corticosteroids were the first substances to be injected locally into the IA space.[2] Hydrocortisone was first used for IA injection in 1951 and was found to lower indicators of the inflammation response, including IA leukocyte count.[3] Since then various corticosteroid suspensions have been used to combat pain and inflammation associated with osteoarthritis (OA) or active rheumatoid arthritis (RA), but the use of corticosteroids in arthritis has been controversial. Animal studies have suggested that multiple corticosteroid injections might alter cartilage protein synthesis and consequently damage the cartilage.[4] These deleterious effects curbed early enthusiasm for IA corticosteroid therapy in arthritis. However other studies suggest that primate joints respond differently than those of rodents and corticosteroids may be useful when used appropriately.[5] IA deliveries provide advantages as compared to other routes for the treatment of active arthritis or OA using corticosteroids or other therapeutic agents such as non-steroidal anti-inflammatory agents (NSAIDs), visco-supplementation etc.

Development of long acting IA delivery system is not an easy task as it has to be based on adequate knowledge of chemical, pharmaceutical and biological disciplines and it is multidisciplinary in nature. There are various factors which influence the fate of drug molecules once they are released from the immobilized depot in the joint cavity, so it is important to understand anatomy and physiology of synovial joints in detail. [1]

1. Synovial joints: The primary role of joints is to provide mobility and stability to the skeleton. The bones of synovial joints are held together by fibrous capsule and accessory ligaments. The inner surface of the capsule is lined by the synovium and the articular surfaces are covered by the hyaline cartilage; further their contact is facilitated by synovial fluid (SF) acting as lubricant.[6] Various components of synovial joints are synovium, synovial fluid, cartilage and bone as shown in **Fig. 1.1**

1.1. Synovium: The synovium consists of two layers, the inner intima layer and a deep subintima. Within the intima are the synovial lining cells of type A and B. One third of the synovial membrane cells are comprised of type-A synviocytes that are macrophages which clear debris within the membrane. Type-B synoviocytes are the most prevalent cells

closely resembling fibroblasts and they synthesize hyaluronic acid (HA), a very long chain glycosaminoglycan.[6]

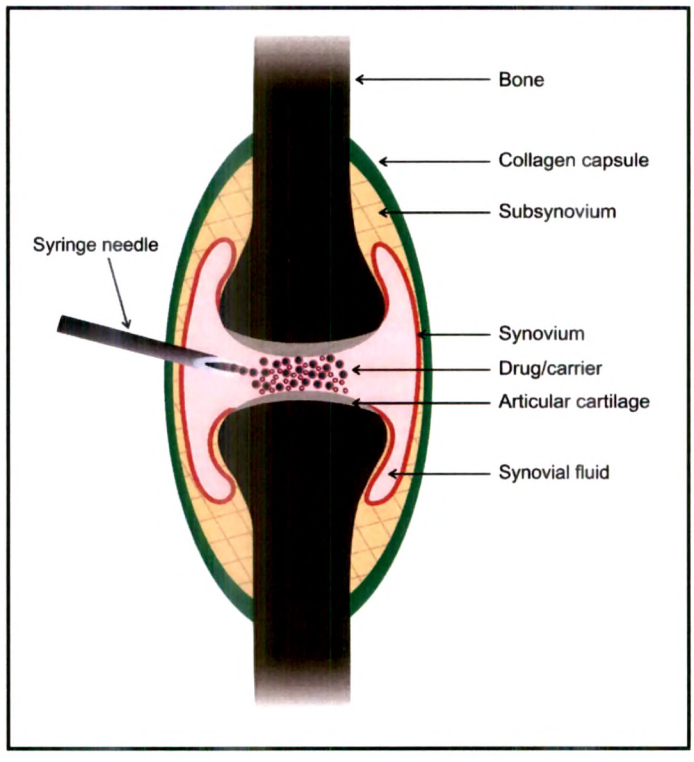

Fig. 1.1: Synovial joint

The synovium performs several functions such as ultra-filtration of plasma and synthesis of HA by type-B synoviocytes from the SF which is essential for the preservation of the low coefficient of friction between the surfaces of articular cartilages and provides the nutrients.[6]

1.2. Synovial fluid

The synovial fluid (SF) is present in the cavity of synovial joint. It is a viscous, non-Newtonian fluid exhibiting thixotropic properties. The average volume of SF in normal human adult knee joint is about 2 ml.[6] This volume can increase up to several hundred milliliters under pathological conditions.[7] SF can be distinguished from plasma by the presence of HA (0.35 g/100 ml) and lubricin (0.005 g/100 ml approx.). These two molecules are the major contributors of SF viscosity and are also important for one of its main functions i.e. to act as lubricant of the joint surfaces.

1.3. Articular cartilage

Articular cartilage provides each moving bony portion of a joint with a smooth, frictionless surface. It is capable of reversible compression, distributing an applied load

homogeneously and minimizing contact stress to the underlying bone. The structure and function of cartilage and its matrix components have been maintained throughout the life by the resident chondrocytes as described earlier in detail in introductory part.

2. Drug transport and distribution process in synovial joints

In the joint cavity, the solute drug molecule, once released from the immobilized depot, may take part in a number of reactions and distribution (equilibrium) processes before it is eventually cleared from the synovial space. These processes, the relative importance of which is determined by the physicochemical properties of the drug substance and the barrier properties of the synovium are shown in schematic diagram **(Fig. 1.2)**. Concomitant to binding of the drug to components of the SF, its transport and distribution into the synovium and articular cartilage and subsequent uptake by synoviocytes and chondrocytes may occur.[1]

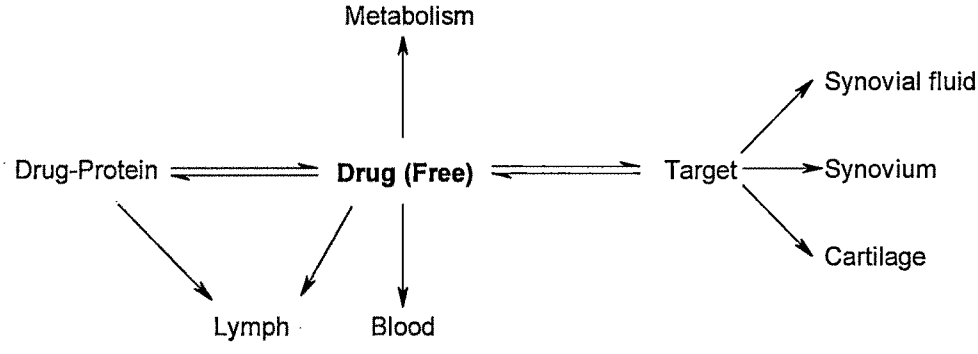

Fig.1.2: Schematic representation of drug transport and distribution processes in the joint.

2.1. Trans-synovial transport

The synovium constitutes the main barrier for drug transport out of the joint cavity due to its complex architecture. The healthy synovial lining is thin (60 μm) and discontinuous without intercellular junctions. Together with the ECM, the synoviocytes function as a permeable, inhomogeneous matrix.[1] The healthy joint is penetrated by capillaries close to the surface of the synovium (modal depth 35 μm in man).

Upon oral drug administration, observed joint C_{max} and T_{max} values are usually lower and occur at later time points respectively, than the corresponding parameters in plasma.[8] For many NSAIDs, plasma/SF drug concentration ratios have been found to reflect plasma/synovial protein concentration ratios. Further, free NSAID concentrations have been found to be similar in the two compartments after attainment of steady state conditions. Often the SF concentrations are more sustained than plasma concentrations

after oral or i.v. administration.[8] Due to continuous drug entrance from the blood compartment, IA elimination half-lives ($t_{1/2}$) estimated from pharmacokinetic profiles obtained following oral administration may tend to underestimate the "true" rate of drug disappearance from the SF after IA administration. By appropriate corrections, however, reasonable estimates of rates of disappearance from joints relevant to IA administration can be extracted from oral pharmacokinetic data as shown in **Table 1.1** below.

As apparent, relatively fast disappearance from the joint is observed for small solutes with $t_{1/2}$ values in the range of about 0.1 to 6 h. In this connection disappearance kinetics from the joint cavity has often been described by simple first-order kinetics.

Table 1.1: Synovial disappearance half-lives ($t_{1/2}$) and molecular weights (MW) of various solutes.[1]

No	Compound	$t_{1/2}$ (h)	MW (g/mol)
1	Albumin	1.23*	$6.7x10^4$
2	Procaine	0.80*	236
3	Diclofenac	5.2	296
4	Salicylic acid	2.4	138
5	Paracetamol	1.1	151
6	Etodolac	4.1	287
7	Ibuprofen	1.9	206
8	Indomethacin	3.3	358
9	Tenoxicam	2.6	337
10	Flurbiprofen	3.4	244
11	Ketoprofen	1.9	254
12	Naproxen	1.6	230
13	Tiaprofenic acid	1.5	260
14	Tolmetin	2.2	257
15	^{14}C-Cortisone	1.3	360

*Values relate to IA administration in animal model (other values from oral dosing)

2.2. Drug transport into cartilage

After IA administration drugs are likely to distribute from the SF into various joint tissues. Calculations on glucose transport, to feed the chondrocytes of the articular cartilage, have revealed that diffusion of this nutrient in the relatively viscous SF might be too slow for reaching the center of larger joints. Instead joint motion is suggested to

generate additional solute transport by convection.[9] Upon attainment of a uniform glucose concentration in the SF, diffusion into cartilage is sufficiently fast to supply the chondrocytes with this nutrient. Similar considerations may be of relevance for drug distribution in the joint cavity. At the SF-cartilage interface two major parameters govern the efficiency of solute transport into the cartilage ECM, that is, the size and the charge of the solute.

3. Pharmacokinetic fate of IA administered drugs[10]

IA injection of aqueous solutions of relatively high doses of drugs for example corticosteroids, local anaesthetics, and NSAIDs results in diffusion of drug rapidly into the blood and consequently duration of drug action is relatively short[8,10,11] First-order transfer rate constants for drug transport out of the synovial space have been reported for various NSAIDs, including diclofenac, etodolac, ibuprofen, indomethacin and tenoxicam.[12] Almost identical transfer rate constants were found with a mean value of $0.32\pm0.12\ h^{-1}$ and hence, corresponding half-lives of approximately 2 h. Thus, prolongation of drug residence time within the joint has to be accomplished by transient immobilization of the injected dose in the form of depot formulations.

Rational design of IA depot formulations requires an in-depth understanding of **(i)** the physicochemical and enzymatic events, which are likely to be operating for drugs injected into the synovial space and **(ii)** the time dependence of these interrelated events each of which influences drug residence time in the joint.

4. Residence time of drug in synovial joints

The short half-life of IA administered drugs can be explained by the synovial ultra-structure which offers little barrier to the diffusion of molecules in and out of the joint. As a consequence of the synovial ultra-structure there is free trans-synovial flux of water and solutes such that the volume of fluid in a joint cavity is completely replaced many times over in 24 h[6]. Small molecules (MW < 10,000 Da) such as lactate, cytokines and most drugs, including NSAIDs, local anaesthetics (LA), diffuse easily through the interstitium and across capillary walls and therefore exist in equilibrium between SF and plasma.[13]

Albumin also diffuses easily through the interstitium; the reflection of albumin by the synovium is negligible for practical purposes.[14] SF is therefore a dialysate of plasma plus HA secreted by type-B synoviocytes. Even with HA, multiple (3-5) weekly injections are needed in order to achieve efficacious OA treatment. There is a need for sustained

release delivery systems if the potential of IA drug administration for the anti-arthritics is to be realised.[15]

4.1. Factors affecting performance of IA drug delivery

The direct delivery of a drug to an affected joint offers the possibility of reaching high drug concentration at the site of action with minimum systemic exposure. Besides the minimum systemic toxicity there are other factors that may contribute to show adverse effects or decreased efficacy of IA delivery as described below:

4.1.1. Size

4.1.2. Shape

4.1.3. Quantity

4.1.4. Charge

4.1.5. Rate of drug release

4.1.6. Number of injections

4.1.1. Size

Concerning the size of the IA administered particles, the discussion on the work done in the field will focus on liposomes, microparticles (microsphere), and nanoparticles emphasizing aspects such as joint retention and phagocytosis as a function of size. Drugs are cleared from SF by lymph drainage, which is largely dependent on the size of the molecule.

The clearance of albumin from SF in knees of OA patients is about 0.04 ml/min, corresponding to a turnover of about 1 h. In other studies the residence time of NSAIDs in SF was shown to be as short as 1-5 h.[1] Bonanomi *et al.* reported that increasing the size of liposomes, ranging from 160 nm to 750 nm in diameter, resulted in a 2.6-fold increase in retention to 48 h post-injection.[16-17] A similar observation was described for liposomes containing methotrexate, for which a mean diameter of 1.2 μm ensured a higher retention, and thus anti-inflammatory action, than 100 nm sized liposomes.[18-19] There is an upper limit (40-250 nm radius) to the size of particles that can escape freely from the joint cavity. Albumin possesses a hydrated molecular domain of 3.55 nm and HA has a radius of gyration of 100-200 nm.[20] Possibly the simplest means of prolonging drug residence time within joints is to formulate a microsphere (MS) of size >250 nm and thereby exploit size inclusion. The fate of biocompatible MS is size dependent, those <10 μm (optimally between 1 and 4 μm) in diameter are phagocytosed by the SF and synovial resident and recruited macrophages, without evoking a neutrophil response. Larger MS (>4 μm) are not

phagocytosed, but are sequestered as subsynovial granulation plaques, surrounded by giant cells.[15,21]

4.1.2. Shape

Not only the size, but also the shape of the particles injected into the joint is important for triggering an immune response. Irregularly shaped microparticles have been demonstrated to promote tissue inflammation in comparison to the round shaped drug delivery systems. In this respect, Liggins *et al.* showed that irregularly, milled chitosan particles induced joint inflammation despite the known articular biocompatibility of this biomaterial.[22] Similar histological observations showing marked inflammation in the synovial membrane and the subsynovial lining were made by Ratcliffe *et al.* for poly-*L*-lactic acid (PLA) and poly(butyl cyanoacrylate) microparticles obtained by simple polymer grinding.[24] Thus, to avoid inflammatory reactions subsequent to the administration of irregularly shaped particles, round-shaped particles are to be preferred for IA drug delivery.[19] This fact was also supported by evidence obtained by injecting IA suspensions. Due to the crystalline nature drugs generate inflammatory conditions leading to crystal induced arthritis.[19]

4.1.3. Quantity

Apart from size and shape the quantity of IA injected drug delivery system, is also important to the appearance of an immune response, as shown in the studies conducted by Nishide *et al.* They demonstrated an enhanced number of white blood cells with increasing amounts of injected microparticles, from 5 to 40 mg per knee.[25] In contrast, no significant dose dependent inflammatory effect was observed for paclitaxel embedded small Poly-Lactide-co-glycolide (PLGA) microparticles after IA administration of 15 to 75 mg of particles per joint in healthy rabbit knees.[19,26]

4.1.4. Charge

Little information is available on the effect of charge on disappearance rates through the synovium. In the study performed by Simkin and Pizzorno, it was found that synovial permeability of magnesium and calcium was lower than for other small neutral molecules investigated.[1,27] Literature survey also revealed that introduction of a quaternary ammonium function on oxicam structures greatly increased their affinity for the cartilaginous tissues.[28] These findings suggest that positively charged molecules may interact with negatively charged sugars present in cartilage and may improve drug residence time with joint cavity.

4.1.5. Release profile

If the drug is released rapidly then efficacy is likely to be approximately to that of free drug and hence it offers no therapeutic advantage. A worse scenario is that the drug is released too slowly resulting in target tissues being exposed to sub-therapeutic dose levels thus having little or no efficacy. Achieving the middle ground, sustained drug release at levels within the therapeutic range *in vivo* may be possible through rational drug delivery design for which rigorous *in vitro* characterization of release kinetics required to be done.[15]

4.1.6. Number of injections

An obvious drawback of IA injection is discomfort, pain and possible risk of infection. Therefore the number of IA injections per year should be reduced to a minimum. These complications coupled with short residence time of the drug in joint cavity underscores the need for the development of sustained release formulations for IA delivery.[1]

5. Drug delivery strategies in joint disease

In RA, the local inflammatory reaction in and around joint tissues promotes an acidic environment. This is partially due to the low levels of oxygen in the synovial tissue and fluid, which appears to induce a shift towards anaerobic glycolysis and lactate formation.[30] In some cases, *p*H values of SF have been reported to be as low as 6.[31-32]. Considering the buffering capacity of SF, a much lower *p*H value in the synovial tissue may be expected. In addition, there seems to be a direct correlation between the low *p*H of the joint tissues and indices of disease severity. The low *p*H has also been associated with local osteoclast activity and bone destruction.[33] For OA, cartilage damage of the joint has been associated with a significant drop of *p*H at the articular cartilage surface, which may contribute to high activities of cathepsins in cartilage destruction.[30] The drug delivery strategies based on the low *p*H value of synovial joint of the RA and OA (such as *p*H sensitive and temperature sensitive gel) and the negative charge of the cartilage (cationic colloidal delivery systems), are in the developmental stage.

6. Literature review

It has been reported that first-order transfer rate constants operate for various NSAIDs, in synovial space.[10] These half-lives vary between 1 and 2 h for cortisone, naproxen or ketoprofen and up to 22-26 h for hyaluronan.[19] In other studies the residence

time of NSAIDs in SF has been shown to be as short as 1-5 h.[23] The clearance of albumin from SF in knees of OA patients was estimated to be about 0.04 ml/min, corresponding to a turnover of about 1 h. So in terms of absorption and distribution into the systemic circulation, the IA route is equivalent to other non-i.v. parenteral routes of administration.[19]

Derendorf *et al.*[34] investigated the pharmacokinetics of glucocorticoid formulations following IA injection. These studies showed that the residence time of poorly soluble glucocorticoids administered as suspension formulations (e.g., rimexolone, triamcinolone acetonide) in the knee joint was longer than for the soluble glucocorticoids administered as solution formulations (e.g. betamethasone hydrogen phosphate disodium).[34] This is due to the fact that the dissolved molecules rapidly undergo clearance from the knee joint, while the solid particles of suspension formulations need to dissolve prior to being cleared from the joint. Thus, the residence time of an IA injected compound in the knee joint depends mainly on its solubility and dissolution rate.[6]

In some cases formulations are available as suspensions or dry powders due to solubility or stability issues related to the drugs. But due to the crystalline nature, these drugs can generate inflammatory conditions upon IA injection, leading to crystal-induced arthritis which is observed in 10 % of the patients, but disappearing within a few days.[35] Along with this, controlled growth of crystals also bears the risk of poor biocompatibility of the suspension formulation upon IA injection.

In case of LipotalonR® a liposomal formulation containing modified dexamethasone i.e. dexamethasone-21-palmitate the solubility of the dexamethasone derivative is reduced due to the covalent bonding of the fatty acid chain, thereby enabling the inclusion of the compound into a liposomal formulation from which the drug is slowly released following IA injection.[6]

The potential of liposomes to increase the IA residence time of drugs was first suggested in 1976 by Shaw *et al.*[36] which represented an important drug delivery system with valuable clinical utility for IA drugs. Bonanomi *et al.* reported that the encapsulation of dexamethasone palmitate in liposomes resulted in improved retention compared to microcrystalline triamcinolone acetonide.[16] Additionally, follow up study showed that increasing the size of these liposomes ranging from 160 nm to 750 nm in diameter, resulted in a 2.6-fold increase in retention to 48 h post-injection.[17]

Hydrocortisone encapsulated liposomes remarkably improved subjective and objective indices of inflammation after 48 h, with a slow return to the pre-injected state

after 2 weeks. So, liposomes have been shown to be very efficient systems in the local treatment of joint diseases in laboratory animals and also in humans.

In the early studies, liposomes showed limited efficacy following IA injection due to rapid release of the drug and its leakage into the circulation. However, Williams *et al.*[18] were able to demonstrate efficacy of liposomes in a rat antigen induced arthritis model by using liposomally conjugated MTX to reduce the rapid loss of MTX from the liposomes and the joint.[18,37]

Williams *et al.* reported that encapsulation of methotrexate in 1.2 μm sized liposomes reduced joint swelling by 26.5 % compared to only 3.5 % of the 100 nm sized liposomes after day 1 of their injection. Moreover, the effect was still notable 20 days after the injection of the liposomes.[18-19] **Fig. 1.3** Shows various treatment strategies for arthritis with their limitations and benefits

Niosomes, which are non-ionic surfactant-based liposomes, were studied for the local delivery of diclofenac sodium to treat arthritis. Radiolabelling study showed that lipogelosomes injected in rabbit arthritic joints showed the longest retention times compared to other formulations. The radioisotope was slowly released in such a way that 67 % of the initially injected radioactivity still remained present 24 h post-injection.[38]

Together with liposomes, another drug delivery system i.e microsphere represents one of the most widely studied means to decrease IA drug clearance. In this respect, Lu *et al.* reported that the mean residence time of flubiprofen got doubled compared to injection of the drug suspension.[40] Similar observations were made for a celecoxib solution compared to the celecoxib embedded chitosan microspheres, where the chitosan microspheres showed a 10-fold increase in the concentration of celecoxib in the joints after IA injection.[41]

Wigginton *et al.* studied pharmacokinetics of methotrexate (MTX), wherein the elimination of MTX (5 mg) from the joint was found to be biexponential over 24 h and the second dose was given after 24 h and the half-life was found to be 0.54 and 2.9 h respectively. However, the authors concluded that MTX was clinically ineffective, primarily because the IA half-life of methotrexate was too short relative to the probable synovial cell cycle generation time. Repeated IA MTX doses produced better results.

Liang *et al.* formulated methotrexate embedded PLA microparticles and tested their pharmacokinetics after IA injection in healthy rabbits. As expected, the concentration of methotrexate in the synovial tissues following IA injection was found to be significantly

higher in the group treated with microparticles compared to the one treated with the drug solution.[42]

Horisawa *et al.*[21] showed PLGA nanoparticles (mean diameter 265 nm) were extensively phagocytosed and subsequently transported through the synovial membrane within 3-7 days. In contrast, microspheres (mean diameter 26 μm) were neither phagocytosed nor transported to the underlying synovial membrane, but they triggered a granulation reaction with multinuclear giant cells.[19]

Nishide *et al.* also supported these findings using poly-*D,L*-lactic acid (PDLLA) microspheres. The authors concluded that microspheres with diameters larger than 20 μm were neither internalised into macrophages, nor did they produce important inflammatory responses when injected IA into healthy rabbit joints.[43]

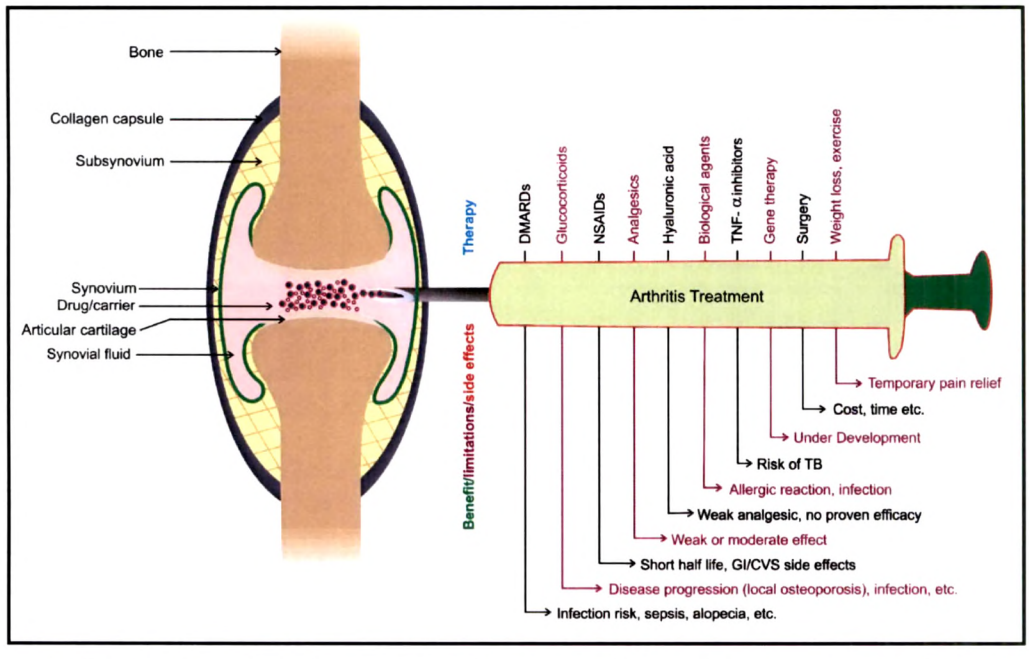

Fig. 1.3: Benefits, limitations and adverse effects of various anti-arthritic agents.

Liggins *et al.* have conducted studies to observe of the effect of size and dose of microspheres on biocompatibility or tolerability in rabbit joints.[45] They have shown that PLGA microspheres of a smaller size range of 1-20 μm produced a greater inflammatory response in rabbit joints than larger sized microspheres (35-105 μm).[37] The phagocytosis of microspheres by synovial macrophages improved the retention of microspheres in the joint and also ensured an increased drug concentration in inflammatory cells and delayed clearance from the joint minimizing drug exposure to cartilage.[28,44]

Ramesh *et al.* pointed out that no drug was detected in the serum of healthy rabbits 7 h after the IA injection of dexamethasone encapsulated in PLA microparticles, thus concluding that dexamethasone release is localised in the joint.[46] In contrast, a small quantity of methotrexate was detected in rabbit plasma 5 min post-injection of PLA microparticles, mostly due to a burst release of the drug from the particles.[47] Moreover, the microparticles, whose size was in the range of 30-100 μm, did not induce acute inflammatory reactions. From the above studies it can be concluded that the most suitable size for microsphere is between 5 and 10 μm. This particle size range ensures capture of the particles by the synovial macrophages, a process that results in a prolonged retention time in the joint and also limits the inflammatory reactions.[19]

Rothenfluh *et al.* demonstrated that in order to achieve efficient carrier penetration into the cartilage, the carrier size plays important role. He coated nanoparticles with collagen-II binding peptide. Fluorescence measurement studies showed 14.9 fold preferential accumulation of 38 nm mean diameter nanoparticles within the cartilage relative to 96 nm diameter nanoparticles. This significant difference was attributed to the 60 nm pore size of the dense collagen network.[48] Rothenfluh *et al.*[48] demonstrated that the peptide decorated nanoparticles concentrated up to 72-fold more in the articular cartilage than nanoparticles displaying scrambled peptide sequence following IA injection in mice. Various drug delivery systems and their benefits or limitations or adverse effects are shown in **fig. 1.4**

Alternative approaches that might also benefit from an IA administration in terms of limitation of non-specific systemic binding, are targeting of the folic acid receptor FRb, which is up-regulated in activated synovial macrophages and targeting of E-selectin, which is an adhesion molecule that is up-regulated on the vascular endothelium of inflammed tissue.[19]

Amongst the physical methods the use of a magnetic field may improve the accumulation of magnetic drug delivery systems at the targeted site. In this respect, Tanaka *et al.*[49] used a 0.2 T permanent magnet implanted into the femur to increase the IA retention time of magnetic liposomes containing TGFb1. It was demonstrated that the presence of a magnet leads to a more efficient retention of the liposomes in the joint and to a significant diminution of cartilage defects up to 12 weeks.[49] Butoescu *et al.* incorporated superparamagnetic iron oxide nanoparticles (SPIONs) in PLGA microparticles loaded with dexamethasone and achieved a joint residence time of at least 3 months.[50] Regarding

the possible toxicity of the SPIONs, Schulze *et al.* demonstrated that 30-40 nm PVA-coated SPIONs were biocompatible with articular and periarticular tissues in sheep.[51]

Other studies on magnetic nanoparticles were conducted by Hellstern *et al.*[51] The authors observed that the nanoparticles are taken up by the reticuloendothelial system, and that the main organs in which the nanoparticles are detected after IA administrations are the liver and the spleen. Magnetic drug delivery systems need further investigation before the initiation of clinical trials to confirm the delivery of sufficient payloads to the joint, long-term retention and efficacy in arthritis or OA animal models.[19]

Thermally responsive elastin like polypeptide gels that can spontaneously aggregate after IA injection represent a simple and innovative way to prolong the IA half-life of a drug. These aggregating elastin like polypeptides form a drug depot resulting in a 25-fold longer half-life than drugs administered with the non-aggregating protein.[47] Research is going on to chemically couple the elastin like polypeptide to proteins, such as the IL-1 receptor antagonist, in order to achieve higher residence times.[47]

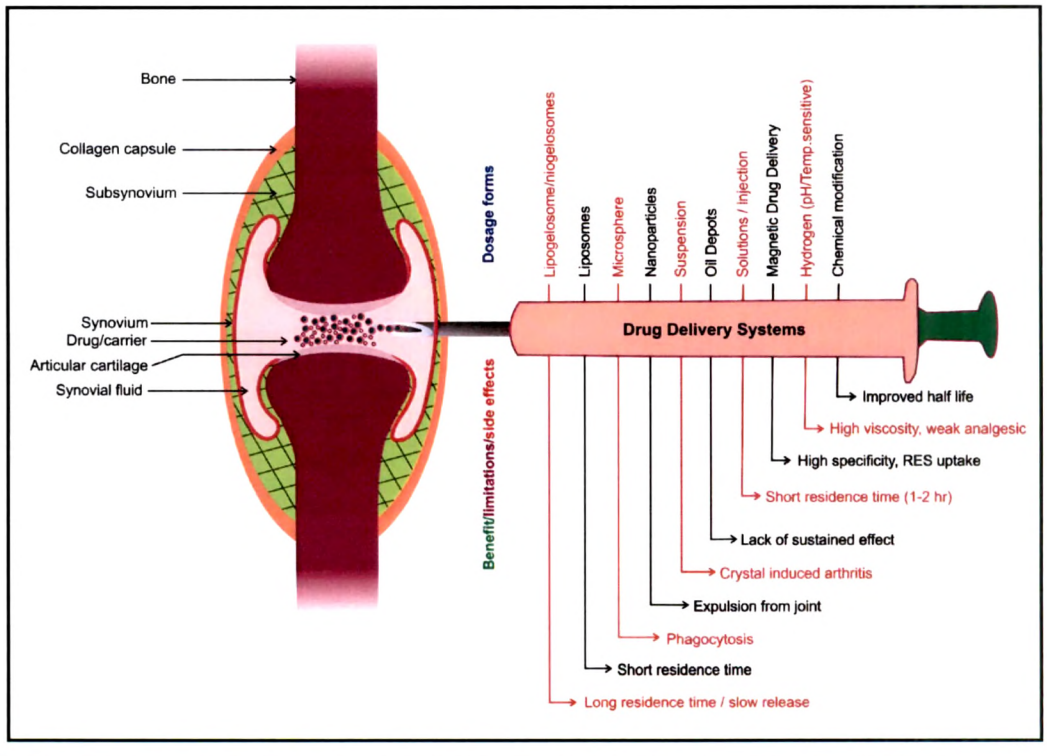

Fig. 1.4: Benefits, limitations and adverse effects of various drug delivery systems

As a possibility, a positively charged colloidal delivery system may be more specific in directing drugs to RA joints because of the EPR (enhanced permeability and retention) effect. The positive charge may help to prolong the retention time of the

delivery system to allow complete release of the payload in the joint. The neutralisation of aggrecan negative charges by a positively charged delivery system may further weaken the mechanical strength of the cartilage. Therefore, it would be ideal if the positive charge of the delivery system resides temporarily in the cartilage only and eventually be cleared from the cartilage or degraded.[30]

Currently available therapy for IA injection includes (i) Celestone®/Soluspan® injection which contains 3 mg/ml betamethasone as betamethasone sodium phosphate and 3 mg /ml betamethasone acetate available as suspension (Mfg By: Schering Corporation), (ii) Depo-Medrol® contains methylprednisolone acetate 20/40/80 mg available in injection, suspension dosage form. (Mfg. by Pharmacia And Upjohn Co), (iii) Lipotalon® contains dexamethasone-21-palmitate manufactured by Merckle Pharmaceuticals.

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Aims and Objectives

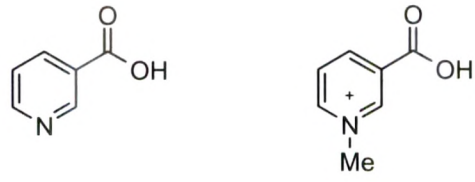
Literature survey revealed that a drug in solution form is rapidly expelled from the joint cavity upon IA administration; so various drug delivery systems such as suspension, liposomes, microspheres etc. have been developed and tested, and have been found to be more effective than simple injection with improved residence time.[1] Cartilage tissue contains negatively charged polysaccharides so drug residence time could be improved upon IA administration if positive charge is present either on the drug moiety or on the drug carrier system. But little information is available on the effect of charge on disappearance rates through the synovium.[1]

In a study by Simkin and Pizzorno, it was found that synovial permeability of magnesium and calcium was lower than that of other neutral and small molecules investigated.[1,2] Literature survey also proved that introduction of a quaternary ammonium function on oxicam structure greatly increased its affinity for the cartilaginous tissues. [3] Quaternary ammonium tropinol esters also show affinity towards cartilage on i.v. administration. These findings suggest that positively charged molecules may interact with negatively charged polysaccharides present in cartilage and may improve drug residence time in the joint cavity.[4]

A positively charged colloidal delivery system may be more specific in directing drugs to RA joints because of the EPR effect. The positive charge may help to prolong the retention time of the delivery system to allow complete release of the payload in the joint. But the neutralisation of aggrecan negative charges by a positively charged delivery system may further weaken the mechanical strength of the cartilage. Therefore, it would be ideal if the positive charge of the delivery system resides only temporarily in the cartilage and get eventually cleared from the cartilage or gets degraded. Enhanced cell penetration of the positively charged delivery system may also raise the undesirable toxicity issues.[5]

The aim of the current work was to develop cationic chemical delivery systems (CDS) of various non-steroidal anti-inflammatory drugs (NSAIDs) to improve drug residence time in joints on IA administration for the treatment of active RA or OA. To develop an ideal chemical delivery system the carrier should be non-toxic, releases drug over a prolonged time and after complete release of the drug must get excreted from the joint without any adverse effects. To fulfill the above requirements it was planned to synthesize CDS of various NSAIDs using nicotinic acid (1) as carrier. Ackermann has

demonstrated that ingested nicotinic acid is partly excreted by the dog as trigonelline (**1A**) which has also been isolated by Linneweh and Reinwein from normal human urine.[6-7]



(1) **(1A)**

Herbert P. *et al.* have shown that normal human subjects excrete only 1-3 milligram of nicotinic acid and its derivatives (amide, glycine conjugate) daily compared to 30-50 mg. of trigonelline.[6] So trigonelline is a major excretory product of nicotinic acid metabolism with proven safety profile. Hence it was planned to develop CDS of various NSAIDs using this carrier. The general structure of the designed CDS is shown in **Fig. 2.1.**

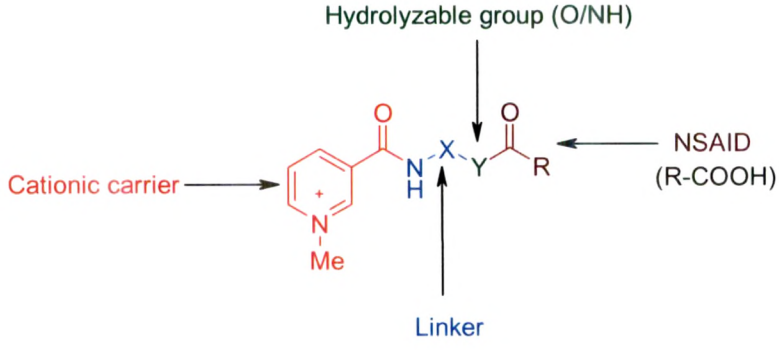


Fig.2.1: Schematic representation of cationic CDS.

The designed CDS contains positively charged pyridinium head linked to the drug through a suitable linker (X). This cationic group will interact with negatively charged polysaccharides present in the cartilage and will improve localization of the drug. Further, the designed CDS contains hydrolyzable functional groups such as ester/amide (Y=O/NH) which will undergo enzymatic and/or acid catalyzed hydrolysis to release the free drug (R-COOH) in a controlled manner.

As shown in **Fig. 2.2** once the CDS is injected by IA route it may adopt two pathways **(i)** it may be expelled from the joint as such and gets excreted or hydrolyzed in blood (*p*H 7.4) to liberate the active drug which might be again available to cartilage tissue by reuptake mechanism. **(ii)** The CDS remains in the joint space and slowly undergoes enzymatic or acidic hydrolysis, as synovial joint contains many enzymes and the *p*H of synovial fluid also gets decreased in inflammatory conditions (*p*H 6). So it was necessary

to study hydrolysis kinetics of the synthesized CDS in phosphate buffers of *p*H 6 and 7.4, and in human serum *in vitro*.

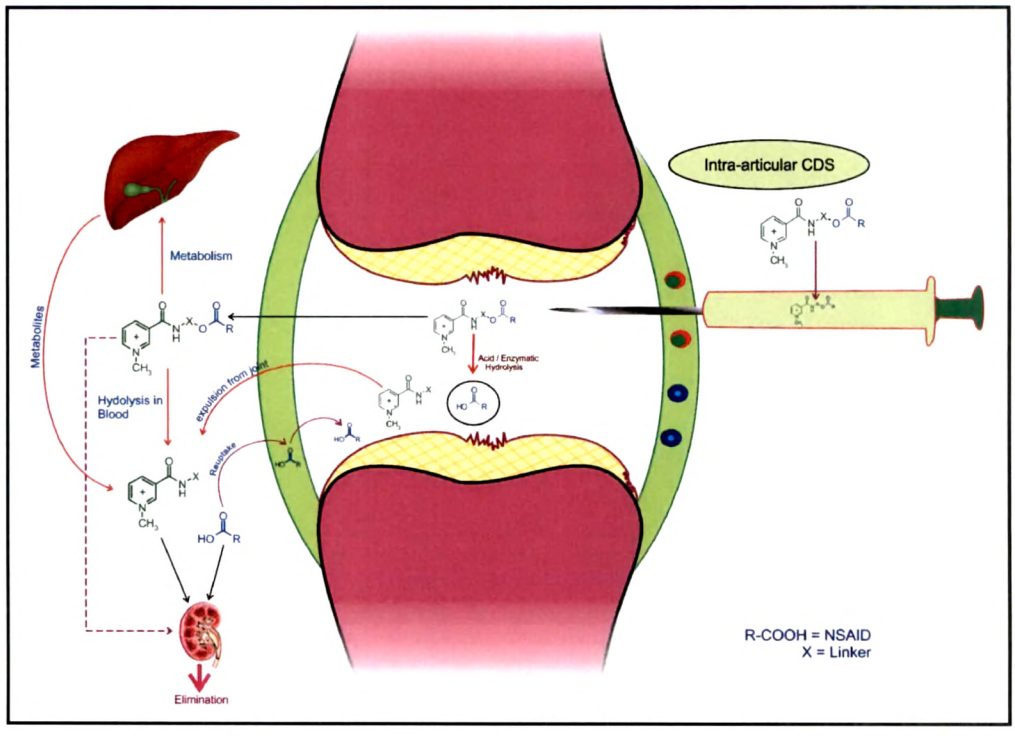


Fig. 2.2: Fate of IA administered CDS

Besides cartilage, the positively charged CDS and cationic metabolites of CDS may be deposited in the liver and gets cleared by the kidney. Therefore, liver and renal toxicity issues are important in development of these systems.[5] So it was planned to study *in vitro* cell cytotoxicity of the developed CDS and compare their relative toxicity with the parent NSAIDs.

Further it was also planned to evaluate joint localization and residence time (half life) of the synthesized CDS and the parent drugs using standard radiolabeling techniques using ^{99m}Technetium as radiolabeling agent.

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3. Results and Discussion

The work carried out towards achieving the proposed plan has been discussed under the following four main headings:

3.1. Chemical studies

3.2. Hydrolyses kinetics

3.3. Cytotoxicity studies

3.4. Biodistribution and gamma imaging studies

3.1. Chemical studies

To synthesize the envisaged CDS, a scheme was planned as given in general **Scheme-1**, wherein nicotinic acid **(1)** was reacted with ethanol in presence of sulphuric acid to obtain ethyl nicotinate[1] **(2)**. Ethyl nicotinate was further treated with aminoalcohols[2] **(3a-3e)** and diamines **(3f-3g)** to obtain various hydroxyalkylnicotinamide (**4a-4e**) and aminoalkylnicotinamide **(4f-4g)** derivatives. These nicotinamide derivatives **(4a-4g)** were coupled with various NSAIDs **(5I-5V)** to get ester **(6aI-6eV)** and amide **(6fI-6gV)** derivatives. The derivatives so obtained were quaternized using methyl iodide in acetonitrile or acetone as a solvent to obtain quaternary ammonium chemical delivery systems **(7aI-7gV)**. The two NSAIDs which were not available commercially but required for the development of CDS i.e. 6-methoxy-2-naphthylacetic acid (6-MNA) **(8)** and biphenylacetic acid (BPA) **(9)** were synthesized as per the reported procedures.[3]

The synthesis of the chemical delivery systems of various NSAIDs has been discussed under the following subheadings:

3.1.1. Chemical delivery systems of ibuprofen **(7aI-7gI)**

3.1.2. Chemical delivery systems of naproxen **(7aII-7gII)**

3.1.3. Chemical delivery systems of indomethacin **(7aIII-7gIII)**

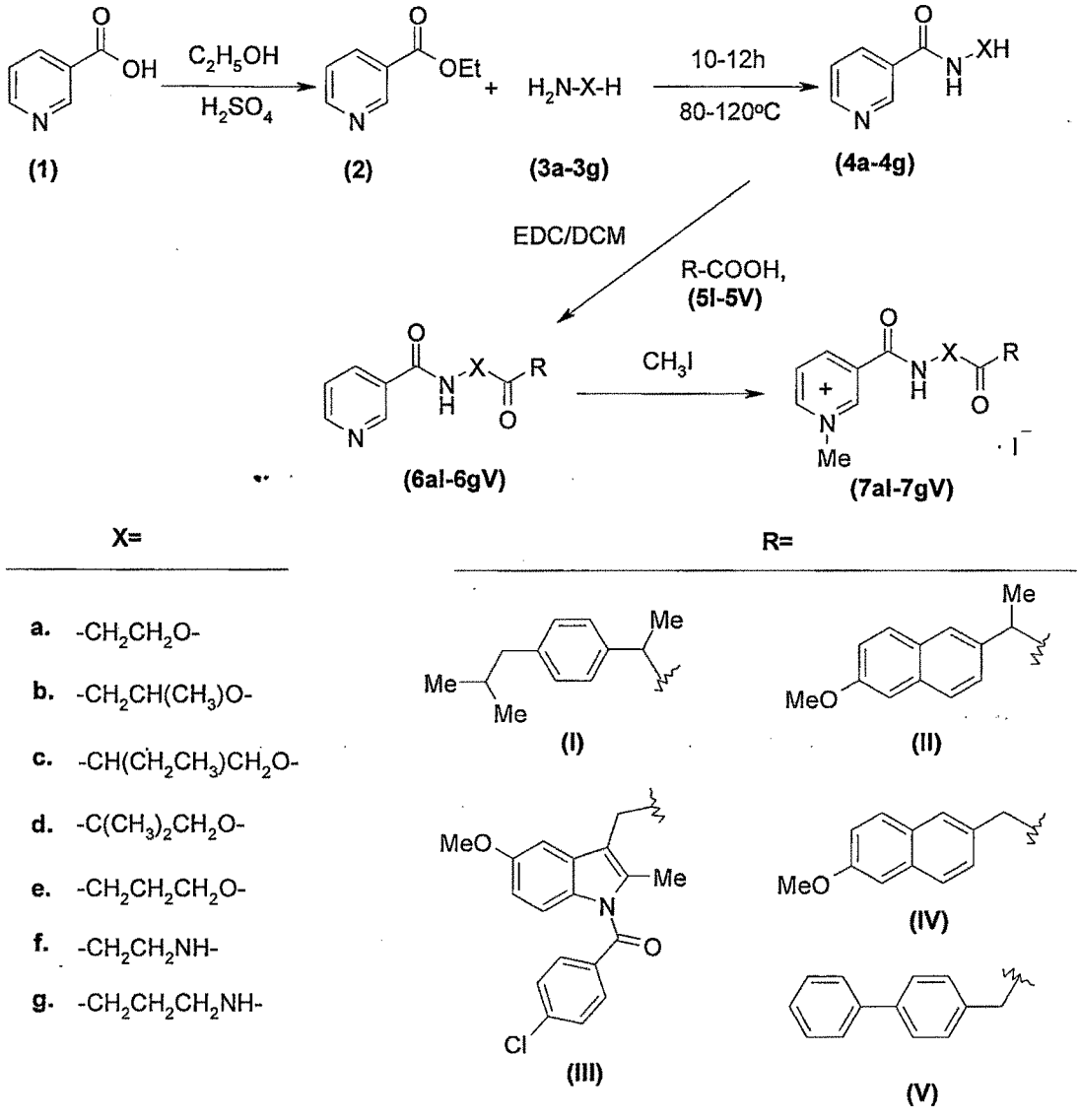
3.1.4. Chemical delivery systems of 6-MNA **(7aIV-7eIV)**

3.1.5. Chemical delivery systems of BPA **(7aV-7eV)**

3.1.1. Chemical delivery systems of ibuprofen (7aI-7gI)

Ibuprofen **(5I)** is a clinically used NSAID belonging to the arylpropionic acid class, hence ibuprofen was chosen as one of the NSAIDs for the present work. The CDS of ibuprofen **(7aI-7gI)** were synthesized as depicted in **Scheme-1**. These CDS fall into two main types, one which contains suitable aminoalcohol moiety **(3a-3e)** as a linker to give hydrolyzable ester derivatives **(7aI-7eI)** and another contains diamine (**3f-3g**) as linker to give amide derivatives **(7fI-7gI)**.

In order to execute **Scheme-1**, various hydroxyalkylnicotinamide (**4a-4e**) and aminoalkylnicotinamide (**4f-4g**) derivatives were synthesized from ethyl nicotinate (**2**) using suitable linkers (**3a-3g**). Ethyl nicotinate (**2**) was treated with aminoalcohols (**3a-3e**) at 80-90°C under stirring for 10-12h and the excess aminoalcohol removed under vacuum



Scheme-1

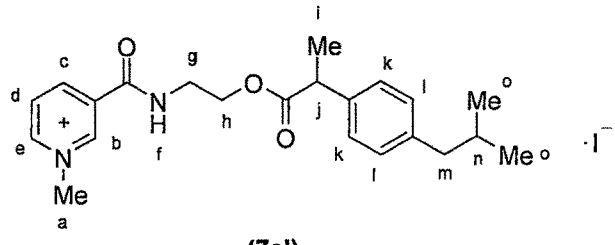
to gave hydroxyalkylnicotinamide intermediates (**4a-4e**). It was observed that majority of these esters were low melting and probably due to this reason, compounds **(4a-4e)** were isolated as liquids. Another series of compounds (**4f-4g**) were synthesized in the same manner using excess of diamines (**3f-3g**) to give aminoalkylnicotinamide intermediates (**4f-4g**). Spectral data of these intermediates **(4a-4g)** is given in **Table 3.1**

Table 3.1: Spectral data of derivatives **(4a-4g)**

Comp.	IR Peaks (cm^{-1})	PMR Peaks (δ)
4a	3270, 1635, 1310, 1064,	9.07 (s, 1H), 8.67-8.65 (d, 1H), 8.45-8.43 (t, 1H), 8.22-8.19 (d, 1H), 7.41-7.37 (m, 1H), 4.74 (s, 1H), 3.70- 3.68 (t, 2H), **3.52-3.47 (m, 2H)**
4b	3285, 1639, 1301, 1122, 702	9.0 (s, 1H), 8.58-8.55 (d, 1H), 8.18-8.12 (m, 2H), 7.33-7.29 (m, 1H), 4.05-3.99 (m, 1H), 3.65 (s, 1H), 3.58-3.25 (m, 2H), **1.19-1.18 (d, 3H)**
4c	3278, 2964, 1638, 1302, 703	9.07 (s, 1H), 8.66-8.65 (d, 1H), 8.22-8.19 (d, 1H), 8.01-7.99 (d, 1H), 7.40-7.36 (m, 1H), 4.04 (bs, 1H), 3.63-3.62 (d, 2H), 3.55 (s, 1H), **1.74-1.53, (m, 2H), 0.97-0.94 (t, 3H)**
4d	3274, 1645, 1372, 702	8.90 (s, 1H), 8.60-8.58 (d, 1H), 8.07-8.04 (d, 1H), 7.32-7.29 (m, 1H), 6.97 (s, 1H), 4.62 (s, 1H), 3.66 (s, 2H), **1.41 (s, 6H)**
4e	3282, 1637, 1305, 702	9.0 (s, 1H), 8.61-8.60 (d, 1H), 8.45-8.43 (m, 1H), 8.17 -8.14 (d, 1H), 7.37-7.34 (t, 1H), 4.44 (s, 1H), 3.72-3.69 (t, 2H), 3.58-3.53 (q, 2H), **1.15-1.79 (m, 2H)**
4f	3348, 3281, 1644, 1307, 825, 706	9.05-9.04 (s, 1H), 8.65-8.63 (d, 1H), 8.22 (bs, 1H), 8.17-8.14 (m, 1H), 7.36-7.32 (m, 1H), 3.50-3.46 (q, 2H), 2.93-2.90 (t, 2H), **2.70 (s, 2H)**
4g	3279, 1644, 1590, 1308, 708	8.81 (s, 1H), 8.35-8.33 (d, 1H), 7.87-7.84 (d, 1H), 7.05-7.02 (m, 1H), 3.22-3.14 (m, 1H), 2.52-2.49 (t, 2H), 2.42-2.40 (t, 2H), 1.54-1.39 (m, 2H), **1.28-1.23 (m, 2H)**

The first member of the ibuprofen series, **(7aI)** was synthesized as per **Scheme-1**. Ibuprofen **(5I)** was coupled with *N*-(2-hydroxyethyl)nicotinamide **(4a)** using ethyl-(dimethylaminopropyl)carbodimide (EDC) as coupling agent in dichloromethane to give the derivative **(6aI)** which on quaternization using methyl iodide in acetonitrile gave a crude yellow semisolid product, which was crystallized from ethyl acetate to give **(7aI)** as a white product.

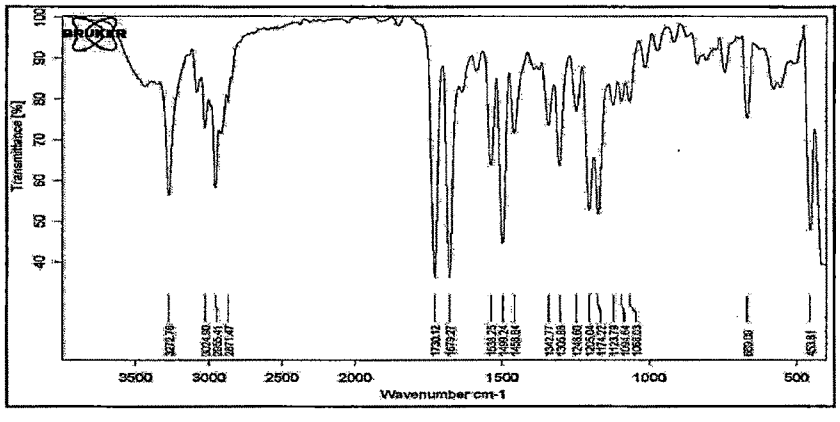
The IR spectrum (**Fig. 3.1 A**) of compound **(7aI)** showed carbonyl stretching of ester and amide groups as sharp intense bands at 1730 cm^{-1} and 1679 cm^{-1} respectively. N-H Stretching of amide was observed at 3272 cm^{-1} and the peaks due to C-O stretching came at 1205 and 1174 cm^{-1}.



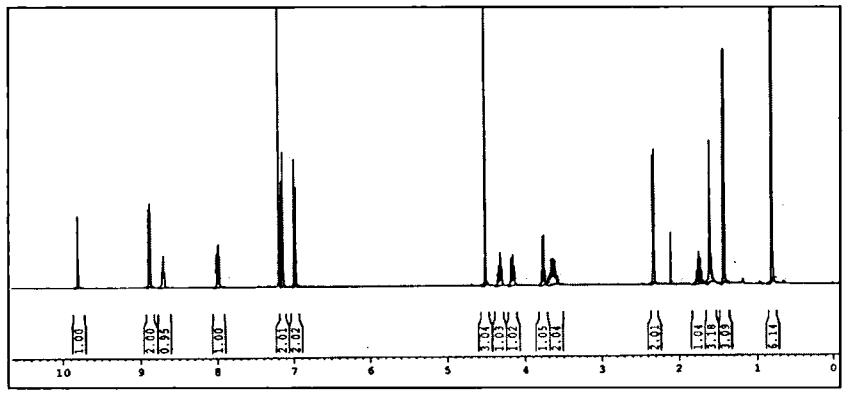
(7aI)

The PMR spectrum (**Fig. 3.1 B**) of the compound **(7aI)**, showed singlet at δ 9.80 for the single proton of pyridinium-H_b, multiplet at δ 8.89-8.87 due to NH_f and -H_e protons with coupling constants equal to 8.0 Hz. Another proton of pyridinium ring (-H_c) was observed at 8.72-8.69 as a multiplet (*J=8.0* Hz) single proton of pyridinium ring appeared at δ 8.0-7.97 as a multiplet (-H_d), (*J=8.0* Hz). The protons of 1,4-disubstituted phenyl ring appeared at δ 7.15-6.97 as a multiplet. Methyl protons ($CH_{3/a}$) of quaternary nitrogen appeared at δ 4.50 as a singlet, The single proton (-CH_n) appeared at δ 1.77-1.70 as a multiplet and methyl protons (-$CH_{3/i}$) at δ 1.43-1.41 as a doublet (*J=7.16* Hz). The six methyl protons ($CH_{3/o}$) appeared at δ 0.81-0.79 as a doublet.

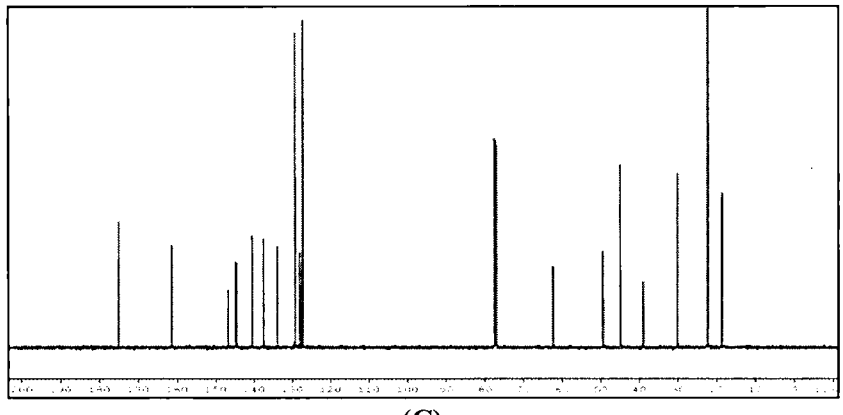
^{13}C-NMR spectrum (**Fig. 3.1 C**) shows peaks at δ 175.0, and 161.35 for *C*=O carbon of ester and amide groups respectively, aromatic carbons appear at 146.82, 144.76, 144.76, 140.51, 137.45, 133.93, 129.29, 128.23, 127.32 and aliphatic carbons appear at 62.40, 49.42, 44.92, 44.89, 38.95, 30.15, 22.37 and 18.65. The H-H coupling was also confirmed using COSY spectrum as shown in **Fig. 3.1 E**



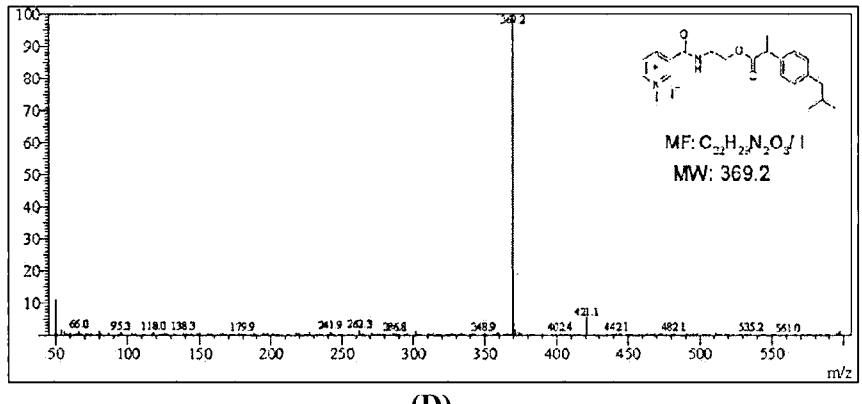
(A)



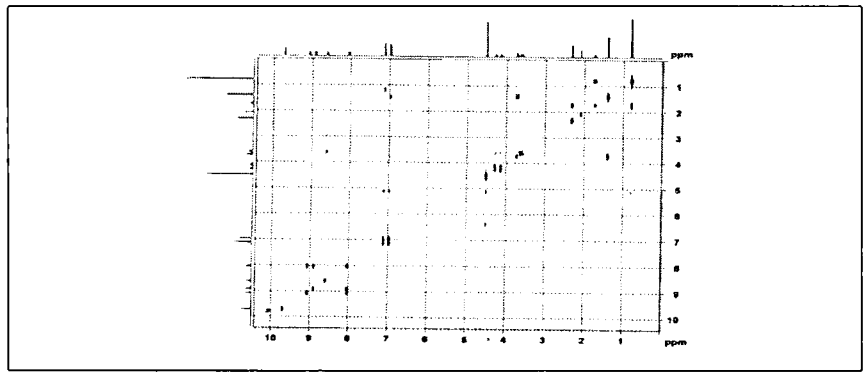
(B)



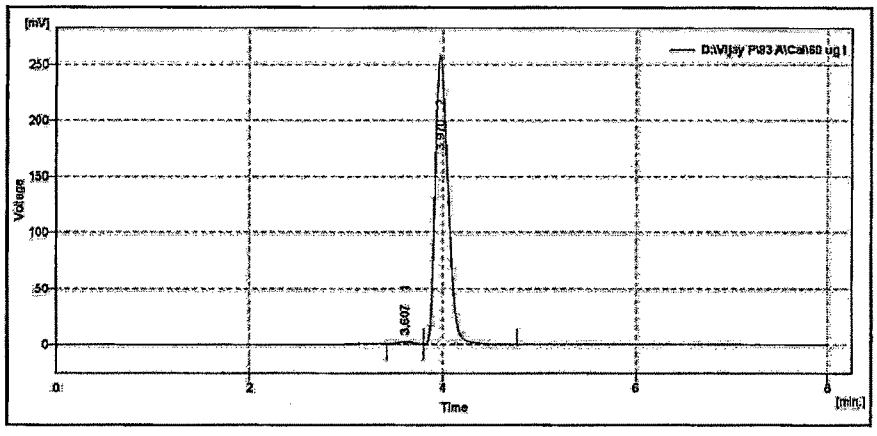
(C)



(D)



(E)

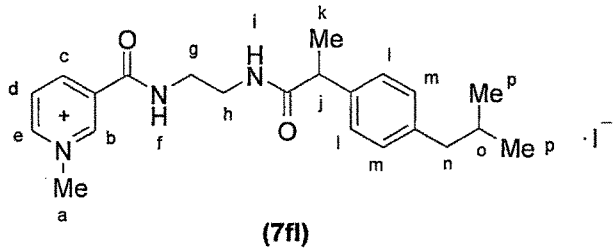


(F)

Fig. 3.1: Spectra of derivative **(7aI)**; IR spectrum **(A)**, ^{1}H-NMR **(B)**, ^{13}C-NMR **(C)**, Mass spectrum **(D)**, COSY **(E)** and HPLC chromatogram **(F)**.

Another series of CDS **(7fI-7gI)** containing diamine moiety **(3f-3g)** as a linker have been synthesized as per the procedure given for compound **(7aI)**. Ibuprofen **(5I)** was coupled with *N*-(2-aminoethyl)nicotinamide **(4f)** using EDC. HCl as coupling agent in dichloromethane to give the intermediate **(6fI)** which on quaternization using methyl iodide in acetonitrile gave a crude yellow semisolid product, which was crystallized from ethyl acetate to give **7fI** as a yellow solid.

The IR spectrum of compound **(7fI)** showed single carbonyl stretching of diamide groups as a sharp intense band at 1645 cm^{-1}, N-H stretching of amide at 3337 cm^{-1}and the peak due to C-N stretching came at 1236 cm^{-1}.

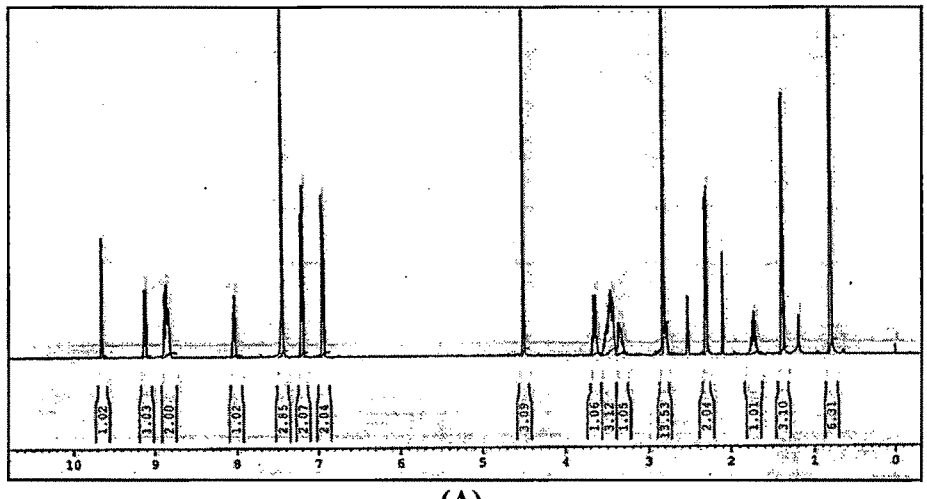


(7fI)

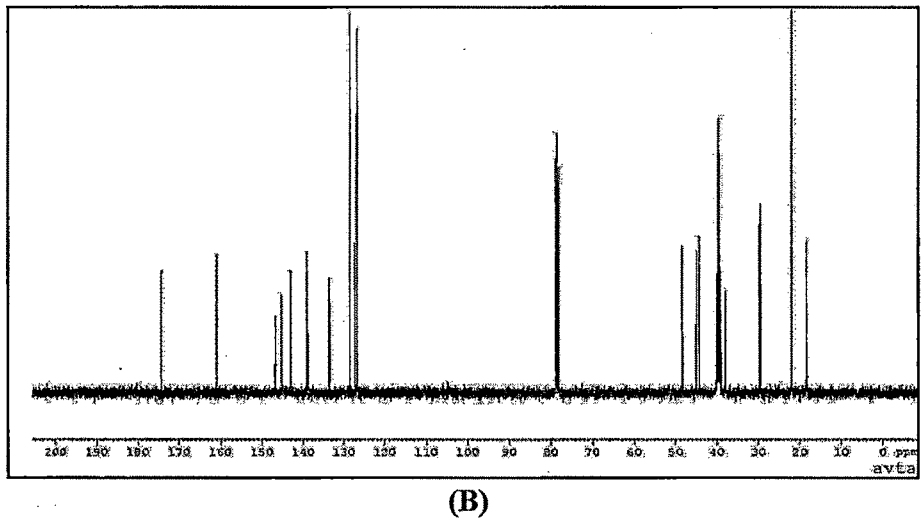
The PMR spectrum **(Fig. 3.2 A)** of the compound **(7fI)**, showed signals at δ 9.64 as a singlet for pyridinium-H_b, multiplet at δ 9.12-9.11 due to pyridinium-H_e, another pyridinium proton-H_c and NH_f at δ 8.88-8.83 as a multiplet with coupling constant equal to 8.0 Hz, triplet due to pyridinium-H_d at δ 8.05-8.03 (*J=8.0* Hz) and broad singlet at δ 7.44 due to NH_i. The protons of phenyl ring (Ar-$H_{2/l}$, *J=8.0* Hz) appeared at 7.21-7.19 and at 6.95-6.93 (Ar-$H_{2/m}$, *J=8.0* Hz) as doublets. Singlet due to methyl protons (-C$H_{3/a}$) of quaternary nitrogen appeared at δ 4.50, quartet due to methine proton (-CH_j) at 3.66-3.64

(*J=7.08* Hz) and methylene protons of ethylenediamine moiety ($CH_{2/g-h}$) at 3.50-3.33 as a multiplet. Doublet due to (-$CH_{2/n}$) appeared at 2.31-2.29 and multiplet at 1.74-1.71 for -$CH_{o,}$ protons (*J=6.70* Hz), Signal at 1.38-1.37 due to methyl protons (-$CH_{3/k}$) with coupling constant equal to *7.08* Hz and six protons of dimethyl group ($CH_{3/p}$) at 0.80-0.79 as a doublet (*J=6.66* Hz) were also observed.

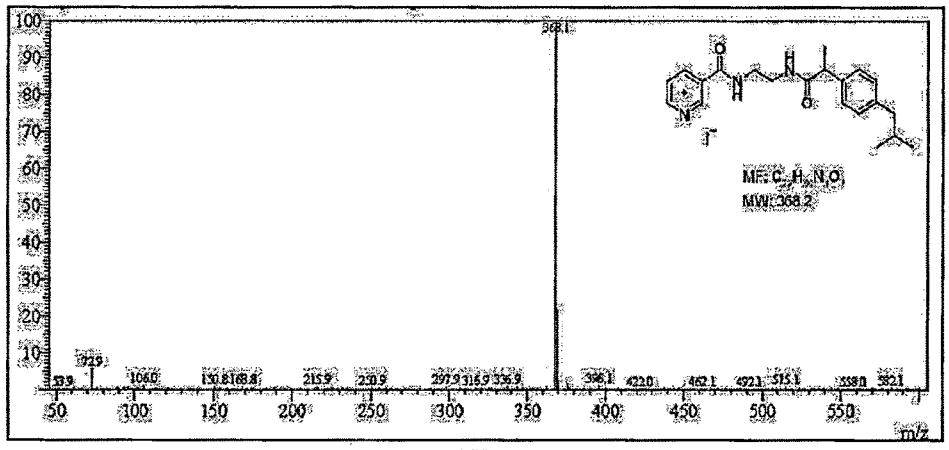
^{13}C-NMR spectrum of **7fl (Fig. 3.2 B)** shows peaks at 174.32, 160.97 for *C*=O carbons, aromatic carbons at 146.73, 145.25, 142.94, 139.13, 139.0, 133.61, 128.53, 127.34, 126.79 and aliphatic carbons at 48.37, 45.0, 44.32, 39.86, 39.02, 37.92, 29.65, 22.03 and 18.37. The mass spectrum **(Fig. 3.2 C)** of **7fl** shows peak at 368.1 (M^+) which is also the base peak. The compound also showed high chromatographic purity (>99%) by HPLC.



(A)



(B)



(C)

Fig. 3.2: Spectra of derivative **(7fI)**; ^{1}H-NMR **(A)**, ^{13}C-NMR **(B)**, and Mass spectrum **(C)**.

Spectral data of the remaining ibuprofen derivatives **(7bI-7eI)** and **(7gI)** is given in **Table-3.1.**

Table 3.1: Spectral data of derivatives **(7bI-eI)** and **(7gI)**

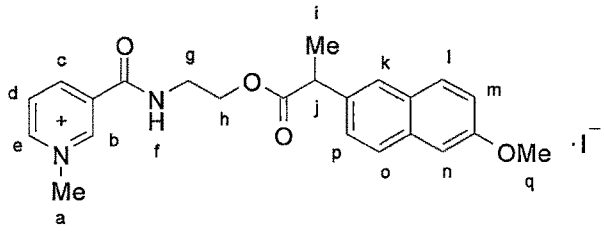
Comp.	IR Peaks (cm^{-1})	PMR Peaks (δ)
7bI	3260, 1725, 1669, 847	9.80 (s, 1H) 8.89-8.87 (d, 2H) 8.72-8.69 (t, 1H), 8.00-7.97 (t,1H) 7.15-7.13 (d, 2H), 6.99-6.97 (d, 2H), 4.50 (s, 3H), 4.35-4.12 (m, 2H), 3.79-3.74 (q, 1H) 3.68-3.60 (m, 2H), 2.33-2.31 (d, 2H), 1.77-1.70 (m,1H), 1.43-1.41 (d,3H), 0.81-0.79 (d,6H)
7cI	3238, 1729, 1668, 834	9.80 (s, 1H), 9.06 (s, 1H) 8.92-8.90 (d, 1H), 8.45-8.43(d, 1H), 8.06-8.03 (t, 1H) 7.20-7.01 (dd, 4H), 4.58 (s, 3H), 4.34-4.29 (m, 2H), 4.22-4.19 (m, 1H), 3.80-3.65 (q, 1H), 1.82-1.73 (m, 2H), 1.72-1.67 (m, 1H), 1.49-1.44 (d, 3H), 0.96-0.93 (t, 3H), 0.89-0.87 (d, 6H)
7dI	3244, 1729, 1670, 746	9.59 (s, 1H), 9.08-9.06 (d, 1H), 8.76-8.74 (d, 1H), 8.05-8.01 (t, 1H), 7.97 (bs, 1H), 7.21-7.03 (dd, 4H), 4.57 (s, 3H), 4.33 (s, 2H), 3.80-3.78 (q, 1H), 2.40-2.38 (d, 2H), 1.82-1.78 (m, 1H), 1.50-1.48 (d, 3H), 1.46 (s, 6H), 0.90 (s, 6H).
7eI	3230, 1725, 1669, 735	10.10 (s, 1H), 9.02-9.0 (d, 1H), 8.90-8.87 (t, 1H), 8.85-8.84 (d, 1H), 8.06-8.03 (t, 1H), 7.21-7.07 (dd, 4H), 4.59 (s, 1H), 4.25-4.13 (m, 2H), 3.76-3.70(q, 1H), 3.54-3.49 (t, 2H), 3.22 (s, 1H), 2.43-2.42 (d, 2H), 2.08-2.02 (m, 2H), 1.86-1.80 (m, 1H), 1.53-1.44 (d, 3H), 0.90-0.87 (d, 6H)

7gI	3268, 1658 850	9.84 (s, 1H), 9.14-9.12 (d, 1H), 9.03-9.00 (d, 1H), 8.81-8.78 (d, 1H), 8.12-8.08 (t, 1H), 7.27-7.06 (dd, 4H), 6.69-6.66 (t, 1H), 4.60 (s, 3H), 4.12-4.09 (q, 1H), 3.75-3.67 (q, 2H), 3.46-3.34 (m, 2H), 2.43-2.40 (d, 3H), 1.85-1.77 (m, 2H), 1.49-1.44 (d, 2H), 1.27-1.21 (m, 1H), 0.89-0.86 (d, 6H)

3.1.2. Chemical delivery systems of naproxen (7aII-7gII)

Another member of the NSAIDs class chosen for developing suitable delivery systems was naproxen **(5II)**. Chemical delivery systems of naproxen were synthesized as per the previously described **Scheme-1**. Naproxen **(5II)** was coupled with *N*-(2-hydroxyethyl)nicotinamide **(4a)** using EDC.HCl as coupling agent in dichloromethane to give the derivative **(6aII)** which on quaternization using methyl iodide in acetonitrile gave a crude yellow semisolid product, which was crystallized from ethyl acetate to give **7aII** as a white solid.

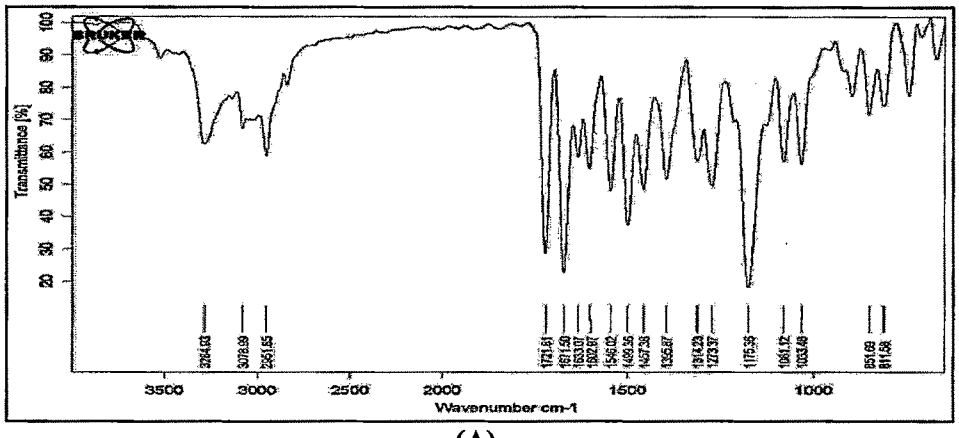
The IR spectrum **(Fig. 3.3 A)** of the compound **(7aII)** showed carbonyl stretching of ester and amide groups as sharp intense bands at 1721 cm^{-1} and 1671 cm^{-1} respectively. N-H stretching of amide was observed at 3284 cm^{-1} and the peaks due to C-O stretching came at 1273 and 1175 cm^{-1}.



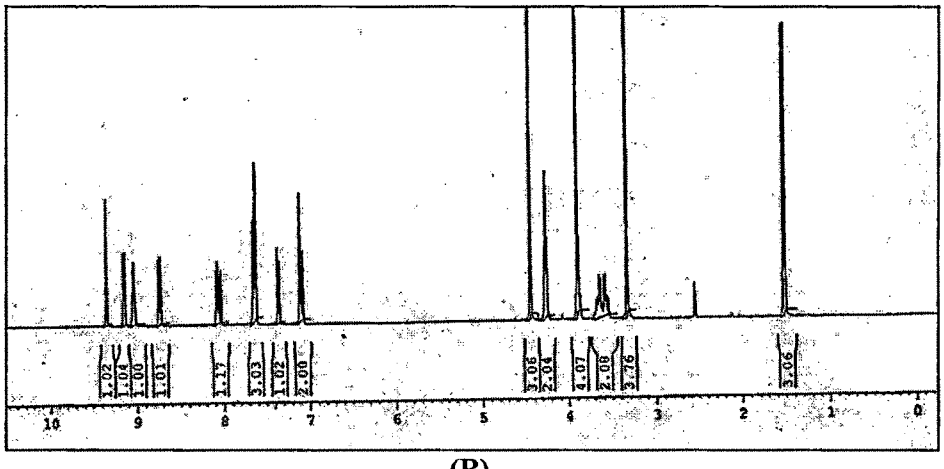
(7aII)

The PMR spectrum **(Fig. 3.3 B)** of the compound **(7aII)** shows singlet of pyridinium-H_b at δ 9.34, doublet of another pyridinium-H_e at δ 9.15-9.13 with coupling constant equal to 8.0 Hz, multiplet at δ 9.04-9.02 due to pyridinium-H_d, singlet at δ 8.74-8.72 due to pyridinium-H_c (*J=8.0* Hz) and peak at δ 8.09-8.03 due to NH_f proton. Naphthalene protons (Ar-$H_{k\text{-}p}$) were observed at δ 7.66-7.07 as multiplets. The signal for methyl protons (-C$H_{3/a}$) of quaternary nitrogen appeared at δ 4.44 as a singlet. The methylene protons (C$H_{2/h}$) appeared at δ 4.28-4.25 as a triplet. Quartet appeared at δ 3.91-3.86 due to methine proton (CH_j) and multiplet at δ 3.67-3.56 due to methylene protons (-C$H_{2/g}$). The singlet at δ 3.33 equivalent to three protons and doublet at 1.52-1.51 for three protons were due to (-C$H_{3/q}$) and (-C$H_{3/i}$) protons respectively.

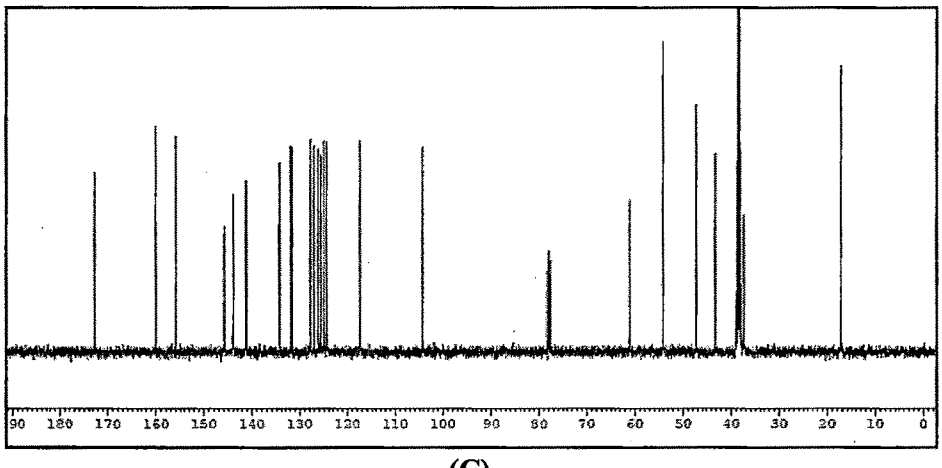
^{13}C-NMR spectrum **(Fig. 3.3 C)** of **7aII** shows peaks at 172.73 and 160.01 for carbonyl carbons of ester and amide functional group respectively, peaks at 155.85, 145.75, 143.94, 141.18, 134.28, 131.94, 127.82, 126.16, 124.97, 117.41, 104.33 appeared due to aromatic carbons and the aliphatic carbons appeared at 61.09, 54.14, 47.23, 43.29, 39.06, 38.85, 38.44, 38.02, 37.33 and 17.20.



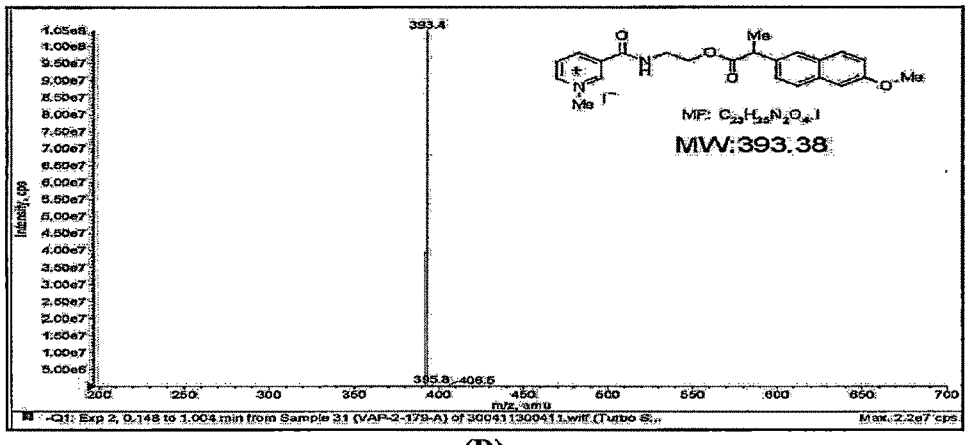
(A)



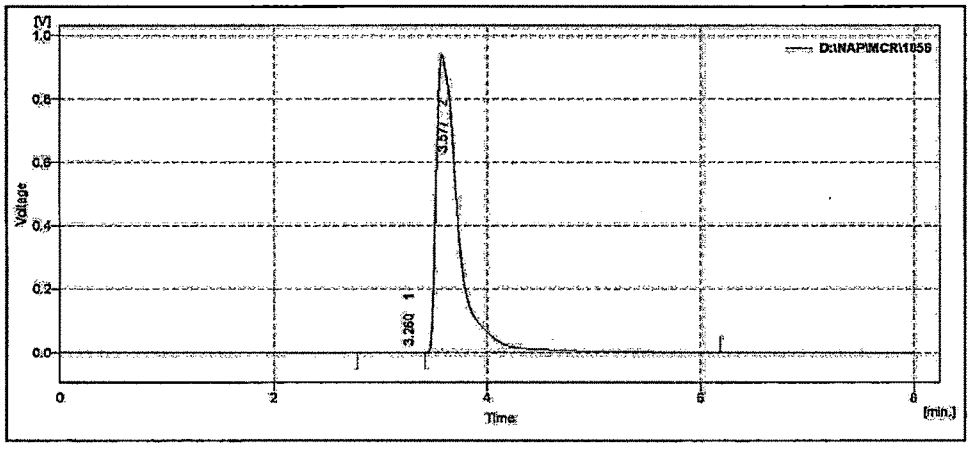
(B)



(C)



(D)



(E)

Fig. 3.3: Spectra of derivative **(7aII)**; IR spectrum **(A)**, ^{1}H-NMR **(B)**, ^{13}C-NMR **(C)**, Mass spectrum **(D)** and HPLC chromatogram **(E)**.

The mass spectrum of compound **(7aII)** shows peak at 393.4 (M^+) which is also the base peak. The compound showed high chromatographic purity (>98.5%) by HPLC. Other derivatives of the series have been synthesized and characterized in the same manner as discussed for compound **(7aII)**. Spectral data of the remaining naproxen derivatives (**7bII-7gII**) is given in **Table-3.2**

Table 3.2: Spectral data of derivatives **(7bII-7gII)**

Comp.	IR Peaks (cm^{-1})	PMR Peaks (δ)
7bII	1721, 1666, 1187, 892	δ 9.08 (s, 1H), 9.02-9.01(d, 1H), 8.81 (bs, 1H), 8.45-8.43 (d, 1H), 7.83-7.79 (m, 1H), 7.59-7.05 (m, 6H), 5.16-5.13 (m, 1H), 4.35 (s, 3H), 3.92 (s, 3H), 3.84-3.82 (q, 1H), 3.37-3.33 (t, 2H), 1.50-1.49 (d, 3H), 1.33-1.32 (d, 3H)

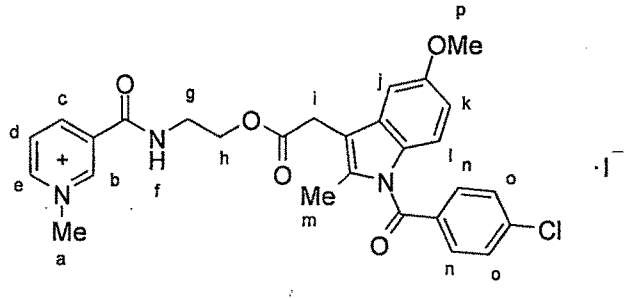
7cII	1727,1668, 1239, 854	9.23 (s, 1H), 8.94-8.87 (dd, 1H), 8.61-8.57 (m, 1H) 8.18-8.13 (dd, 1H), 7.85-7.75 (m, 1H), 7.61-6.97 (m, 6H), 4.40 (s, 3H), 4.34 (s, 3H), 3.91-3.88 (d, 4H), 1.74-1.65 (m, 1H), 1.54-1.51 (d, 3H), 1.27-1.23 (q, 1H), 0.95-0.90 (t, 3H)
7dII	1730,1658, 1372, 854	9.29 (s, 1H) 9.11-9.10 (d, 1H), 8.65-8.63 (d, 1H), 8.28 (s, 1H), 8.05-8.01 (m, 1H), 7.66-7.07 (m, 6H), 4.40 (s, 3H), 3.90 (s, 3H), 3.37-3.34 (m, 1H), 3.02 (s, 2H), 1.51-1.48 (d, 3H), 1.38-1.35 (d, 6H)
7eII	1716,1667, 1266	9.44 (s, 1H), 9.15-9.13 (d, 1H), 9.06-9.03 (m, 1H), 8.91-8.89 (d, 1H), 8.23-8.19 (m, 1H), 7.76-7.11 (m, 6H), 4.44 (s, 3H), 4.15-4.11 (q, 2H), 3.91-3.89 (q, 1H), 3.88 (s, 3H), 3.40-3.35 (t, 2H), 1.89-1.85 (p, 2H), 1.52-1.50 (d, 3H)
7fII	1665, 1210, 857	δ 9.17 (s, 1H), 9.01-8.99 (d, 1H), 8.88-8.85 (m, 1H), 8.61-8.59 (d, 1H), 8.10-8.07 (m, 1H), 7.99-7.94 (m, 1H), 7.77-7.07 (m, 6H), 4.36 (s, 3H), 3.89 (s, 3H), 3.74-3.72 (q, 1H), 3.53-3.39 (m, 2H), 3.31-3.24 (t, 2H), 1.50-1.44 (d, 3H)
7gII	1673, 1269, 857	δ 9.52 (s, 1H), 9.15-9.14 (d, 1H), 8.95-8.93 (d, 2H), 8.14-8.10 (m, 1H), 7.84-7.81 (m, 1H), 7.70-7.65 (m, 3H), 7.47-7.44 (d, 1H), 7.11-7.09 (d, 2H), 4.49 (s, 3H), 3.90 (s, 3H), 3.79-3.77 (q, 1H), 3.38-3.37 (m, 2H), 3.24-3.17 (m, 2H), 1.78-1.75 (p, 2H), 1.52-1.50 (d, 3H)

3.1.3. Chemical delivery systems of indomethacin (7aIII-7gIII)

In order to synthesize quaternary ammonium derivatives of indomethacin **Scheme-1** was employed. Indomethacin **(5III)** was coupled with various hydroxyl- and aminoalkyl nicotinamide derivatives **(4a-4g)** using EDC as a coupling agent to give the derivatives **(6aIII-gIII)** which on quaternization by methyl iodide gave quaternary ammonium derivatives **(7aIII-7gIII)** of indomethacin. Derivative **(7aIII)** was synthesized using *N*-(2-hydroxyethyl)nicotinamide **(4a)** and indomethacin **(5III)** to give **6aIII** as an intermediate

which on quaternization by methyl iodide gave yellow semisolid which was further crystallized from ethanol to obtain **7aIII** as a yellow solid.

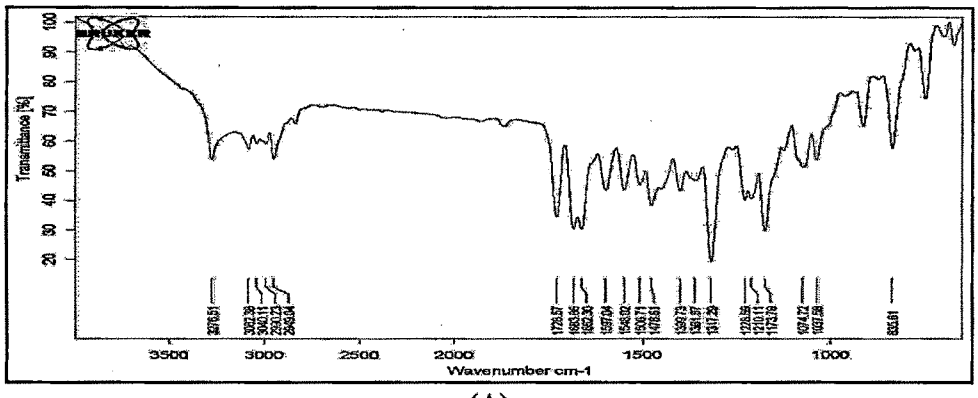
In the IR spectrum **(Fig. 3.4 A)** of compound **(7aIII)** the *C=O* stretchings were observed at 1728, 1683 and at 1662 cm^{-1} for the ester and amide carbonyl groups respectively. *N-H* stretching was observed at 3276 cm^{-1} and the peaks due to *C-O* stretching appeared at 1228 and 1173 cm^{-1}.



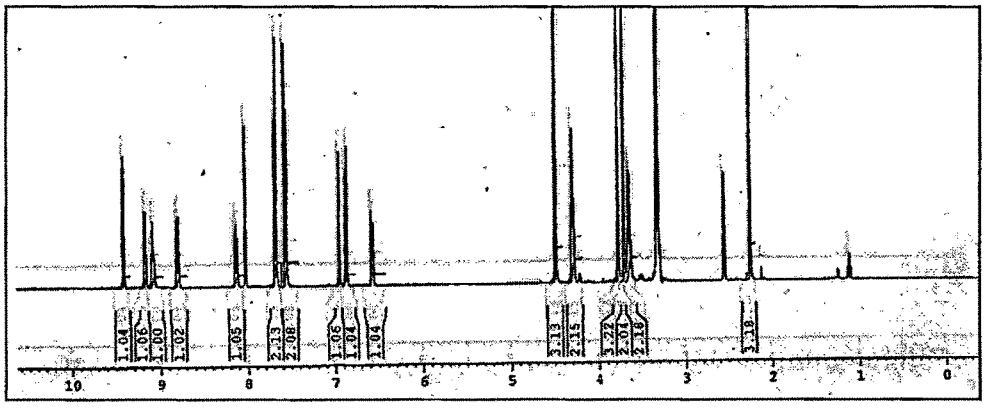
(7aIII)

PMR Spectrum **(Fig. 3.4. B)** showed signal at δ 9.41 as a singlet for pyridinium-H_b, multiplet at δ 9.18-9.16 due to pyridinium-H_e with coupling constant equal to 8.0 Hz, multiplet at δ 9.08 due to pyridinium-H_d, (*J=8.0*) and a signal at δ 8.81-8.79 due to pyridinium-H_c, (*J=8.0* Hz). The NH_f proton appeared at δ 8.15-8.11 as a triplet. The aromatic protons of phenyl ring appeared at δ 7.69-7.55 (Ar-H_n), and at δ 7.57-7.55 (Ar-H_o), as doublets and multiplets due to indole protons (Ar-H_{j-l}) appeared at δ 6.95-6.56. The methyl protons on quaternary nitrogen (-C$H_{3/a}$) appeared at δ 4.49 as a singlet. Signals at δ 4.31-4.28 were observed due to methylene protons (-C$H_{2/h}$) as triplet. Methoxy protons (-C$H_{3/p}$) showed signal at δ 3.77 as a singlet and the methylene (-C$H_{2/i}$) protons appeared as singlet at δ 3.71. Other methylene protons (-C$H_{2/g}$) appeared at δ 3.67-3.63 as multiplet and the methyl protons (-C$H_{3/m}$) appeared as a singlet at δ 2.25.

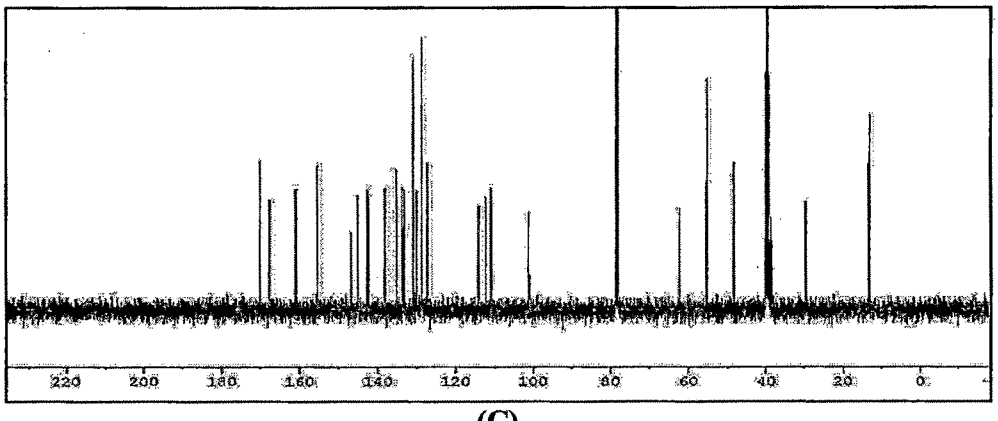
^{13}C-NMR spectrum **(Fig. 3.4 C)** shows peaks at δ 170.17, 167.63, 160.93 due to C=O carbons, aromatic carbons appear at 155.32, 146.84, 145.19, 142.58, 138.16, 135.25, 133.61, 130.88, 130.07, 128.80, 127.30, 114.26, 112.21, 111.03, 101.27 and aliphatic carbons appear at 62.31, 55.33, 48.29, 40.25, 40.04, 39.83, 39.20, 38.55, 29.59 and 13.20. The mass spectrum of compound **(7aIII)** shows peak at 520.60 (M^+) which is also the base peak. The compound also showed high chromatographic purity by HPLC.


Transmittance [%]
Wavenumber cm-1
3500
3000
2500
2000
1500
1000

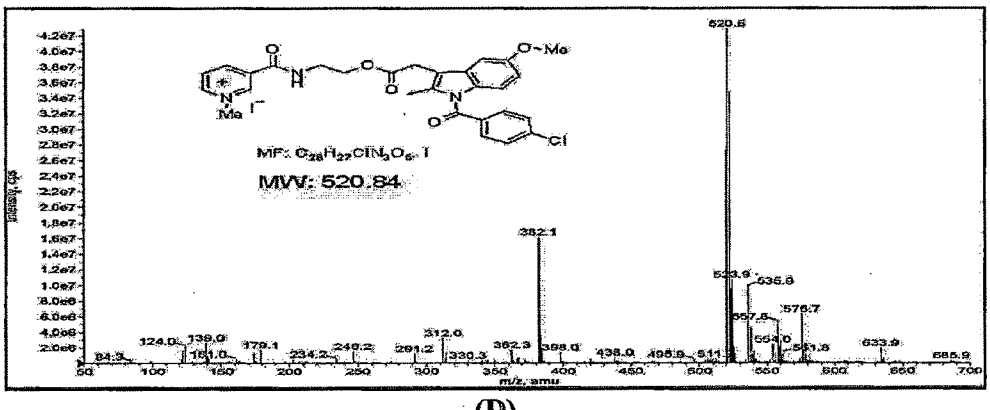
(A)



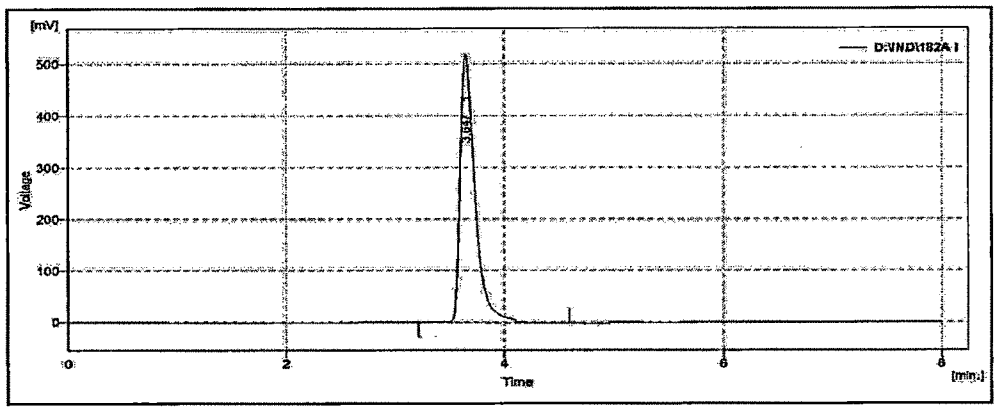
(B)



(C)


MF: C28H22ClN3O5 . I
MW: 520.84
520.6
382.1
536.8
576.7

(D)



(E)

Fig. 3.4: Spectra of derivative **(7aIII)**; IR spectrum **(A)**, ^{1}H-NMR **(B)**, ^{13}C-NMR **(C)**, Mass spectrum **(D)** and HPLC chromatogram **(E)**.

Other derivatives of the series have been synthesized and characterized in the same manner as compound **(7aIII)**. Spectral data of the remaining indomethacin derivatives (**7bIII-7gIII)** is given in **Table 3.3.**

Table 3.3: Spectral data of the derivatives **(7bIII-7gIII)**

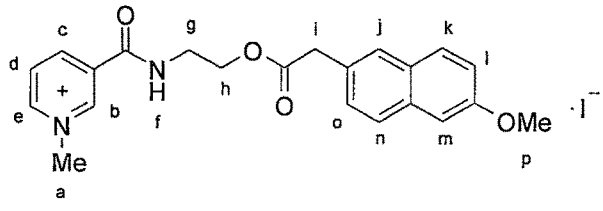
Comp.	IR Peaks (cm^{-1})	PMR Peaks (δ)
7bIII	3439, 1729, 1675,1323, 754	9.54 (s, 1H) 8.95-8.93 (d, 1H), 8.73-8.71 (d, 1H), 8.61-8.58 (m, 1H), 7.92-7.88 (m, 1H), 7.67-7.63 (d, 2H), 7.48-7.45 (d, 2H), 6.97-6.96 (d,1H), 6.89-6.87 (d, 1H), 6.55-6.52 (dd, 1H), 4.47 (s, 3H), 3.80 (s, 3H), 1.36-1.30 (d, 3H)
7cIII	3436, 1733, 1672, 1322,754	9.51 (s, 1H), 8.97-8.95 (d, 1H), 8.74-8.72 (d, 1H), 8.28 (bs, 1H), 7.93-7.89 (m, 1H), 7.67-7.65 (d, 2H), 7.48-7.46 (d, 2H), 6.94-6.93 (dd, 2H), 6.48-6.45 (dd,1H), 4.48 (s, 3H), 4.33 (s, 3H), 3.83 (s, 3H), 1.27-1.24 (m,1H)
7dIII	3436, 1733, 1675, 1322,752	9.53 (s, 1H), 9.0-8.98 (d, 1H), 8.70-8.68 (d, 1H), 7.69-7.93 (t, 1H), 7.87 (s, 1H), 7.64-7.62 (d, 2H), 7.47-7.44 (d, 2H), 6.95 (s,1H), 6.88-6.86 (d,1H), 6.56-6.54 (d,1H), 4.50 (s, 3H), 3.78 (s, 3H), 1.49 (s, 6H)
7eIII	3288, 1711, 1630, 1316, 827	9.53 (s, 1H), 9.20-9.18 (d, 1H), 9.07-9.04 (m, 1H), 8.98-8.96 (d, 1H), 8.21-8.17 (m, 1H), 7.67-7.66 (d, 2H), 7.55-7.54 (d, 2H), 6.98 (s, 1H), 6.92-6.89 (d, 1H), 6.67-6.64 (d, 1H), 4.50 (s, 3H), 3.80 (s, 3H), 1.99-1.93 (p, 2H)

7fIII	3273,1663, 1312,751	9.34 (s, 1H), 9.14-9.13 (d, 1H), 8.83 (s, 1H), 8.73-8.71 (d, 1H), 8.04-8.02 (m, 1H), 7.71-7.52 (d, 2H), 7.54-7.52 (d, 2H), 6.99 (s, 1H), 6.92-6.90 (d, 1H), 6.55-6.53 (d, 1H), 4.45 (s, 3H), 3.77 (s, 3H), 2.26 (s, 3H)
7gIII	3299, 2929, 1668, 754	9.5 (s, 1H), 9.18-9.17 (d, 1H), 9.0-8.98 (m,1H), 8.95-8.93 (d, 1H), 8.19-8.15 (m, 1H), 7.97-7.95 (t, 1H), 7.70-7.52 (dd, 4H), 7.09-6.62 (m, 3H), 4.48 (s, 3H), 3.79 (s, 3H), 3.33 (s, 3H), 3.26-3.22 (q, 2H), 1.81-1,75 (m, 2H)

3.1.4. Chemical delivery systems of 6-MNA (7aIV-7eIV)

Nabumetone is a non-acidic, NSAID devoid of local gastric irritation. But as such this is not active biologically. It gets metabolized to 6-methoxy-2-naphthylacetic acid (6-MNA) **(5IV)**, which acts as anti-inflammatory agent. So, it was planned to prepare CDS of 6-MNA the active metabolite of nabumetone. The required NSAID i.e. 6-MNA **(5IV)** was synthesized as per the reported procedure.[3] Synthesis of 6-MNA has been described in Section-III (Section 3.1). As discussed earlier in **Scheme-1**, same procedure has been adopted for the synthesis of CDS (**7aIV-7eIV**) of 6-MNA **(5IV)**. The first derivative **(7aIV)** was synthesized by coupling 6-MNA **(5IV)** with *N*-(2-hydroxyethyl)nicotinamide **(4a)** using EDC.HCl as the coupling agent to give the derivative **(6aIV)** as an ester which on quaternization using methyl iodide gave the final compound **(7aIV)** as yellow solid.

In the IR spectrum **(Fig. 3.5 A)** of the synthesized derivative (**7aIV**) strong bands at 1735 and 1672 cm^{-1} due to *C=O* stretching of ester and amide groups respectively were observed.

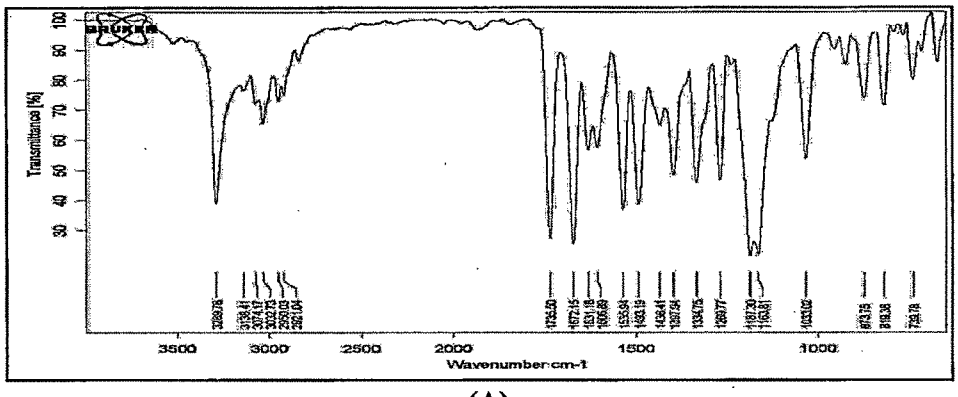


(7aIV)

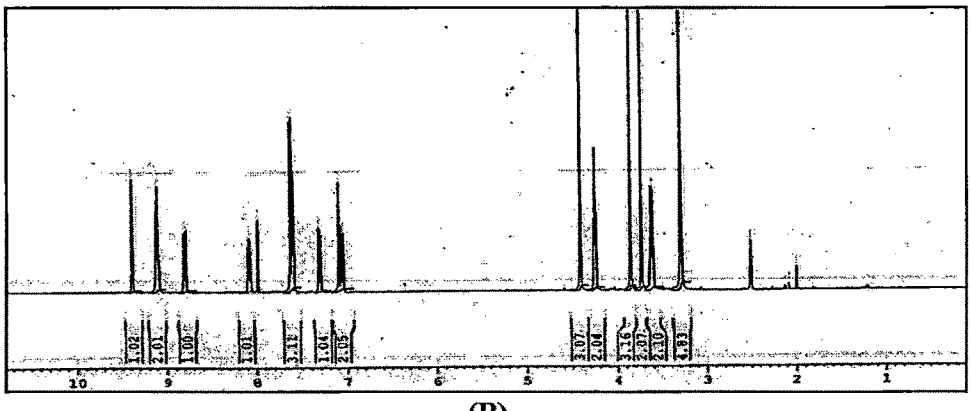
The PMR spectrum **(Fig. 3.5 B)** of the compound (**7aIV**) showed signal at δ 9.38 as a singlet due to pyridinium-H_b proton. Another pyridinium proton (-H_e) merged with amide proton (NH_f) was observed at δ 9.11-9.08 as a multiplet with coupling constant equal to 8.0Hz. Multiplets at δ 8.80-8.78 and δ 8.09-8.06 appeared due to pyridinium-H_c and pyridinium-H_d respectively. Naphthalene protons (Ar-$H_{j\text{-}o,}$) were observed at δ 7.62-

7.04 as multiplet. Methyl protons on quaternary nitrogen (-C$H_{3/a}$) appeared at δ 4.41 as a singlet and methylene protons (-C$H_{2/g}$) at δ 4.26-4.23 as a triplet. Peaks were observed at δ 3.85 as singlet due to methoxy protons (-C$H_{3/p}$) and at δ 3.73 and 3.64-3.60 due to methylene protons (Ar-C$H_{2/i}$) and (-C$H_{2/h}$) respectively.

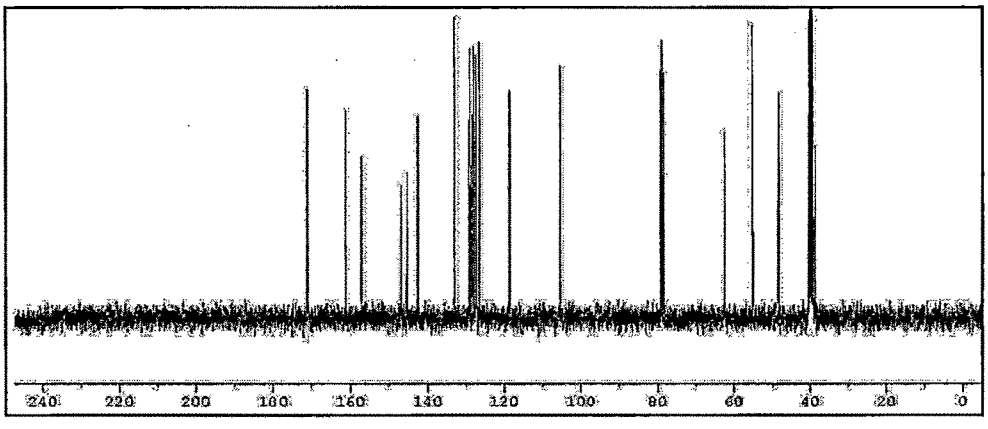
^{13}C-NMR spectrum (**Fig. 3.5** C) shows peaks at 171.17 and 161.22, due to carbonyl carbons. Peaks at 157.03-105.44 were observed for aromatic carbons. Aliphatic carbons appeared at 62.28-38.60. Mass spectrum **(Fig. 3.5 D)** shows peak at 379.0 (M+) which is also the base peak. The compound also showed high chromatographic purity.



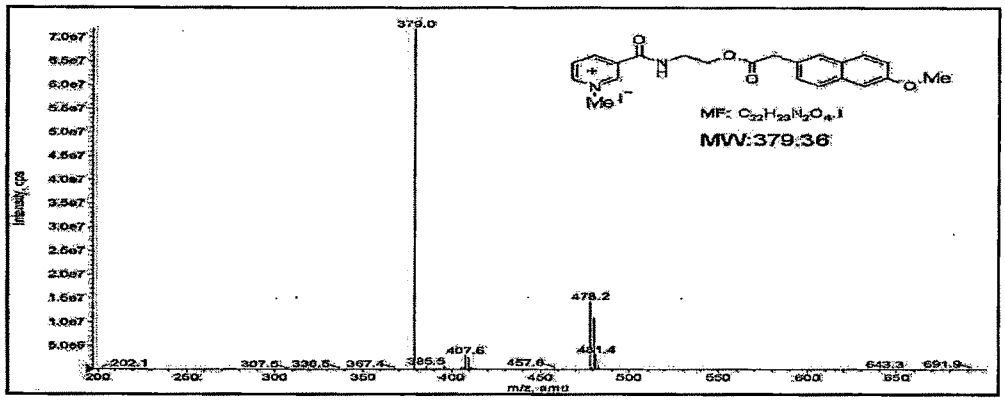
(A)



(B)



(C)



(D)

Fig. 3.5: Spectra of derivative **(7aIV)**; IR spectrum **(A)**, ^{1}H-NMR **(B)**, ^{13}C-NMR **(C)**, mass spectrum **(D)**.

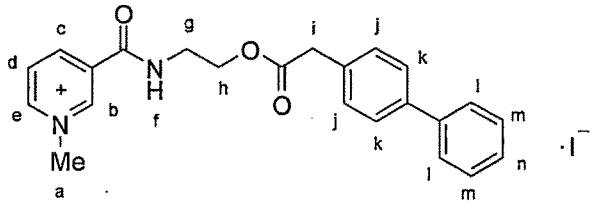
Other derivatives of the series have been synthesized and characterized in the same manner as compound **(7aIV)**. Spectral data of the remaining 6-MNA derivatives **(7bIV-7eIV)** is given in **Table-3.4.**

Table 3.4. Spectral data of derivatives **(7bIV-7eIV)**

Comp. No.	IR Peaks (cm^{-1})	PMR Peaks (δ)
7bIV	1724,1668, 1264, 852	9.26 (s, 1H), 74-8.73 (d, 1H), 8.61-8.59 (d, 1H), 8.55-8.53 (m, 1H), 7.72-7.69 (m, 1H), 7.58-7.00 (m, 3H), 7.35-7.32 (m, 1H), 7.06-7.0 (m, 2H), 5.26-5.22 (m,1H), 4.27 (s, 3H), 3.88 (s, 3H), 3.74 (s, 2H), 3.64-3.53 (m, 2H), 1.32-1.30 (d, 3H)
7cIV	3246, 1727, 1667, 1264, 850,	9.30 (s, 1H), 8.76-8.74 (d, 1H), 8.62-8.60 (d, 1H), 8.27-8.25 (m, 1H), 7.73-7.70 (m, 1H), 7.58-7.53 (m, 3H), 7.37-7.27 (m, 1H), 7.14-7.0 (m, 2H), 4.41-4.31 (m, 2H), 4.29 (s, 3H), 3.90-3.89 (m, 1H), 3.88 (s, 3H), 3.77 (s, 2H), 1.84-1.69 (m, 2H), 0.99-0.95 (t, 3H)
7dIV	3267, 1737, 1660, 1329, 857	9.36 (s, 1H), 9.12-9.10 (d, 1H), 8.75-8.73 (d, 1H), 8.34 (s, 1H), 8.10-8.05 (t, 1H), 7.66-7.08 (d, 3H), 7.35-7.33 (d, 1H), 7.17-7.08 (m, 2H), 4.41 (s, 3H), 4.33 (s, 2H), 3.90 (s, 3H), 3.78 (s, 2H), 1.42 (s, 6H)
7eIV	1724,1666, 1265, 850	9.45 (s, 1H), 9.15-9.13 (d, 1H), 9.13-9.10 (m, 1H), 8.93-8.91 (d, 1H), 8.23-8.19 (m, 1H), 7.75-7.73 (d, 2H), 7.68 (s, 1H), 7.38-7.36 (d, 1H), 7.23 (s, 1H), 7.14-7.12 (d, 1H), 4.44 (s, 3H), 4.17-4.14 (t, 2H), 3.88 (s, 3H), 3.78 (s, 2H), 3.46-3.41 (q, 2H), 1.94-1.91 (m, 2H)

3.1.5. Chemical delivery systems of BPA (7aV-7eV)

Fenbufen is a potent anti-inflammatory agent that is metabolized into 4-hydroxy-4-biphenylbutanoic acid and 4-biphenylacetic acid **(5V)**. 4-Biphenylacetic acid (BPA) **(5V)** is an active metabolite of fenbufen with three times more anti-inflammatory activity than the parent drug.[4] Synthesis of 4-biphenylacetic acid has been described in Section-3 (Section 3.1). The chemical delivery systems of BPA were prepared according to **Scheme-1,** in which *N*-(2-hydroxyethyl)nicotinamide **(4a)** was coupled with BPA **(5V)** using EDC. HCl as coupling agent to give the derivative **(6aV)** which on quaternization using methyl iodide gave a residue which was crystallized from chloroform: methanol to give **(7aV)** as a yellow solid.

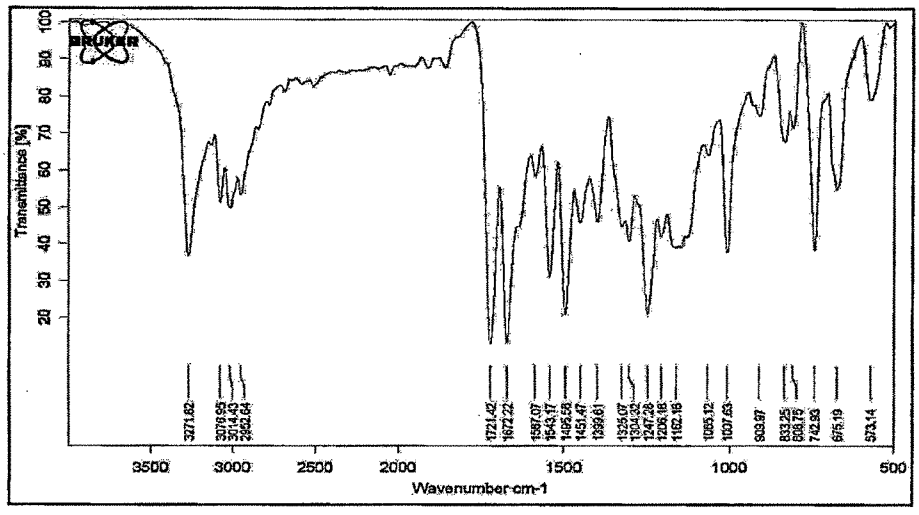


(7aV)

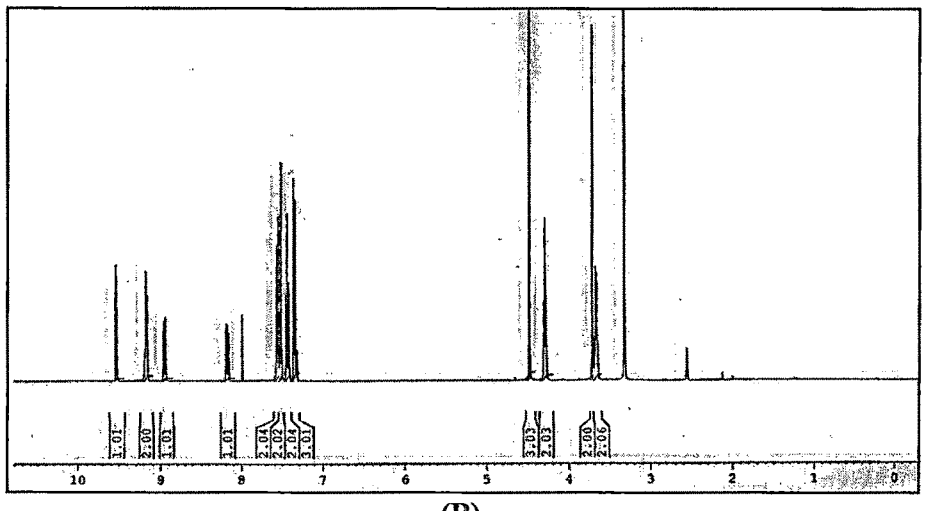
IR spectrum (**Fig. 3.6 A)** of compound **(7aV)** showed C=O stretching at around 1721 and 1672 cm^{-1} due to ester and amide carbonyl functional groups respectively. The N-H stretching and deformation peaks were observed around 3271 and 1495 cm^{-1}.

The PMR spectrum (**Fig. 3.6 B)** of the compound **(7aV)** showed singlet at δ 9.53 due to proton of pyridinium (-H_b) ring. Other signal appeared at δ 9.19-9.16 as a multiplet due to pyridinium-H_e merged with NH_f, proton with coupling constant equal to 8.0 Hz. The multiplet at δ 8.96-8.94 was due to pyridinium-H_c. Other proton of pyridinium-H_d appeared at δ 8.19-8.16 as mltiplet. Nine biphenyl ring protons were observed at 7.57-7.32 as a multiplet (Ar-$H_{j\text{-}n}$). The methyl protons of quaternary nitrogen (-C$H_{3/a}$) appeared at δ 4.47 as a singlet, methylene protons (-C$H_{2/h}$) at δ 4.30-4.28 as a triplet (*J=4.0* Hz) and a peak at δ 3.72 due to methylene protons (Ar-C$H_{2/i}$) appeared as singlet. Other methylene protons (-C$H_{2/g}$) appeared at δ 3.72-3.65 as a multiplet (*J=4.0* Hz)

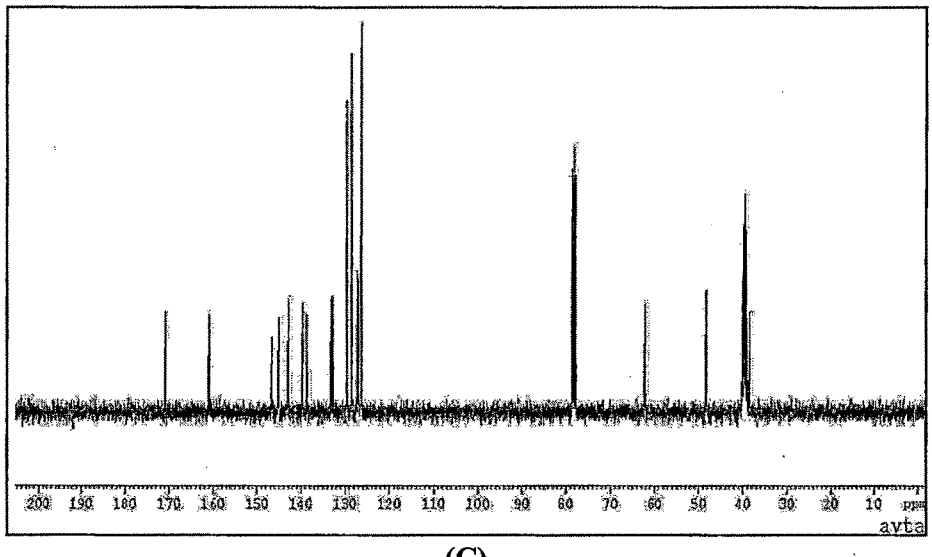
^{13}C-NMR shows (**Fig. 3.6 C)** peaks at δ 170.95 and 160.84 for carbonyl carbons of ester and amide functional groups. Aromatic carbons appeared at 146.41-126.18 and the aliphatic carbons appeared at 62.24, 48.41, 40.29, 39.87, 39.10, 38.89 and 38.45. The mass spectrum of compound **(7aV)** shows peak at 375.90 (M^+) which is also the base peak. The derivative **(7aV)** was found to be pure and chromatographic purity by HPLC was more than 98.90 %.


BRUKER
Transmittance [%]
Wavenumber cm-1
3371.82
3078.59
3014.43
2952.64
1721.45
1672.22
1587.07
1543.17
1495.58
1451.47
1399.61
1335.07
1304.02
1247.28
1206.18
1162.16
1065.12
1037.63
969.97
833.25
808.19
742.93
675.19
573.14

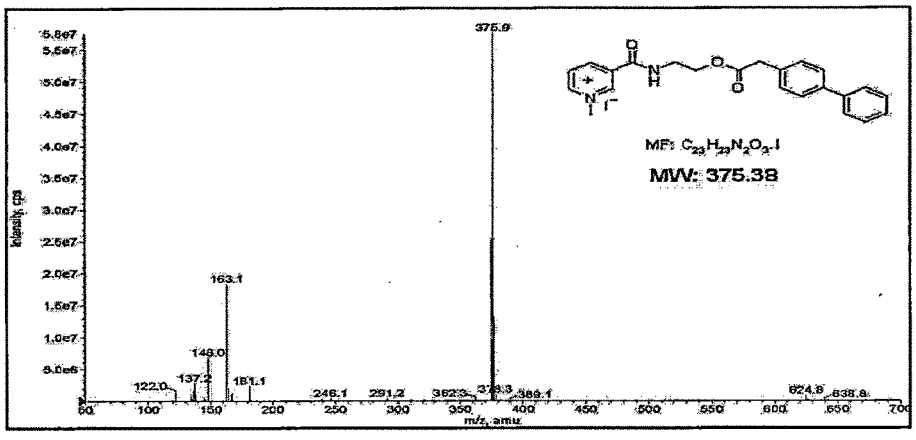
(A)



(B)


avta

(C)



(D)

Fig. 3.6: Spectra of derivative **(7aV)**; IR spectrum **(A)**, ^{1}H-NMR **(B)**, ^{13}C-NMR **(C)**, Mass spectrum **(D)**.

Other derivatives of the series have been synthesized and characterized in the same manner as compound **(7aV)**. Spectral data of the remaining BPA derivatives **(7bV-7eV)** is given in **Table 3.5.**

Table 3.5: Spectral data of derivatives **(7bV-7eV)**

Comp.	IR Peaks (cm^{-1})	PMR Peaks (δ)
7bV	3236, 1725, 1667,744	9.46 (s, 1H), 8.79-8.73 (m, 2H), 8.75-8.73 (d, 1H), 8.62-8.59 (d, 1H), 7.87-7.85 (t, 1H), 7.48-7.19 (m, 9H), 5.17-5.12 (m, 1H), 4.30 (s, 3H), 3.48-3.46 (t, 2H), 1.99 (s, 2H) 1.24-1.23 (d, 3H)
7eV	3247, 1727, 1665,743	9.54 (s, 1H), 8.79-8.76 (m, 2H), 8.32-8.30 (d, 1H), 7.86-7.82 (t, 1H), 7.49-7.19 (m, 9H), 4.33 (s, 3H), 4.29-4.18 (m, 2H), 3.65 (s, 2H), 1.97-1.1.94 (d, 2H), 1.79-1.64 (m, 1H), 0.92-0.89 (t, 3H)
7fV	3436, 1728, 1668,743	9.52 (s, 1H), 8.84-8.82 (d, 1H), 8.70-8.68 (d, 1H), 7.93 (bs, 1H), 7.89-7.86 (t, 1H), 7.53-7.31(m, 9H), 4.41 (s, 3H), 4.37 (s, 2H), 3.74 (s, 2H), 1.52 (s, 6H)
7gV	3391, 1725, 1660,741	9.93 (s, 1H), 8.98-8.96 (m, 2H), 8.86-8.85 (d, 1H), 8.01-7.97 (t, 1H), 7.57-7.26 (m, 9H), 4.51 (s, 3H), 4.23-4.20 (t, 2H), 3.70 (s, 2H), 3.58-3.53 (q, 2H), 3.09 (bs, 1H), 2.08-2.05 (m, 2H)

3.2. Hydrolyses kinetics study

The synthesized chemical delivery systems (CDS) have to be injected by IA route, so once they are deposited into the joint cavity they must be hydrolyzed enzymatically or non-enzymatically to liberate the active NSAID to elicit the desired pharmacological action. As mentioned previously the administered CDS contain hydrolysable ester and amide groups which either undergo hydrolysis in the joint cavity or expelled in the intact form from joints which will be ultimately hydrolyzed in the systemic circulation and generate free NSAIDs. The free NSAID again appear in the joint cavity by reuptake mechanism or get excreted from the body. Hence, it is important to study the hydrolysis kinetics of synthesized CDS at various *p*H conditions and in serum. This *in vitro* study will give idea about the fate of CDS upon IA administration.

The *p*H of joint cavity gets decreased up to *p*H 6 in inflammatory conditions while the *p*H of normal human blood is around 7.4. All the synthesized CDS were evaluated *in vitro* for their stability at 37±1 °C in buffers of *p*H 6.0 and 7.4 which simulated the *p*H of the joint cavity and the blood. To get an idea about the enzymatic susceptibility of CDS towards serum esterase, *in vitro* hydrolyses studies were performed in pooled human serum (80/90 %) at 37±1 °C for all the CDS.

The studies so performed are discussed under the following five headings:

3.2.1. Hydrolyses studies of CDS of ibuprofen (7aI-7gI)

3.2.2. Hydrolyses studies of CDS of naproxen (7aII-7gII)

3.2.3. Hydrolyses studies of CDS of indomethacin (7aIII-7gIII)

3.2.4. Hydrolyses studies of CDS of 6-MNA (7aIV-7eIV)

3.2.5. Hydrolyses studies of CDS of BPA (7aV-7eV)

3.2.1. Hydrolyses studies of CDS of ibuprofen (7aI-7gI)

High performance liquid chromatography (HPLC) methods were developed for the analysis of CDS. The CDS **(7aI-7gI)** were analyzed using a mobile phase consisting of acetonitrile and monobasic potassium phosphate buffer (KH_2PO_4) (15 mM). The ratio of mobile phase was adjusted for each of these CDS to obtain retention time of 3-6 min. Analytical method was validated for linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) as described in literature.[5-7] A stock solution of CDS in buffer solution or in water was prepared. Dilutions were made representing samples from 60 ng/ml to 60 µg/ml and analyses of the samples were performed at 220 nm. Each experiment was repeated thrice, and LOD and linearity determined as shown in **Fig. 3.2.1** Analysis of the data within this

range offered Equation-1. Graphical representation of the data proved the linearity of data as shown in **Fig. 3.2.2**

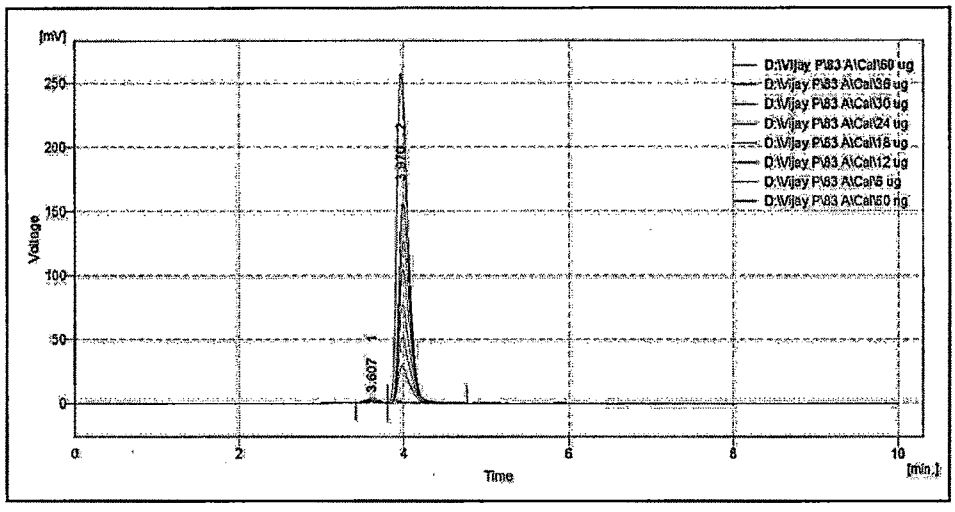


Fig. 3.2.1: Calibration and linearity chromatogram of derivative **(7aI)**

Table 3.2.1: The absorbance readings of **(7aI)** for the studies in buffers

Concentration (µg/ml)	Mean area (mV) ±SD*
6	269.0±7.07
12	481.0±12.82
18	718.5±14.12
24	969.5±16.26
30	1172.5±16.36
36	1437.0±22.82

*n=3

$$A = 38.88x + 24.7 \quad \text{.....(1)}$$

(R^2=0.999)

Where, A=concentration (µg/ml). X= area

For the determination of half life of disappearance of CDS, the derivative **(7aI)** in phosphate buffer solution was kept at 37±1°C at *p*H 6.0 and 7.4. After different time intervals aliquots (1.0 ml) were withdrawn from the test solution and appropriate dilutions were made using mobile phase and injected into HPLC system (20 µl) and analyzed at 220 nm in triplicate and area (mV) was measured. There are various methods used for the determination of order of the reaction but graphical method is the most common one.[8]

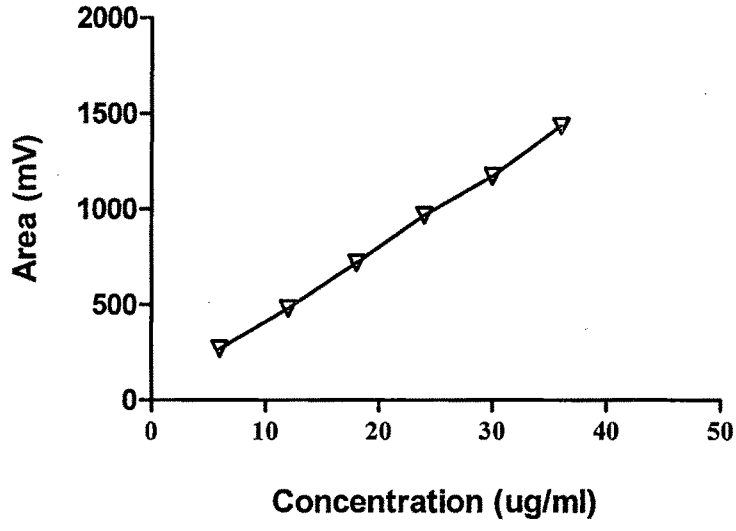


Fig. 3.2.2: Calibration plot for the estimation of **(7aI)**

According to this method, on plotting the concentration factor (y-x) versus t, if a straight line is obtained then the reaction is of zero order, if log (y-x) versus t gives a straight line then reaction is first order, if 1/(y-x) against t offers a straight line then the reaction is second order and if a straight line is obtained by plotting $1/(y-x)^2$ against t then the reaction is of third order.

The areas obtained from the study were plotted according to the above given criteria and correlation coefficients were determined. These were found to be 0.989, 0.995, 0.961 and 0.900 for zero, first, second and third order kinetics respectively as shown in **Fig. 3.2.3**

From these values it has been concluded that the synthesized CDS followed first order kinetics and as the overall concentration of the CDS is very small in the medium, so it is pseudo-first order kinetics. Comparing the above obtained linear equation with the general equation for the psuedo-first order kinetics, $\log Z_0/ (Z_0 - Z) = -k \times t/2.303$ (Z_0 is initial concentration of a substance, Z is the constant fraction degrading from it in time interval 't', k is the rate constant and t is time), we get the rate constant *k* for Sample **(7aI)** as given below:

$$k = m \times 2.303$$
$$= -0.051 \times 2.303,$$
$$= -0.1174$$

Table 3.2.2: Data presentation of (7aI) for calculation of rate constant (k) at *p*H 6.0

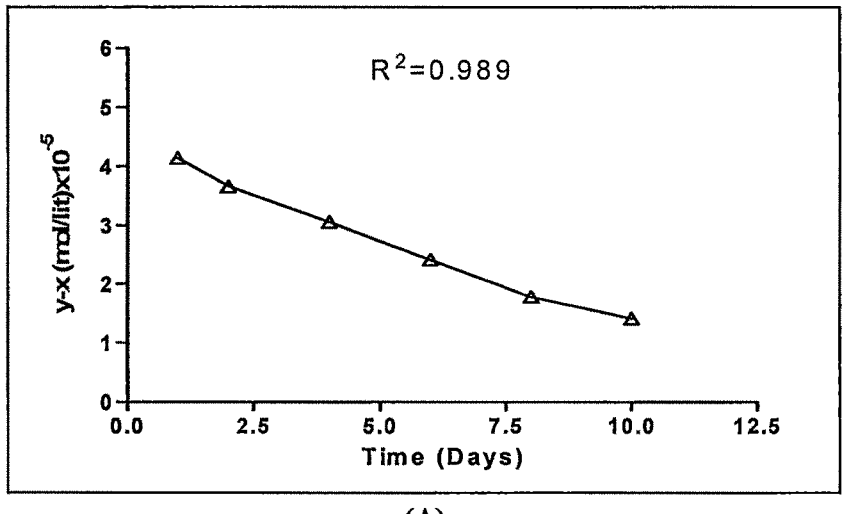
Time (days)	Area (mV) mean±SD (n=3)	Conc. (µg/ml) y	Amt. Degraded (µg/ml) x	y-x (µg/ml)	y-x (mol/lit)
1	824.33±26.10	20.56	0	20.56	4.14×10^{-05}
2	731.66±22.54	18.18	2.38	18.18	3.66×10^{-05}
4	615.33±23.45	15.19	5.37	15.19	3.06×10^{-05}
6	490.33±15.03	11.97	8.59	11.97	2.41×10^{-05}
8	368.66±12.03	8.847	11.71	8.84	1.78×10^{-05}
10	298.66±14.26	7.04	13.52	7.046	1.41×10^{-05}

Time (days)	Area (mV) mean±SD (n=3)	y-x (µg/ml)	Log (y-x) (mol/lit)	1/(y-x)	$1/(y-x)^2$
1	824.33±26.10	20.56	-4.38	24131.2	582316751.3
2	731.66±22.54	18.18	-4.43	27294.3	744977554.7
4	615.33±23.45	15.19	-4.51	32670.3	1067345826
6	490.33±15.03	11.97	-4.61	41440.6	1717326761
8	368.66±12.03	8.84	-4.74	56098.9	3147084090
10	298.66±14.26	7.046	-4.84	70432.5	4960730244

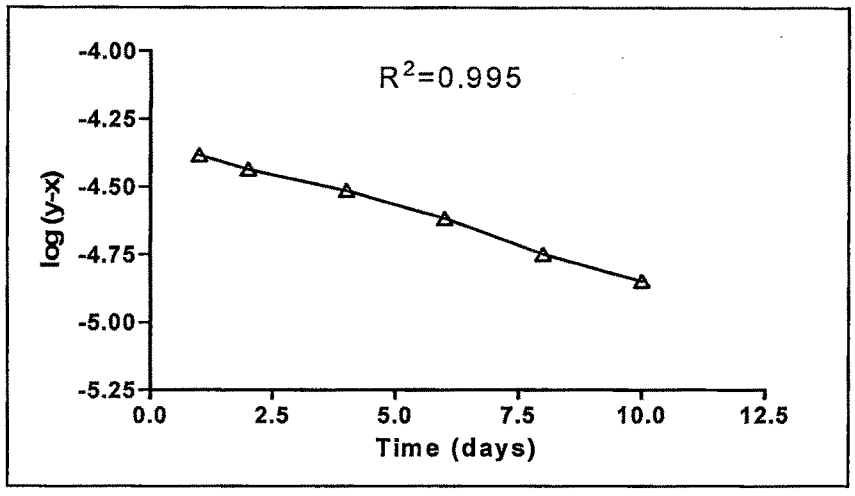
x= Concentration of derivative **(7aI)** at various time intervals which got hydrolyzed

y= initial concentration of the derivative at 0 h, i.e. 20.56 µg/ml

y-x= Concentration of derivative left intact in the solution at various time intervals



(A)



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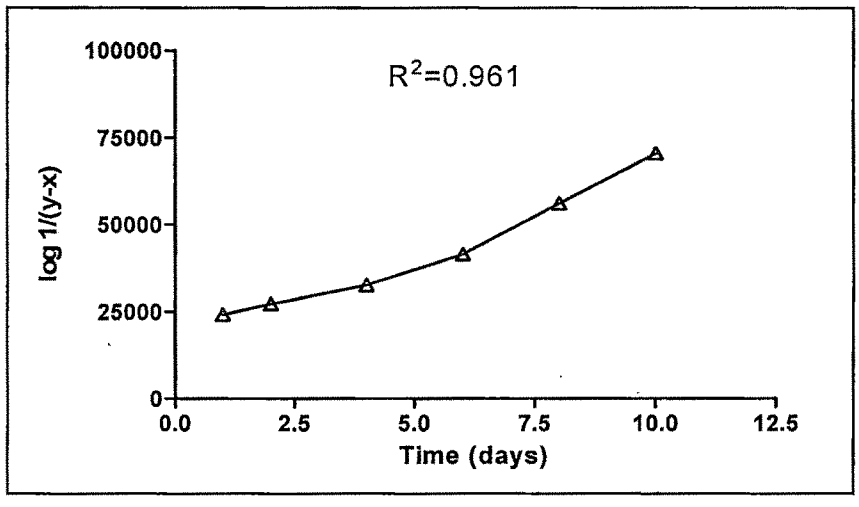
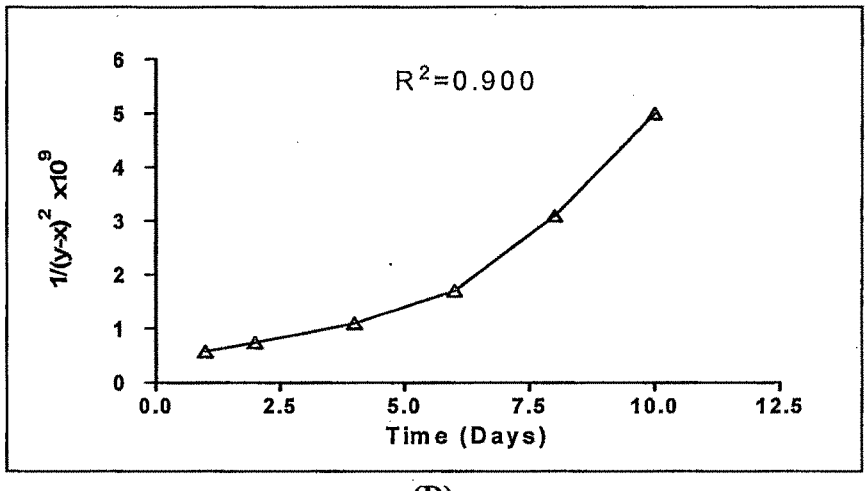


(C)



(D)

Fig. 3.2.3: Graphical presentation for calculated (y-x) vs t **(A)**, log (y-x) vs t **(B)**, 1/(y-x) vs t **(C)**, and $1/(y-x)^2$ vs t **(D)** for sample **(7aI)** at *p*H 6.0

Further calculations were done to find out half-life using first order half-life equation as given below:

$$t_{1/2} = 0.693/k \quad \text{.....(2)}$$

The same procedure as described above was followed for rest of the derivatives **(7bI-7fI)**, and the calibration and chromatographic conditions have been shown in **Fig. 3.2.4** and **Table 3.2.3**

Stability study in human serum has been carried out using the same calibration curve as used in stability study determination in phosphate buffers. Working solutions of test compounds were prepared by dissolving the compounds in water and small amount of DMSO (20-40 µl). Whenever required followed by sonication for 2 min. Human serum samples were kept at 37 °C for 5 min before the addition of the test compound to initiate the reaction. Aliquots of the incubation mixtures (100 µL) were transferred to another tube containing 400 µL of cold acetonitrile/1 N HCl (90/10, v/v) to stop the reaction.[9] Samples were then centrifuged at 2800 rpm for 10 min.

Control incubations in the absence of the test compound were also performed. The analyses of test compounds were performed by the same method as indicated above and additionally guard column has been used. In all experiments, the stability of the synthesized CDS was determined as the percentage of the remaining compound at the given time point of interest (assuming 100 % of the compound is present at time 0). The slope of the linear regression from log (y-x) versus time plot (-k) was determined and the *in vitro* human serum half-life was determined using the above given equation (2) and the data obtained has been shown in **Table 3.2.4.**

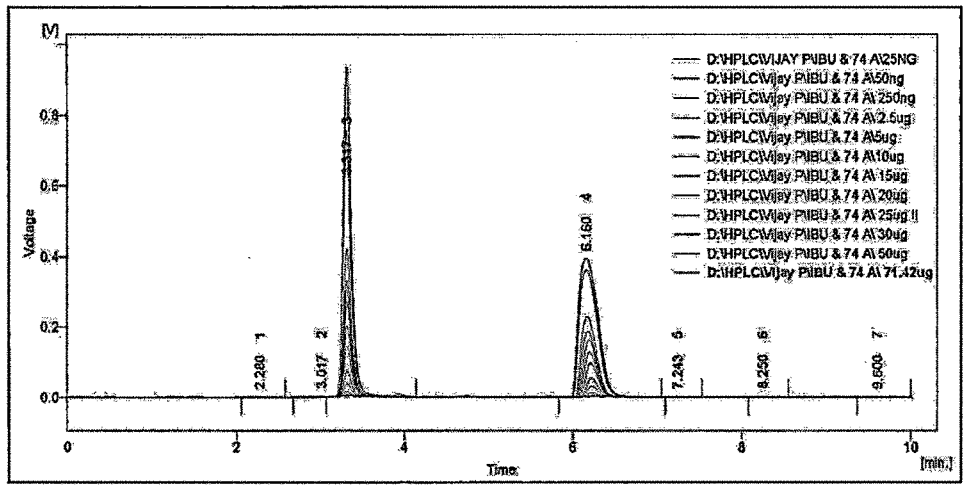


Fig. 3.2.4: Calibration and linearity chromatogram of **7fI**, (RT=3.31 min) and the parent drug **5I** (RT=6.16)

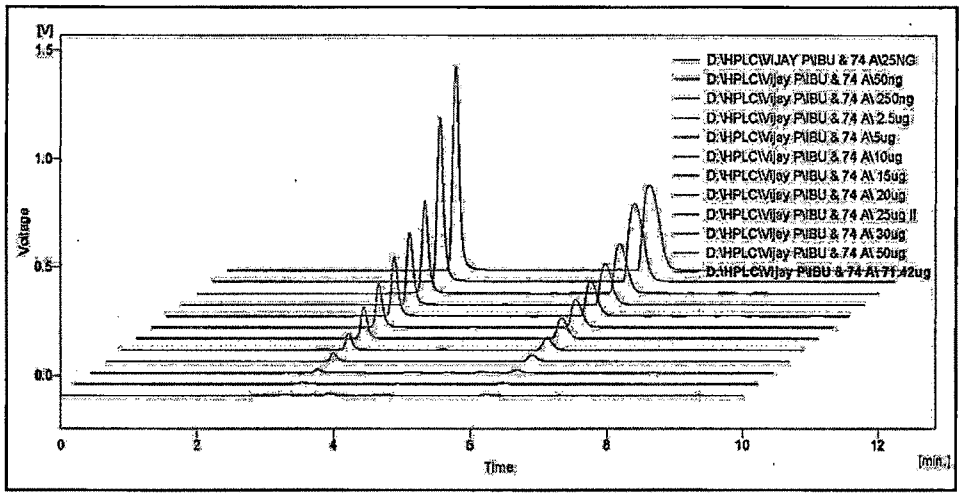


Fig. 3.2.5: 3D Calibration and linearity chromatogram of CDS **(7fI)**

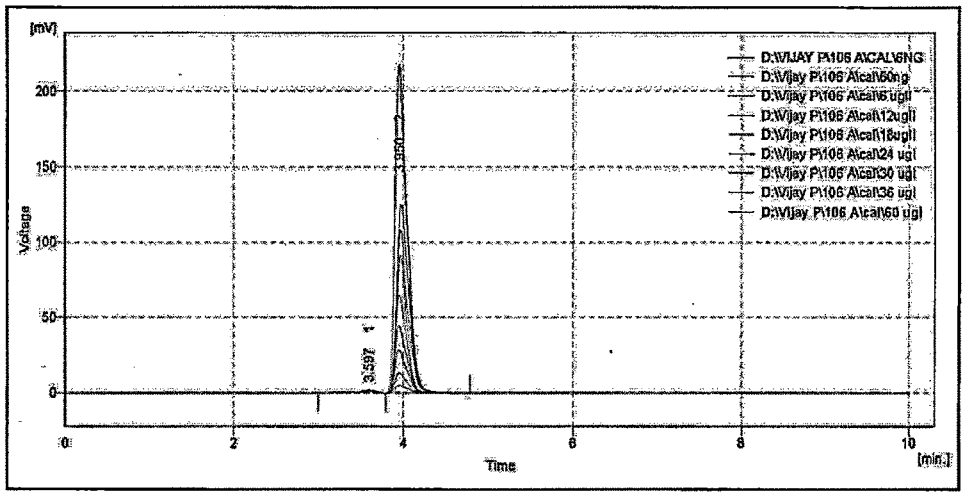


Fig. 3.2.6: Calibration and linearity chromatogram of CDS **(7bI)**

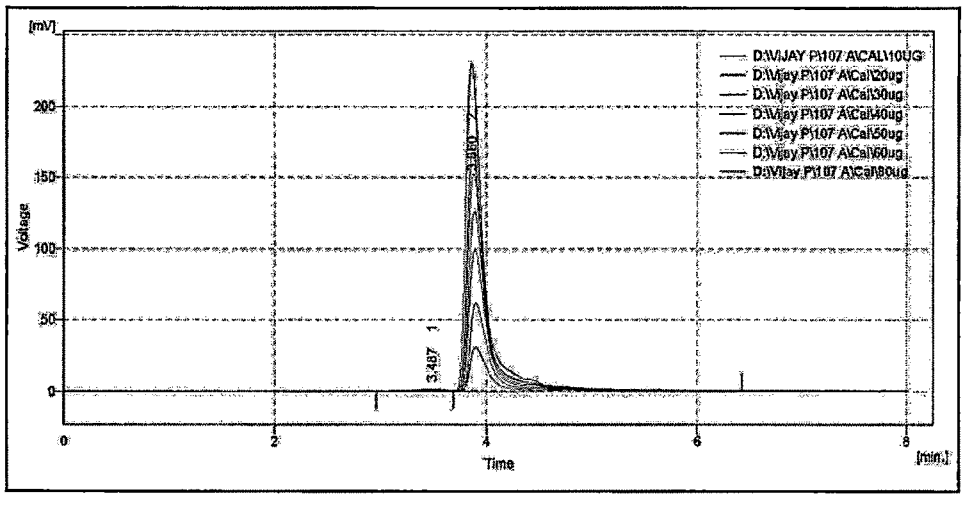


Fig. 3.2.7: Calibration and linearity chromatogram of CDS **(7dI)**

Table 3.2.3: Chromatographic data for CDS **(7aI-gI)**

Compound	RT (min)	Linearity (µg)	λ max (nm)	Mobile phase PB:ACN*	Flow rate (ml/min)
7aI	3.90±0.1	6-60	220	9:1	0.75
7bI	3.97±0.1	6-60	220	9:1	0.75
7cI	3.60±0.1	5-50	220	8:2	0.75
7dI	3.64±0.1	5-50	220	8:2	0.75
7eI	3.50±0.1	6-36	220	9:1	0.75
7fI	3.33±0.1	2.5-50	220	2:8	0.75
7gI	3.60±0.1	6-48	220	2:8	0.75

*PB= Phosphate buffer (15 mM): ACN= Acetonitrile

Table 3.2.4: Half lives of disappearance for CDS **(7aI-7gI)** in phosphate buffers *p*H 6.0, 7.4 and in the human serum.

Compound	Half life of disappearance of various CDS		
	***p*H 6.0 (h)**	***p*H 7.4 (h)**	**Human serum (min)**
7aI	141.01	156.87	8.45
7bI	167.76	133.68	15.30
7cI	164.33	177.63	12.84
7dI	137.28	184.55	18.47
7eI	85.37	92.55	10.88
7fI	432.0	446.40	38.10
7gI	384.0	412.80	28.44

The studies in buffers (*p*H 6.0 and 7.4) indicated that all of the drug derivatives **(7aI-7gI)** showed very high stability in both the *p*H conditions with the CDS having diamide bond found to be more stable as compared to CDS with ester bond. The half-lives ranged from 85.37-446.4 hours. The long half-lives of the derivatives (7**aI-7gI**) assured that these CDS would be hydrolyzed slowly giving prolonged release of the parent NSAID but a very long half life also is not described as it would adversely contribute to the therapeutic effect of the parent NSAID due to insufficient therapeutic concentration at the site of action.

On the other hand stability studies in human serum showed very fast hydrolysis of CDS and the half-lives varied from 8.45-38.10 min. This could be due to enzyme

catalyzed hydrolysis of the CDS. Interestingly CDS without ester bond **(7fI-7gI)** showed moderate stability over the CDS with ester bond.

From the above studies it could be concluded that the synthesized CDS upon IA administration may release active drug in a controlled manner due to the coupled action of *p*H and enzymes (synovial fluid has *p*H 6.0 and increased activity of hydrolyzing enzymes during inflammation) and may show improved therapeutic effect.

3.2.2. Hydrolyses studies of CDS of naproxen (7aII-7gII)

Hydrolysis study of CDS of naproxen has been carried out in the same way as discussed for CDS of ibuprofen **(7aI-7gI)**. HPLC method has been developed to determine half life of disappearance of the synthesized CDS in phosphate buffers of *p*H 6 and 7.4, and in human serum. Half-lives of disappearance of CDS **(7aII-7gII)** were calculated and given in **Table 3.2.7.** The chromatographic conditions used for all the CDS were listed in **Table 3.2.6** below. For calibration plot and linearity determination, mobile phase composition was adjusted for each CDS so that the retention time (RT) of each CDS would fall in the range of 3-6 min as shown in **Fig. 3.2.8** below. Calibration curve shows linearity with r^2=0.999 as shown in **Fig. 3.2.9** and **Table 3.2.5**

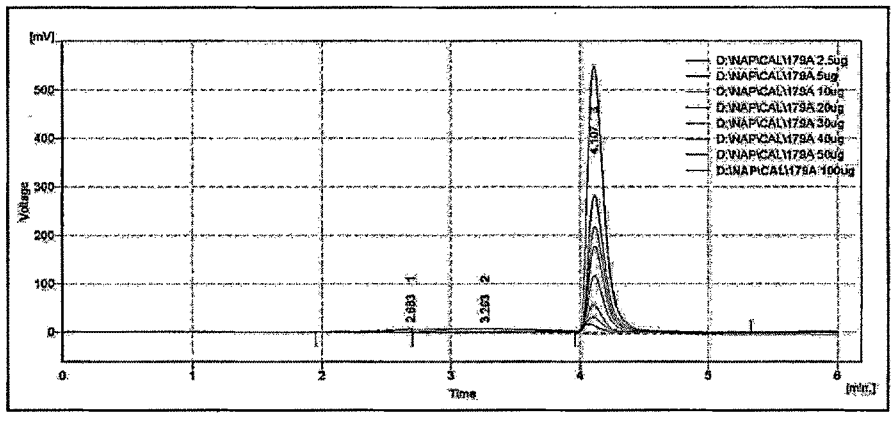


Fig. 3.2.8: Calibration and linearity chromatogram of derivative **(7aII)**

Table 3.2.5: The absorbance readings of **7aII** for the studies in buffers

Concentration (µg/ml)	Mean area (mV) ±SD*
5.0	264±08
10.0	504±14
20.0	1026±19
30.0	1563±16
40.0	2038±22
50.0	2609±26

*n=3, A= 51.92x + 7.40.....**(3)**, (R^2=0.999)

From these results it could be concluded that pyridinium salts were quite stable in phosphate buffer *p*H 6 as well as 7.4; the primary route of hydrolysis of these compound may be ester/amide bond cleavage.

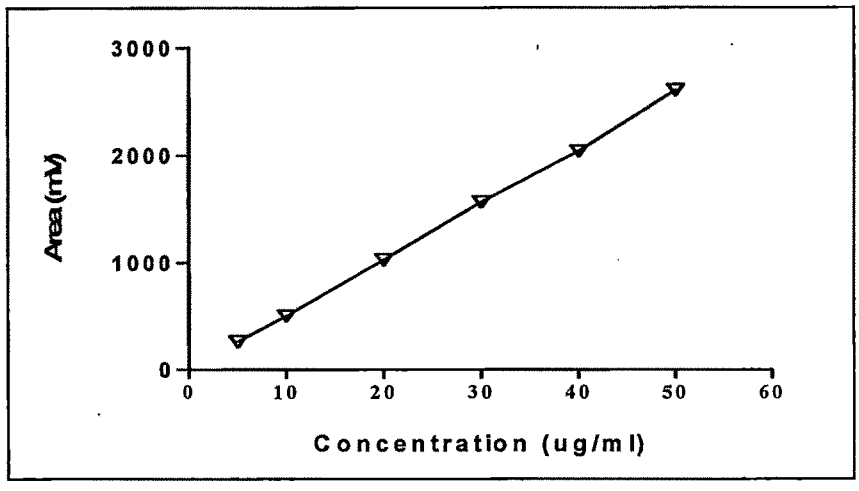


Fig. 3.2.9: Calibration plot for the estimation of **7aII**

Table 3.2.6: Chromatographic data of CDS **(7aII-7gII)**

Compound	RT (min)	Linearity (µg)	λ max (nm)	Mobile phase PB (15mM):ACN*	Flow rate (ml/min)
7aII	4.11±0.1	5-50	229.0	8:2	0.75
7bII	3.73±0.1	5-50	229.0	8:2	0.75
7cII	3.68±0.1	5-50	229.0	8:2	0.75
7dII	3.71±0.1	5-50	229.0	8:2	0.75
7eII	3.77±0.1	5-50	229.0	8:2	0.75
7fII	3.66±0.1	5-50	229.0	8:2	0.75
7gII	3.64±0.1	5-50	229.0	8:2	0.75

Table 3.2.7: Half lives of disappearance of CDS in various conditions

Compound	Half life of disappearance of CDS 7aII-7gII		
	***p*H 6.0 (h)**	***p*H 7.4 (h)**	**Human serum (min)**
7aII	52.58 h	72.58	8.10
7bII	144.88	192.36	6.40
7cII	168.40	204.18	5.30
7dII	132.50	228.31	10.12
7eII	491.41	578.35	4.06
7fII	127.2	134.4	14.18
7gII	276.1	252.97	11.36

All the synthesized CDS have shown rapid enzymatic hydrolysis in human serum to cleave ester/amide bond present in CDS **(7aII-7gII).** Again from the above studies it could be concluded that the synthesized CDS upon IA administration will release the active drug in a controlled manner over a long period of time.

3.2.3. Hydrolyses studies of CDS of indomethacin (7aIII-7gIII)

Hydrolysis study of a CDS of indomethacin has been carried out in the same way as discussed for CDS **(7aI-7gI).** HPLC method has been developed to determine half-lives of disappearance of the synthesized CDS in phosphate buffers of *p*H 6 and 7.4 and in human serum. The chromatographic conditions used for all the CDS have been listed in **Table 3.2.9** below. Calibration plot and linearity were determined as shown in **Fig. 3.2.11**

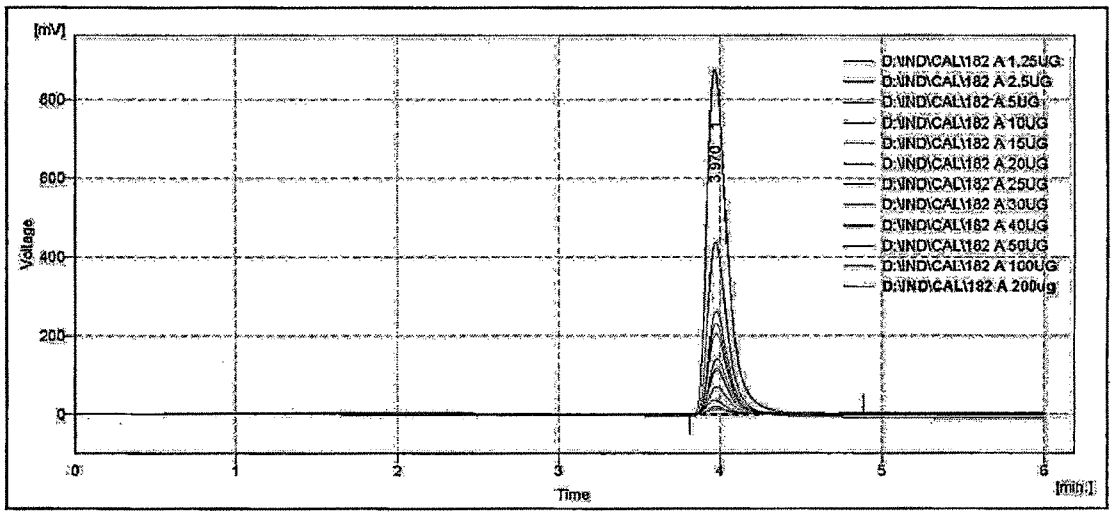


Fig. 3.2.10: Calibration and linearity chromatogram of derivative **(7aIII)**

Table 3.2.8: The absorbance readings of **7aIII** for the studies in buffers

Concentration (µg/ml)	Mean area (mV) ±SD*
2.5	158±08
5.0	297±11
10.0	593±14
20.0	1113±21
30.0	1689±24
40.0	2288±31

*n=3,

$A = 56.35x + 13.25$.....**(4)**, (R^2=0.999)

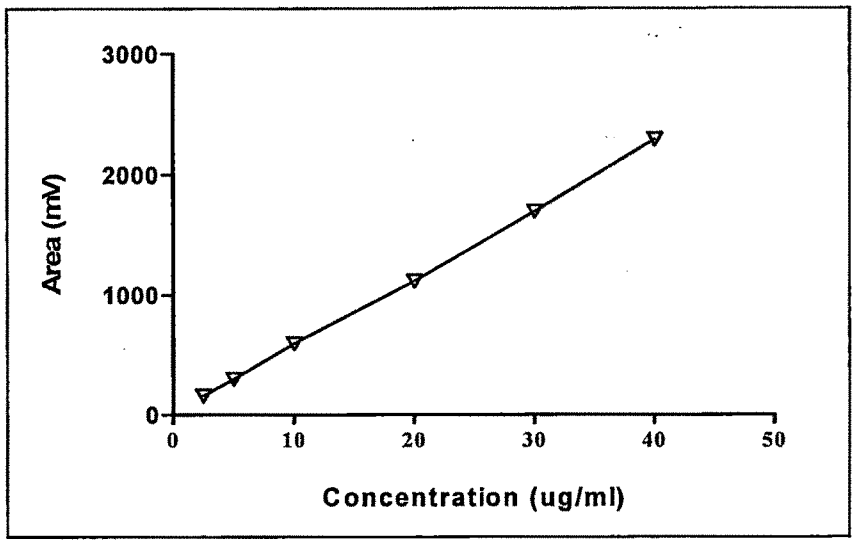


Figure 3.2.11: Calibration plot for the estimation of **7aIII**

Table 3.2.9: Chromatographic conditions for CDS **(7aIII-7gIII)**

Compound	RT (min)	Linearity (µg)	λ max (nm)	Mobile phase PB(15mM):ACN*	Flow rate (ml/min)
7aIII	3.98±0.1	2.5-40	229.0	8:2	0.75
7bIII	3.73±0.1	2.5-40	229.0	8:2	0.75
7cIII	3.76±0.1	2.5-40	229.0	8:2	0.75
7dIII	3.74±0.1	2.5-40	229.0	8:2	0.75
7eIII	3.71±0.1	2.5-40	229.0	8:2	0.75
7fIII	3.81±0.1	2.5-40	229.0	8:2	0.75
7gIII	3.78±0.1	2.5-40	229.0	8:2	0.75

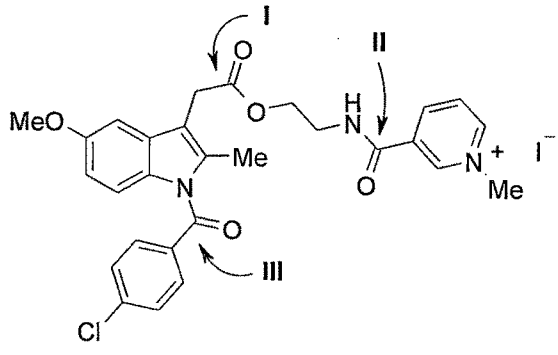
*ACN=Acetonitrile

From these results it could be concluded that indomethacin CDS were quite stable in phosphate buffer of *p*H 6.0 as well as 7.4, but interestingly compared to CDS of ibuprofen **(7aI-7gI)** and naproxen **(7aII-7gII)** these CDS showed significantly low stability in phosphate buffer at *p*H 6.0 and 7.4, except for **7fIII-7gIII**.

This instability may be due to presence of extra hydrolysable groups in indomethacin CDS **(7aIII)** which may contribute to the low stability of these CDS. Suspected sites **(I-III)** which are prone to hydrolysis are shown in the structure given below.

Table 3.2.10: Half lives of disappearance of CDS **(7aIII-7gIII)** in phosphate buffers of *p*H 6.0 and 7.4 and in human serum (80 %).

Compound	Half life of disappearance of various CDS		
	***p*H 6.0 (h)**	***p*H 7.4 (h)**	**Human serum (min)**
7aIII	29.11	38.35	18.40
7bIII	76.85	81.47	16.10
7cIII	41.84	52.70	22.55
7dIII	56.37	69.74	39.50
7eIII	32.44	49.61	46.74
7fIII	135.88	147.53	55.80
7gIII	141.7	129.20	34.19



(7aIII)

The primary route of hydrolysis of these CDS may be ester bond cleavage. Indomethacin CDS were also prone to hydrolysis in human serum but are more stable than CDS of ibuprofen and naproxen, may be due to steric hinderance due to bulky indomethacin moiety.

3.2.4. Hydrolyses studies of CDS of 6-MNA (7aIV-7eIV)

Hydrolysis study of a CDS of 6-MNA **(5IV)** has been carried out in the same way as discussed for CDS **(7aI-7gI)**. HPLC method has been developed to determine half-lives of disappearance of the synthesized CDS in phosphate buffers of *p*H 6.0 and 7.4, and in human serum. The chromatographic conditions used for all the CDS have been listed in **Table 3.2.12** below. Calibration plot and linearity were determined and mobile phase composition adjusted for each CDS so that RT should fall in the range of 3-6 min as shown in **Fig. 3.2.13** below.

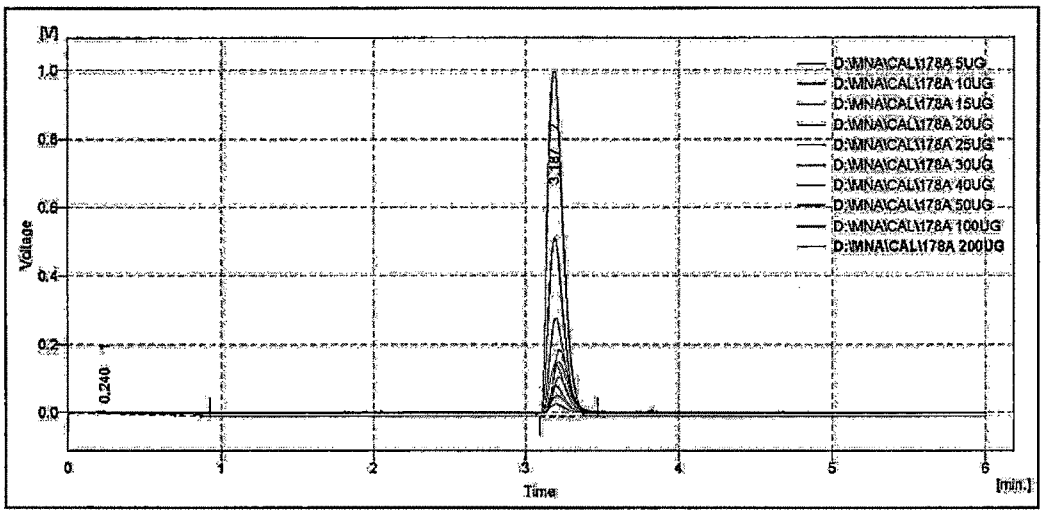


Fig. 3.2.12: Calibration and linearity chromatogram of derivative **(7aIV)**

Table 3.2.11: The absorbance readings of **(7aIV)** for the studies in buffers

Concentration (µg/ml)	**Mean area (mV)** ±SD*
10.0	358±09
15.0	551±11
20.0	746±14
30.0	1111±19
40.0	1520±23
50.0	1973±26

*n=3,

A= 39.93x–55.03.....**(5)**

R^2=0.998)

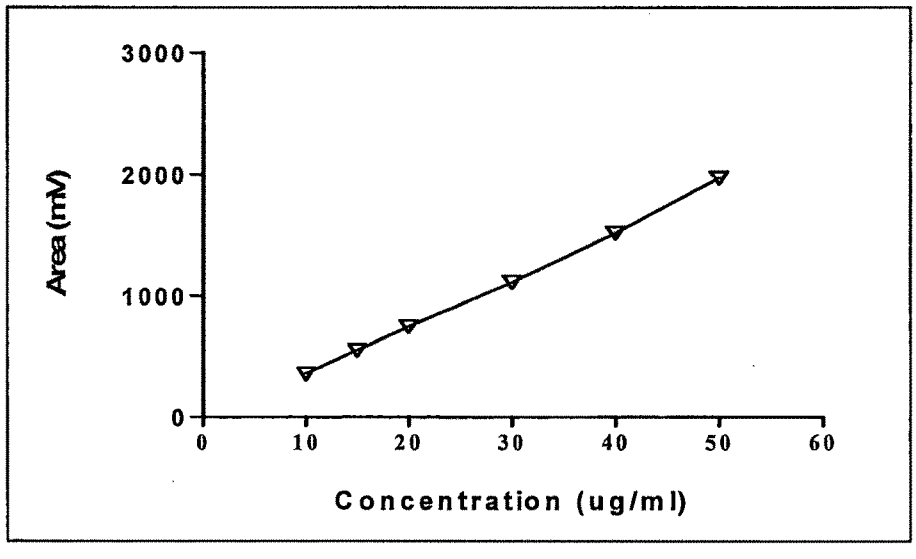


Fig. 3.2.13: Calibration plot for the estimation of **(7aIV)**

Table 3.2.12: Chromatographic conditions for CDS **(7aIV-7gIV)**

Compound	RT (min)	Linearity (µg)	λ max (nm)	Mobile phase PB:ACN*	Flow rate (ml/min)
7aIV	3.18±0.1	10-50	226.0	8:2	1.0
7bIV	3.65±0.1	10-50	226.0	8:2	0.75
7cIV	3.63±0.1	10-50	226.0	8:2	0.75
7dIV	3.64±0.1	10-50	226.0	8:2	0.75
7eIV	3.66±0.1	10-50	226.0	8:2	0.75

Table 3.2.13: Half life of disappearance of CDS **(7aIV-gIV)** in phosphate buffers of *p*H 6.0 and 7.4 and in human serum (80 %).

Compound	Half life of disappearance of various CDS		
	***p*H 6.0 (h)**	***p*H 7.4 (h)**	**Human serum (min)**
7aIV	252.11	276.30	18.41
7bIV	220.40	271.50	34.80
7cIV	204.30	283.56	22.63
7dIV	271.12	148.80	32.41
7eIV	180.48	218.40	16.09

3.2.5. Hydrolyses studies of CDS of BPA (7aV-7eV)

Hydrolysis study of a CDS of BPA **(5V)** has been carried out in the same way as discussed for CDS **(7aI-7gI)**. HPLC method has been developed to determine half-lives of disappearance of the synthesized CDS in phosphate buffers of *p*H 6 and 7.4, and in human serum.

The chromatographic conditions used for all the CDS have been listed in **Table 3.2.15** below. Calibration plot and linearity were determined and mobile phase composition adjusted for each CDS so that RT should fall in the range of 3-6 min as shown in **Fig. 3.2.15** below.

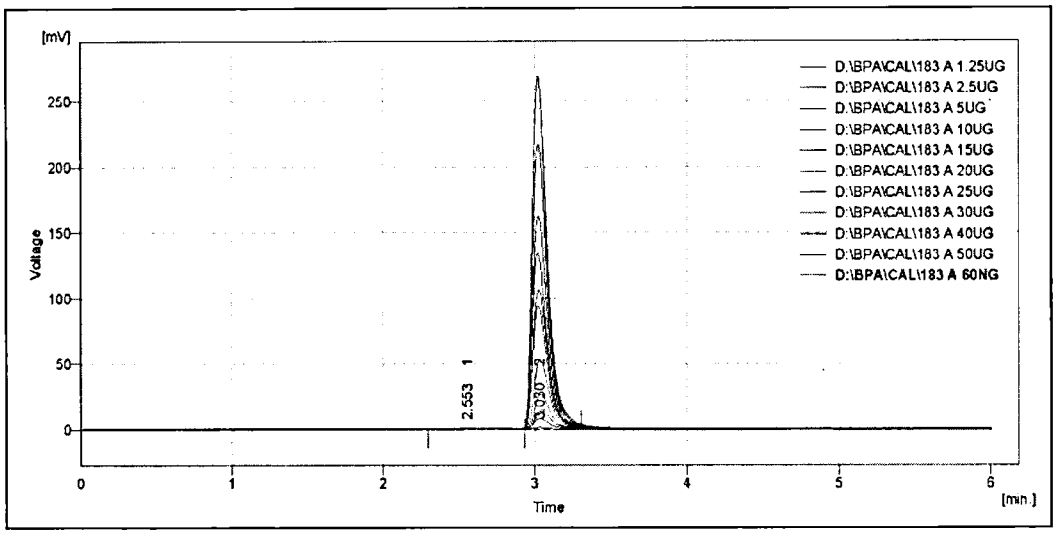


Fig. 3.2.14: Calibration and linearity chromatogram of derivative **(7aV)**

Table 3.2.14: The absorbance readings of **(7aV)** for the studies in buffers

Concentration (µg/ml)	Mean area (mV) ±SD*
2.5	99±06
5	184±10
10	367±16
20	741±19
30	1125±22
40	1504±26
50	1865±33

*n=3,

A= 37.43x–1.43.....**(4)**,

(R^2=0.999)

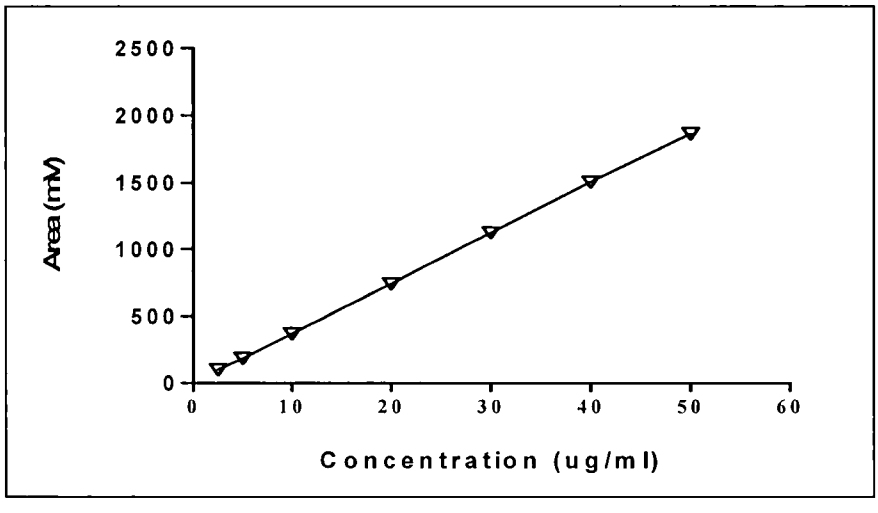


Fig. 3.2.15: Calibration plot for the estimation of **(7aV)**

Table 3.2.15: Chromatographic conditions for CDS **(7aV-gV)**

Compound	RT (min)	Linearity (µg)	λ max (nm)	Mobile phase PB(15mM):ACN*	Flow rate (ml/min)
7aV	3.02±0.1	2.5-50	228.0	8:2	1.0
7bV	4.18±0.1	2.5-50	228.0	8:2	0.75
7cV	4.13±0.1	2.5-50	228.0	8:2	0.75
7dV	4.15±0.1	2.5-50	228.0	8:2	0.75
7eV	4.22±0.1	2.5-50	228.0	8:2	0.75

Table 3.2.16: Half life of disappearance of CDS **(7aV-gV)** in phosphate buffer *p*H 6.0, 7.4 and in human serum (80 %).

Compound	$t_{1/2}$ in Phosphate buffer and human serum		
	***p*H 6.0 (h)**	***p*H 7.4 (h)**	**Human serum (90%) (min)**
7aV	180.12	276.03	26.88
7bV	218.11	230.40	34.51
7cV	244.8	304.8	18.55
7dV	224.81	324.10	30.77
7eV	194.4	267.81	44.07

The studies in buffers (*p*H 6.0 and 7.4) indicated that all the CDS **(7aI-7gV)** showed very high stability in phosphate buffer at both the *p*H conditions. CDS having diamide bond were found to be more stable as compared to CDS with ester bond. The minimum and maximum values of half life of disappearance were shown in **Table 3.2.17** below.

The long half-lives of the CDS **(7aI-7gV)** in phosphate buffers assured that these CDS would be hydrolyzed slowly giving prolonged release of the parent drug but a very long half life is also not desirable. On the other hand stability studies in human serum showed very fast hydrolysis of CDS and the half-life values fall in the range of 4.06-55.80 min. This could be due to enzyme catalyzed hydrolysis of the CDS. Interestingly CDS without ester bond **(7fI-7gIII)** showed moderately high stability over the CDS with ester bond **(7aI-7eV)**. This may be due to highly specific hydrolyzing nature of esterase enzymes towards ester bond.

Table 3.2.17: Comparison of half lives of disappearance of CDS **(7aI-7gV)**

CDS	Series	Half life of disappearance in PB		Human serum (min-max, min)
		*p*H 6 (min-max h)	*p*H 7.4 (min-max h)	
7aI-7eI	Ester	85.37-167.76	92.55-184.55	8.45-18.47
7fI-7gI	Amide	384-432	412.80-446.40	28.44-38.10
7aII-7eII	Ester	52.58-491.41	72.58-578.35	4.06-10.12
7fII-7gII	Amide	127.2-276.1	134.4-252.97	11.36-14.18
7aIII-7eIII	Ester	29.11-76.85	38.35-81.47	16.10-46.74
7fIII-7gIII	Amide	135.88-141.7	129.20-147.53	34.19-55.80
7aIV-7eIV	Ester	180.48-271.12	148.80-283.56	16.09-34.80
7aV-7eV	Ester	180.12-244.8	230.40-324.10	18.55-44.07

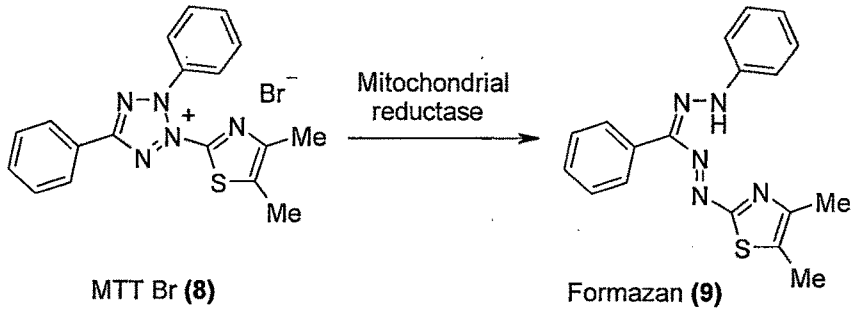
From the above studies it could be concluded that all the synthesized CDS upon IA administration might release active drug in a controlled manner due to the coupled action of *p*H and enzymes.

3.3. Cytotoxicity studies (MTT assay)

As mentioned earlier cationic molecules get accumulated in the liver also along with cartilage and cleared by the kidney. Therefore, liver and renal toxicity issues have to be addressed in order to develop these CDS.[10] Hence, this study was undertaken to evaluate the *in vitro* cellular response to CDS on exposure. *In vitro* assays for xenobiotic toxicity are recently carefully considered by key government agencies (e.g. NIH, FDA etc), mainly to reduce the use of animals in research and to assess human risks. Rat Liver cell line (BRL-3A) has been chosen for the present study.[11]

The MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT to formazan dyes giving a purple color. Its application allows assessing the viability (cell counting) and the proliferation of cells (cell culture assays). It can also be used to determine cytotoxicity of potential medicinal agents and toxic materials, since these agents would stimulate or inhibit cell viability and growth. The assay is based on the reduction of soluble yellow tetrazolium salt (MTT Br) to insoluble Purple formazan crystals by metabolically active cells as shown below. Only live cells can take up tetrazolium salt. The enzyme, NADPH dehydrogenase present in the mitochondria of live

cells can convert internalized tetrazolium salt to purple coloured formazan crystals, which is directly proportional to the number of live cells.



MTT Br **(8)** Formazan **(9)**

The compound can be quantified by measuring its absorbance at a fixed wavelength (between 500 and 600 nm, usually 570nm) by a microplate reader (spectrophotometer). The percent viability of the cells can be calculated using the formula given below.

$$\text{Cell viability (\%)} = At/Ac \times 100 \quad \textbf{(1)}$$

Where:

At= absorbance of sample (test) at 570 nm;

Ac= absorbance of control at 570 nm

MTT assay was performed on the synthesized CDS and their IC_{50} concentration compared with parent drugs as shown in **Fig. 3.3.1**

Table 3.3.1: MTT assay result of compound **(5l)**

Sample Concentration (µg/ml)	**% Cell viability ± SD**	**% Cell Death**
Control	100±3.01	--
46.87	79.10±5.04	20.90
93.75	79.03±5.08	20.97
187.5	71.53±4.11	28.47
375	62.14±3.20	37.86
750	37.01±3.05	62.99
1500	27.98±2.08	72.01

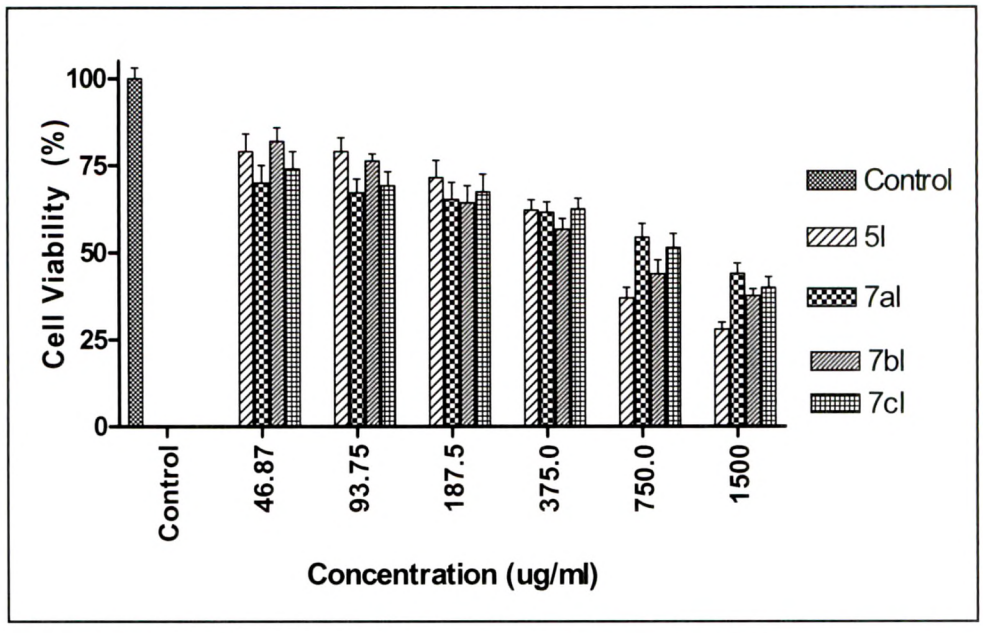


Fig. 3.3.1: Effect of various concentrations of compounds on cell viability

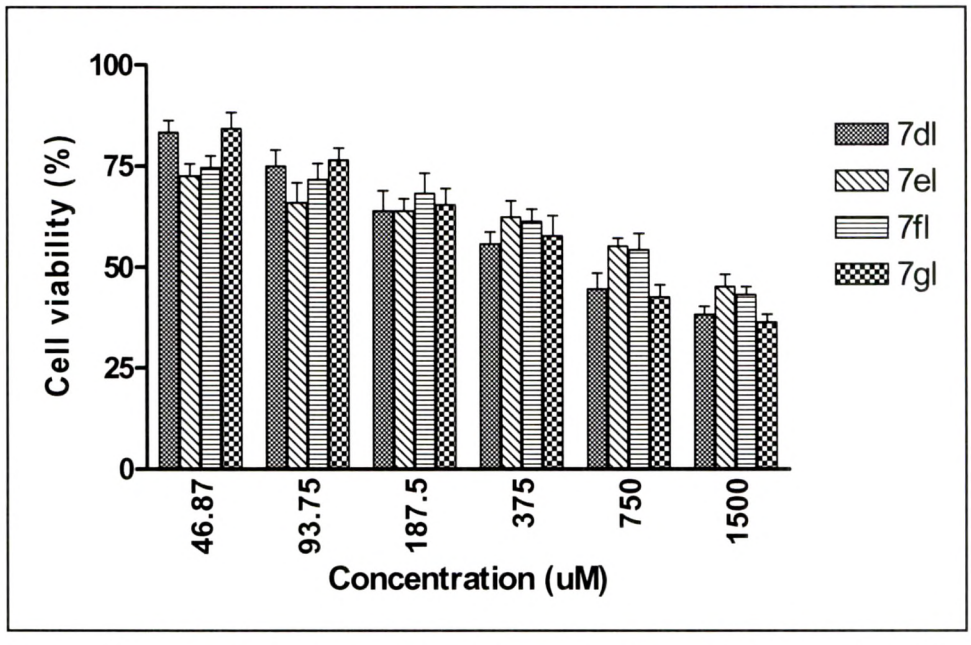
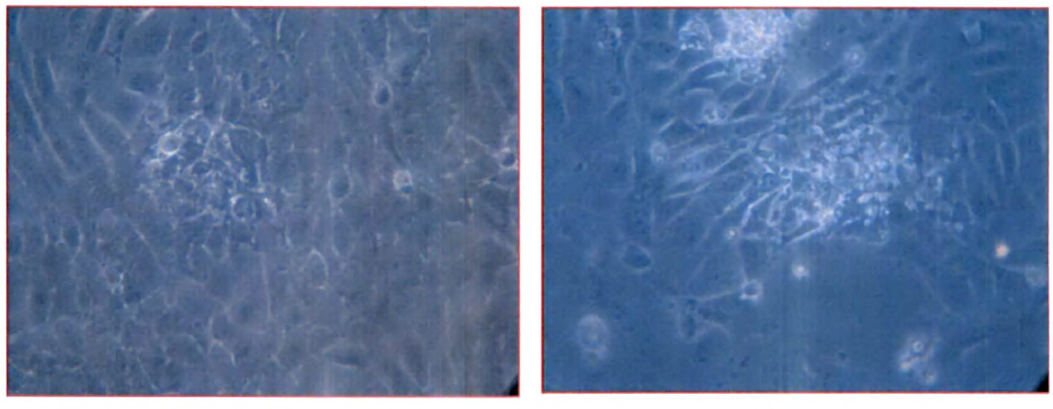


Fig. 3.3.2: Effect of various concentrations of compounds **(7dI-7gI)** on cell viability

Along with cell viability, morphology of the cells was also studied to check whether any cell necrosis occurred or not. The photograph of normal cells and drug treated cells shows slight cell necrosis in CDS treated cells at high concentration as compared to normal cells.

Fig. 3.3.3: Photograph of normal rat Liver cell line (BRL-3A)



(A) **(B)**

Fig. 3.3.4: Photograph of rat Liver cell line (BRL-3A) treated with CDS **(7aI)**.
(A) at low concentration (46.48 µM), **(B)** at high concentration (1500 µM)

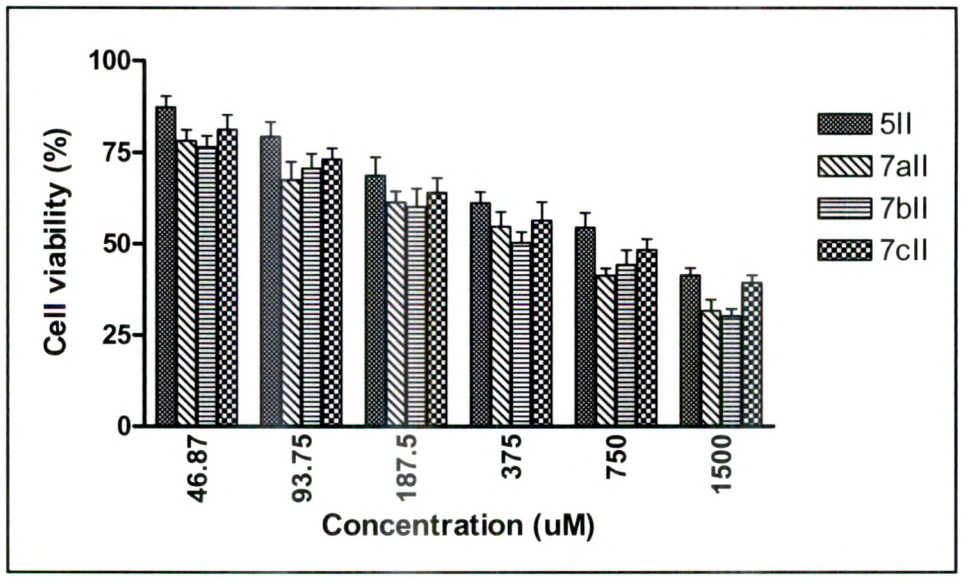


Fig. 3.3.5: Effect of various concentrations of compounds **(5II, 7aII-7cII)** on cell Viability

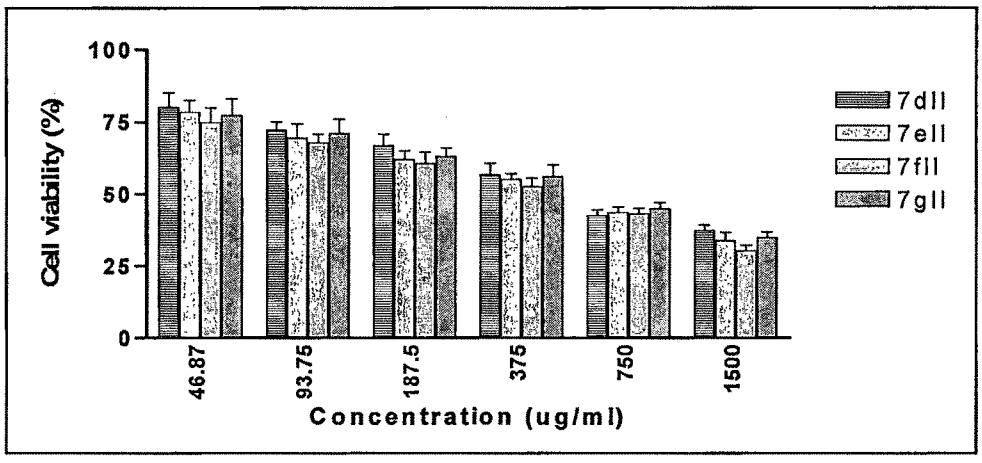


Fig. 3.3.6: Effect of various concentrations of compounds **(5II, 7dII-7gII)** on cell viability

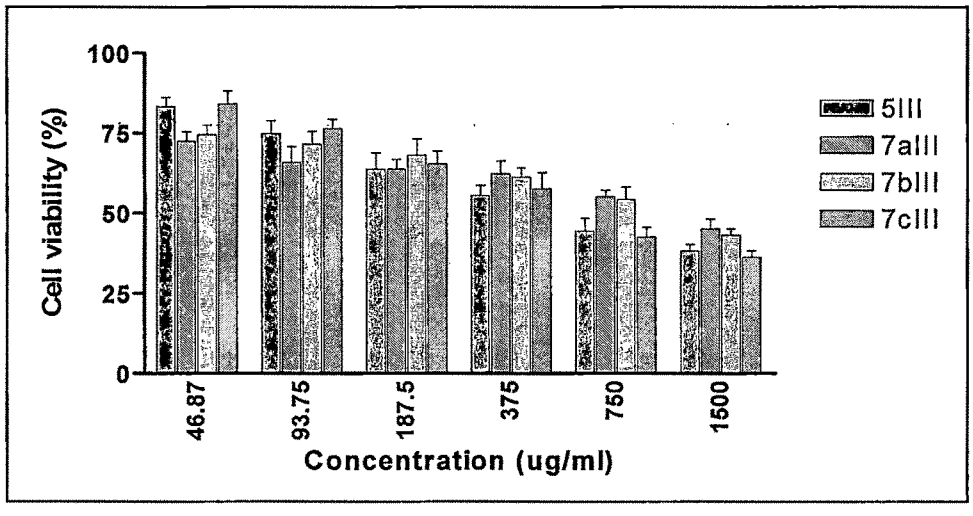


Fig. 3.3.7: Effect of various concentrations of compounds **(5III, 7aIII-7cIII)** on cell viability.

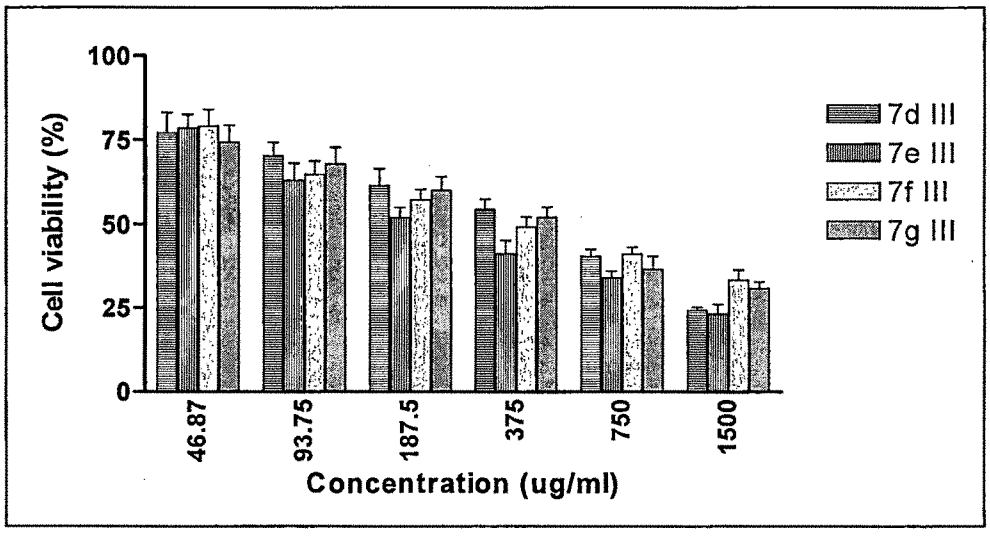


Fig. 3.3.8: Effect of various concentrations of compounds **(7dIII-7gIII)** on cell viability

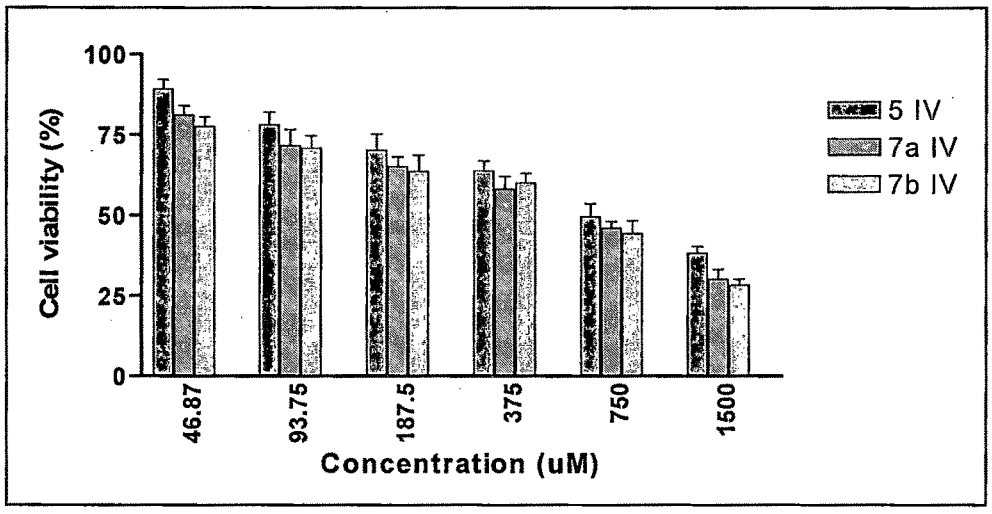


Fig. 3.3.9: Effect of various concentrations of compounds **(5IV, 7aIV-7bIV)** on cell viability

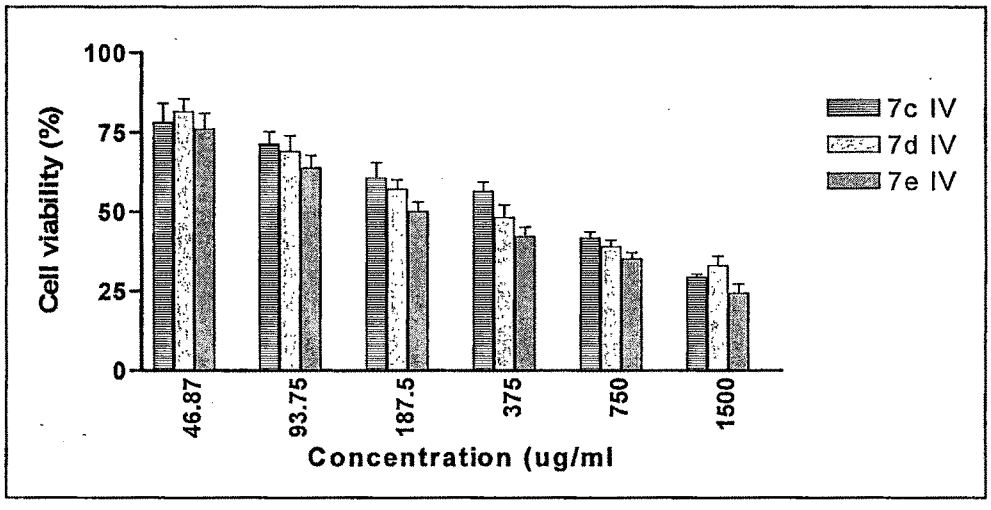


Fig. 3.3.10: Effect of various concentrations of compounds **(7cIV-7eIV)** on cell viability

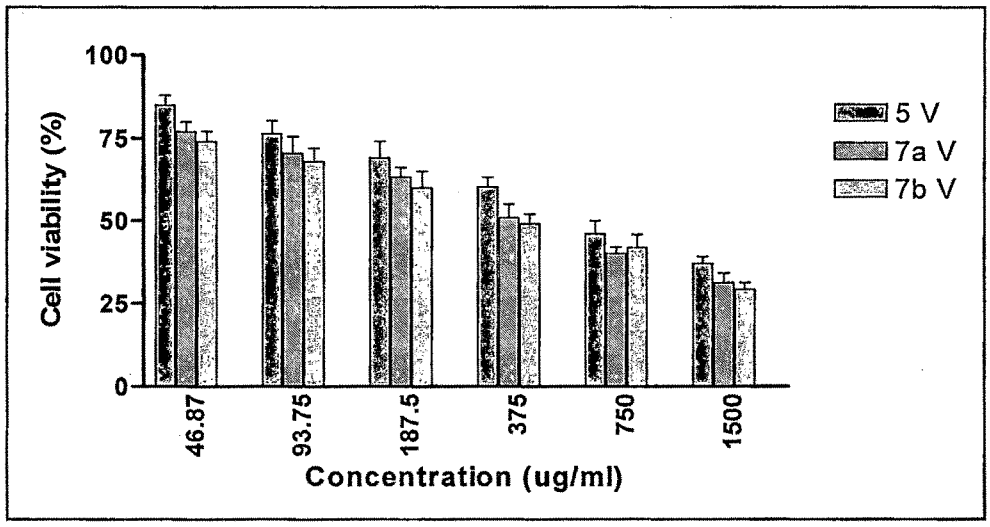


Fig. 3.3.11: Effect of various concentrations of compounds **(5V, 7aV-7bV)** on cell viability

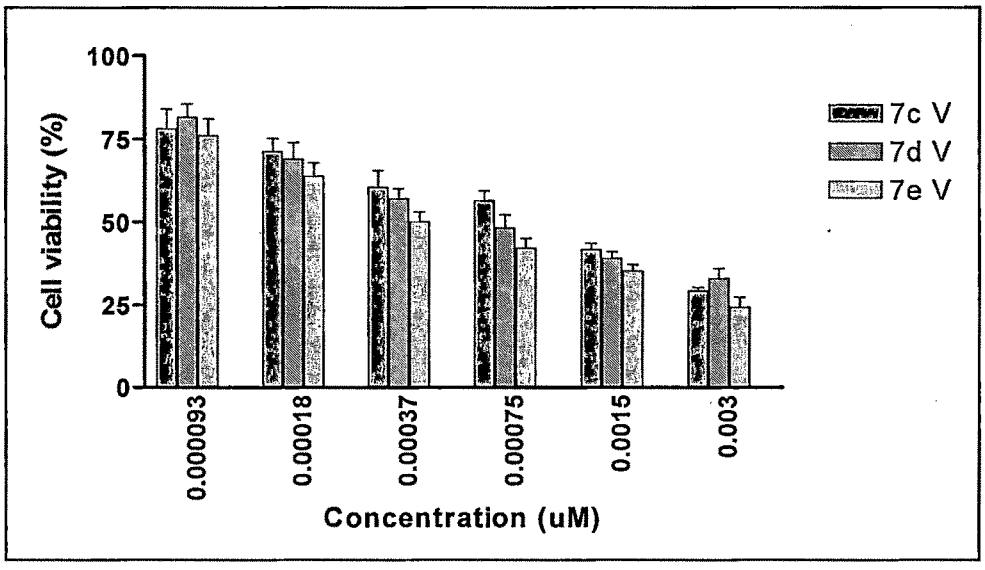


Fig. 3.3.12: Effect of various concentrations of compounds **(7cV-7eV)** on cell viability

From the *in vitro* cell line studies it was concluded that there is no significant effect of pyridinium carrier system on cell toxicity. But as compared to the parent drugs all the synthesized CDS showed higher cell death which may be due to charge on the CDS. All the cell membranes have negative charge and they may interact with positively charged molecules affecting the cell permeability which may result in cell death or retardation of cell growth. Such type of interaction may also result in cell necroses which occurs at higher concentrations of CDS but the parent NSAIDs lack this effect as shown in **Fig. 3.3.4.**

3.4. Bio-distribution and gamma imaging studies

This study was carried out with the aim to assess the residence time of NSAIDs and synthesized CDS in joint cavity upon IA administration. Little information is available in the literature on the effect of charge on residence time of drugs in joint cavity. For such a study to be performed, radiolabeling of the synthesized derivatives was required to be done for their localization/quantification in the joint cavity. To study these aspects the NSAID and the CDS were labeled using ^{99m}Tc. Nearly 80 % of all radiopharmaceuticals, used in nuclear medicine are ^{99m}Tc-labeled compounds. The reason for such a predominant position of ^{99m}Tc in clinical use is its extremely favorable physical and radiation characteristics. It has a 6-hour (approximately) half-life and emits soft monochromatic γ-rays of 140 keV power which do not affect the patient adversely. ^{99m}Tc

is readily available in a sterile, pyrogen free and carrier free state from ^{99}Mo-^{99m}Tc generators.

Technetium can exist in eight oxidation states namely, -1 to +7, and the stability of these oxidation states depends on the type of ligands and chemical environment. The +7 and +4 states are the most stable states. The lower oxidation states are normally stabilized by complexation with ligands. The radioactive form of technetium (^{99m}Tc) is available as sodium pertechnetate salt (^{99m}Tc-$NaTcO_4$). In this salt, technetium is present in a stable state so, it does not easily complex with various ligands. To make a complex, the oxidation state has to be reduced to +5 or less from its original +7 state. For this purpose various reducing agents like stannous chloride, stannous citrate, stannous tartrate and sodium borohydride with concentrated hydrochloric acid and ferrous sulphate with dithionite are used. Among these, stannous chloride in acidic medium is the most widely used reducing agent. After reduction of technetium to lower oxidation state, the *p*H of the medium has to be raised because complexes in general are less stable in acidic media and more stable in neutral/alkaline media. But, the *p*H of the medium cannot be raised to a more basic side otherwise the stannic form of tin (which gets formed in the medium) and the reduced forms of technetium precipitate out. So, a compromise has to be made and a proper *p*H adjustment of the solution has to be done so that there is no precipitation and the complex also remains stable.

The reduced ^{99m}Tc can combine with different ligands like -COO-, -CONH, -OH, -NH_2, -CO-, -COOR and -SH to afford complexes which may be stable depending on the medium and the complexing agent. For a new compound acting as a complexing agent for ^{99m}Tc, its labeling efficiency has to be evaluated. If the new compound is a good complexing agent then it would give a stable complex under the given conditions and the labeling efficiency would be approximately 100 % but, if the complex is not very stable then this efficiency would be much less.[12-13]

To assess the stability, the solution of the complex is run on ascending instant thin layer chromatography (ITLC) in three different solvent systems separately, as mobile phases i.e. acetone (100 %), saline (0.9 %) and a mixture of pyridine-acetic acid-water (PAW) (3.5:5:1), and detection of the complex is carried out using γ-camera. In acetone (100 %) and saline (0.9 %) solvent systems the ^{99m}Tc-bound complex would remain at the base of application of the spot and the uncomplexed ^{99m}Tc would travel along with the solvent front while in the PAW solvent system the complex would travel along with the solvent front while the uncomplexed ^{99m}Tc would remain at the base. By subtracting the

activity obtained from acetone/saline run ITLC from the activity of PAW-run ITLC (activity of the solvent front region) the net amount of activity obtained from the labeled compound could be obtained which would provide the labeling efficiency. Stability of the labeled complex in human serum could also be determined based on the above said principle. After incubating the labeled complex with serum, samples were withdrawn at different time intervals and run on ITLC using the above described three mobile phases separately. Any increase in reduced/hydrolysed ^{99m}Tc activity is an indication of breakdown of the complex in human serum. 6-MNA **(5IV)** was chosen as the parent NSAID for all the studies as it has a long half-life. Due to its long half life it would be easy to find out its concentration in the whole body if the drug would leave the joint.

3.4.1. Optimization of radiolabeling of CDS and parent drug

The radiolabling of the compounds with reduced ^{99m}Tc was carried out as per the direct labeling method. The radiolabeling was optimized by taking three factors into account i.e. effect of *p*H on complex formation, incubation time and stannous chloride concentration.[14]

Table 3.4.1: Effect of *p*H on radiolabeling efficiency of compounds **(5IV, 7aI-7bI, 7aIV)**

*p*H	% Radiolabeled compound			
	5IV	**7aI**	**7bI**	**7aIV**
5.5	63.08±3.1	67.18±5.0	61.29±2.1	72.80±4.1
6.0	71.06±4.0	77.04±4.1	75.11±4.3	84.05±3.0
6.5	84.38±4.3	88.05±4.3	90.01±3.0	96.51±5.1
7.0	**94.44±5.1**	**95.88±5.0**	**94.86±5.1**	**95.61±4.2**
7.5	89.01±4.7	91.07±3.7	91.76±4.0	94.46±5.0
8.0	80.36±3.0	84.53±4.1	85.60±4.1	90.47±3.1

The *p*H of the labeled complex was increased from 5.5 to 8 and its effect on labeling efficiency was studied. The radiolabeled complexes were incubated for various time periods (5-40 min) and the effect of incubation time on labeling efficiency was determined keeping other variables constant. The effect of stannous chloride dihydrate ($SnCl_2.2H_2O$) concentration (50-200 µg) on the labeling efficiency was also studied to obtain the optimum concentration needed for maximum labeling. From the data it was concluded that maximum radiolabeling efficiency was obtained at *p*H 7.0, with incubation time of 30 min and at 150 µg stannous chloride concentration.

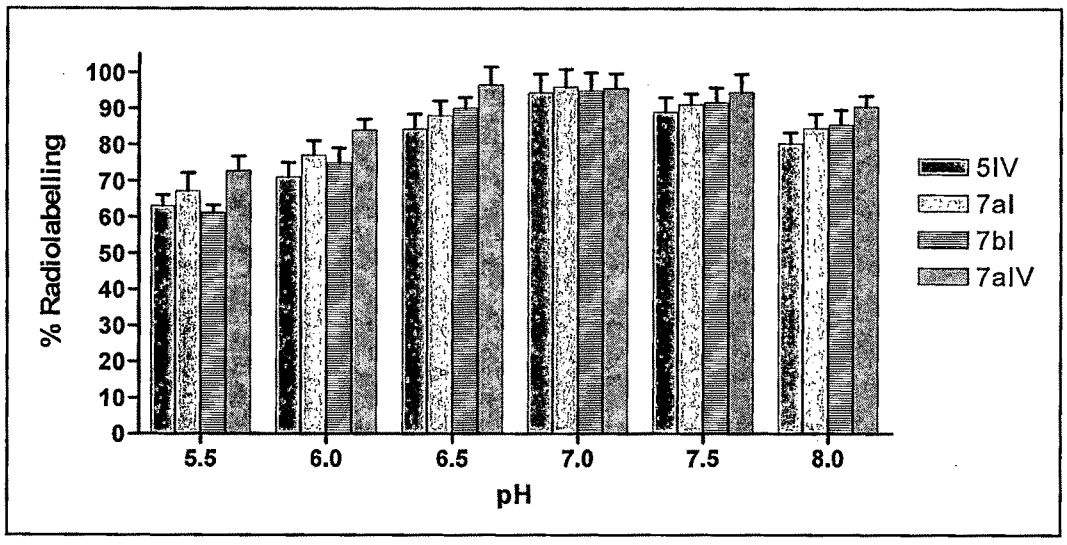


Fig. 3.4.1: Effect of *p*H on radiolabeling efficiency of compounds **(5IV, 7aI-7bI, 7aIV)**

Table 3.4.2: Effect of incubation time on radiolabeling efficiency of derivatives **(5IV, 7aI-7bI, 7aIV)**

Incubation Time (min)	% Radiolabeled compound			
	5IV	7aI	7bI	7aIV
5.0	60.12±3.0	63.17±2.3	59.01±3.1	64.05±3.0
10	68.06±4.1	82.51±3.1	78.56±2.0	84.15±2.1
20	88.33±3.2	89.06±4.1	87.96±3.3	89.96±3.4
30	95.06±2.0	94.08±4.3	**96.86±4.3**	**94.57±4.2**
40	**96.40±3.0**	**95.09±5.0**	96.63±5.1	91.05±4.3

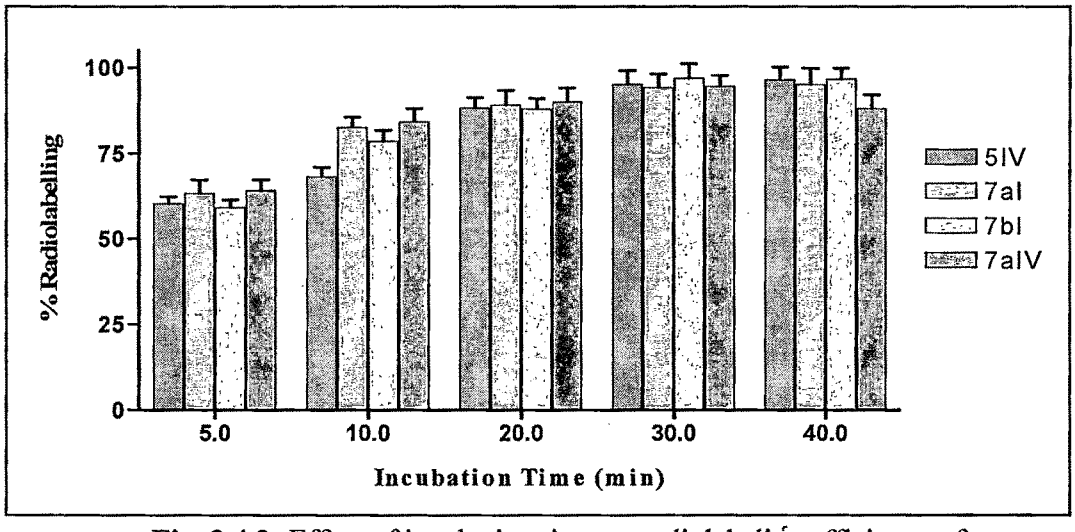


Fig. 3.4.2: Effect of incubation time on radiolabeling efficiency of compounds **(5IV, 7aI-7bI, 7aIV)**

Table 3.4.3: Effect of $SnCl_2.2H_2O$ concentration on radiolabeling efficiency of derivatives **(5IV, 7aI-7bI, 7aIV)**

$SnCl_2$ (µg)	5 IV			7aI			7bI		
	% B	% F	% C	% B	% F	% C	% B	% F	% C
50	79.89	18.13	1.88	78.77	19.33	1.9	81.54	15.87	2.41
100	84.55	14.33	1.11	83.78	13.87	1.76	87.71	11.64	0.58
150	94.87	4.61	0.51	**96.04**	3.37	0.39	93.84	5.06	0.69
200	**95.01**	4.1	0.88	95.1	3.77	1.12	**94.88**	4.09	1.02

$SnCl_2$ (µg)	7aIV		
	% B	% F	% C
50	84.1	14.67	1.23
100	89.55	8.46	1.97
150	**94.91**	4.36	0.72
200	93.45	5.45	1.04

% B= % bound; % F= % Free; % C= % colloid

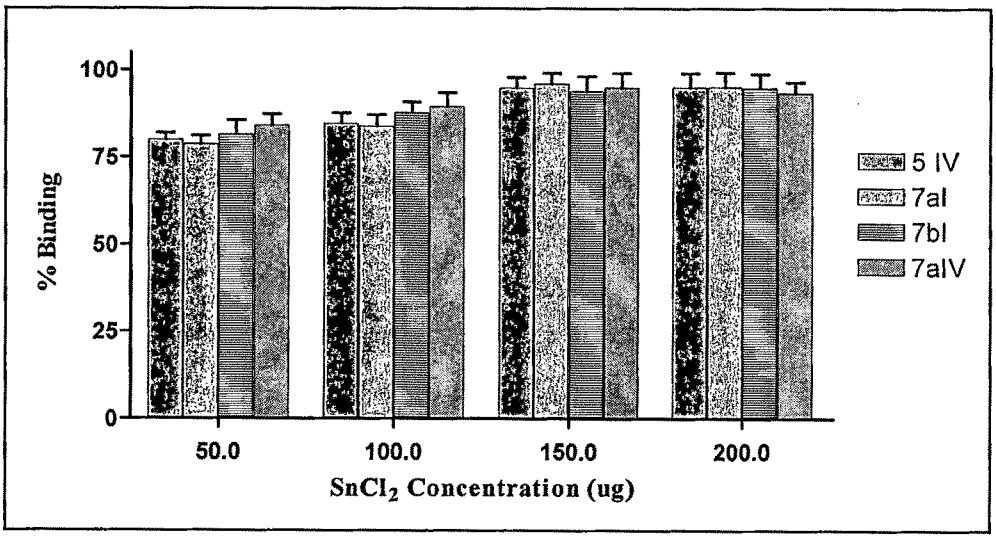


Fig. 3.4.3: Effect of $SnCl_2.2H_2O$ concentration on radiolabeling efficiency of compounds **(5IV, 7aI-7bI, 7aIV)**

3.4.2. *In vitro* stability study of ^{99m}Tc- labeled complexes in saline.

The *in vitro* stability study[15] of radiolabeled complexes was determined in sodium chloride (0.9 %) by ascending thin layer chromatography. The ^{99m}Tc-labeled compound solution (0.1 ml) was prepared and mixed with normal saline (1.9 ml) and incubated (37± 1°C). ITLC was performed at different time intervals (2.0, 4.0, 6.0 and 24 hours) as

described above, in acetone to assess the stability of the complex. Any decrease in percentage of ^{99m}Tc-labeled complex was considered as its degree of degradation. As shown in **Table 3.4.4.** the complexes of the parent drug (5IV) and the CDS **(7aI-7bI, 7aIV)** were reasonably stable up to 24 h in normal saline.

Table 3.4.4: Stability study of compounds **(5IV, 7aI-7bI, 7aIV)** in saline

Compound	**% Radiolabeling Efficiency** (in saline)			
	5 min	**02 h**	**06 h**	**24 h**
5 IV	96.16±2.0	93.38±4.2	88.52±5.2	86.75±4.0
7aI	94.47±4.3	93.24±3.3	91.30±3.3	85.53±4.6
7bI	95.63±4.3	93.35±2.1	90.16±2.4	87.17±3.4
7aIV	94.89±4.2	91.22±3.8	86.11±4.9	79.19±3.2

3.4.3. *In vitro* stability study of ^{99m}Tc- labeled complexes in human serum.

The *in vitro* stability of radiolabelled complexes was tested in human serum. The study was accomplished by incubating an aliquot of 0.1ml of labeled complex prepared in saline (0.9 %) and mixed with 1.9 ml of serum and incubated at 37±1°C. ITLC was performed at different time intervals (0, 2, 6, and 24 h) as described above, in acetone to assess the stability of the complex. Any decrease in percentage of ^{99m}Tc-labeled complex was considered as its degree of degradation. It is evident from **Table 3.4.5.** that the complexes of the parent drug **(5IV)** and the CDS **(7aI-7bI, 7aIV)** were sufficiently stable up to 6 hours and reasonably stable up to 24 hours.

Table 3.4.5: Stability study of compounds **(5IV, 7aI-7bI, 7aIV)** in human serum

Compound	**% Radiolabeling Efficiency** (in human serum)			
	5 min	**02 h**	**06 h**	**24 h**
5IV	96.56±2.0	92.38±4.1	89.52±5.2	81.70±4.0
7aI	94.48±4.3	91.29±3.5	89.30±3.3	82.03±4.3
7bI	94.06±4.3	92.37±2.3	88.16±2.4	83.77±3.4
7aIV	94.37±4.2	91.82±3.7	90.11±4.1	80.11±3.8

3.4.4. Gamma imaging studies

IA residence time of synthesized CDS and parent drug were studied in inflammatory condition. Animals were divided into two groups, Group-A (standard) was administered by IA the parent NSAID i.e 6-MNA **(5IV)**. Another group, Group-B (Test) was administered by IA the synthesized CDS, 3 h after induction of inflammation in the rat paw. Inflammation was induced in the right hind paw of the rats by injecting carrageenan (0.1 ml, 1 % w/v in normal saline) into the subplantar region of the paw. The animals were anaesthetized, fixed on a board and images were taken 2 h, 6 h and 24 h after the administration of the radiolabeled complexes by IA route.

Table 3.4.6: Percentage of radioactivity in knee after IA administration

Time (h)	% Radioactivity in ROI*			
	5IV	**7aIV**	**7bIV**	**7aIV**
1	100±4.0	100±5.1	100±3.0	100±4.3
2	58.47±3.1	78.14±4.0	76.54±3.1	81.09±4.1
6	42.06±4.3	63.57±4.1	60.78±3.0	64.37±3.0
24	13.44±3.0	43.41±4.0	40.96±4.1	42.05±3.1

*Radioactivity counts converted in to percentage and initial counts taken as 100 %; (ROI=Region of interest)

The gamma images obtained are shown in **Fig. 3.4.7-3.4.11** below and radioactivity was measured for whole body as well as for the region of interest (ROI) i.e. knee. Initially, injection was given in both the knees of the animal for the estimation and validation of radioactivity. Subsequently, after establishing the experiment, only one knee in all the remaining animals was injected with the radiolabelled complex for the purpose of localization of the derivatives.

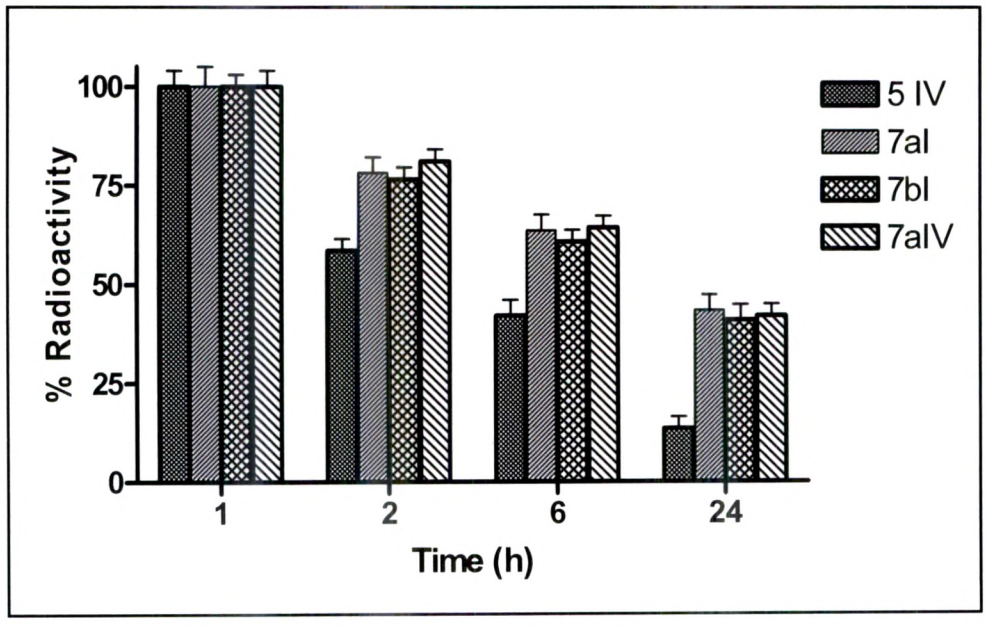
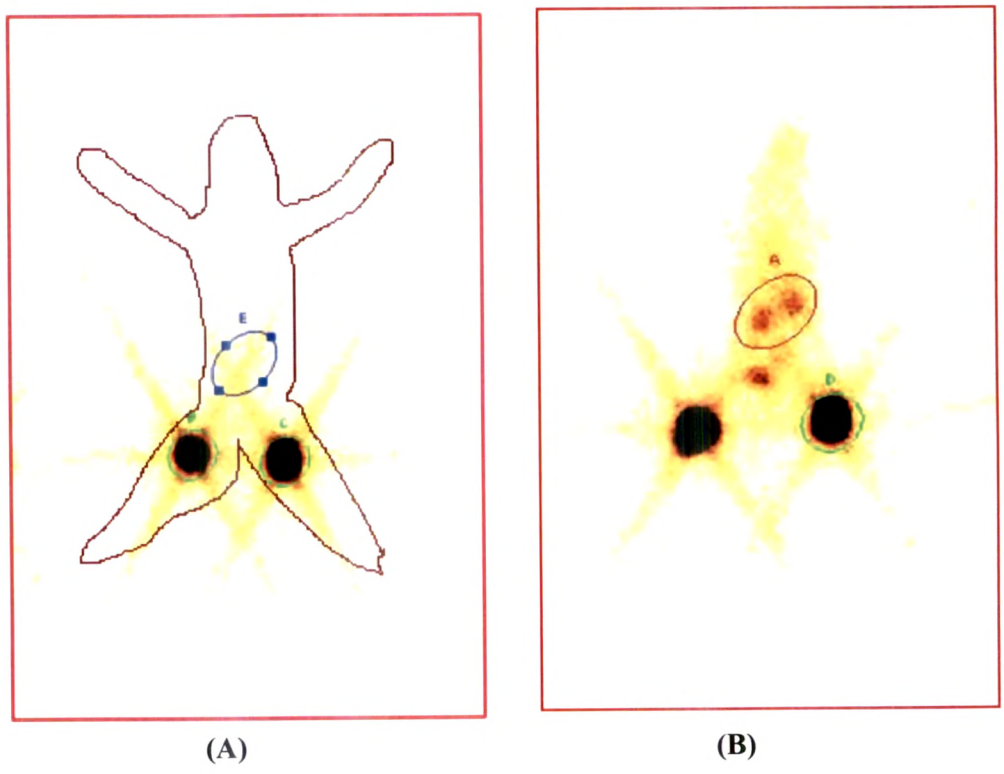


Fig. 3.4.4: Effect of time on Radioactivity after IA injection in knee



(A) **(B)**

Fig. 3.4.5: **A:** Gamma camera image of rats after IA injection of **(5IV)** 1h

B: Gamma camera image of rats after IA injection of **(5IV)** 6h

Fig. 3.4.6: Gamma camera image of rats after IA injection of compounds (A=**5IV**, B=**7aI**, C=**7bI** and D=**7fI**) * 2 h (In figure there are total four animals **A-D** injected drug on right knee.

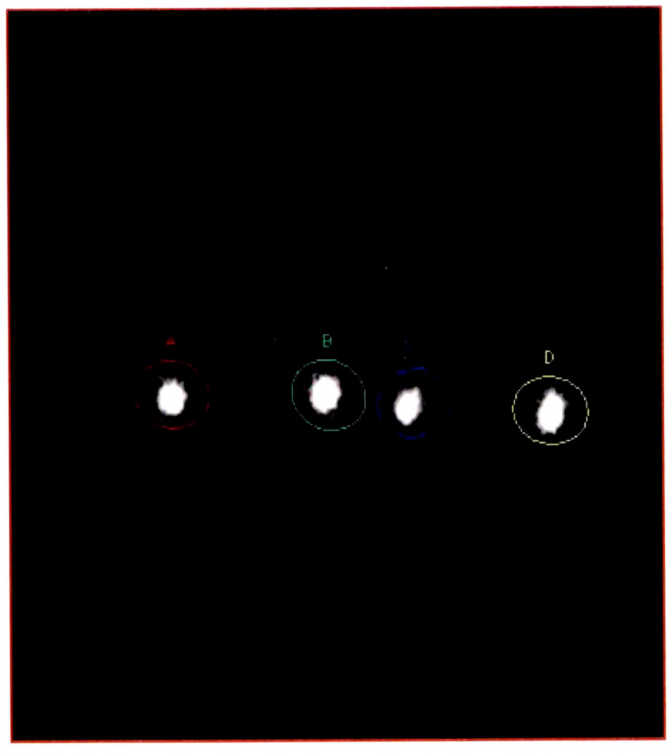


Fig. 3.4.7: Gamma camera image of rats after IA injection of compounds after 6 h (IA injection of CDS, A=**5IV**, B=**7aI**, C=**7bI** and D=**7fI**)

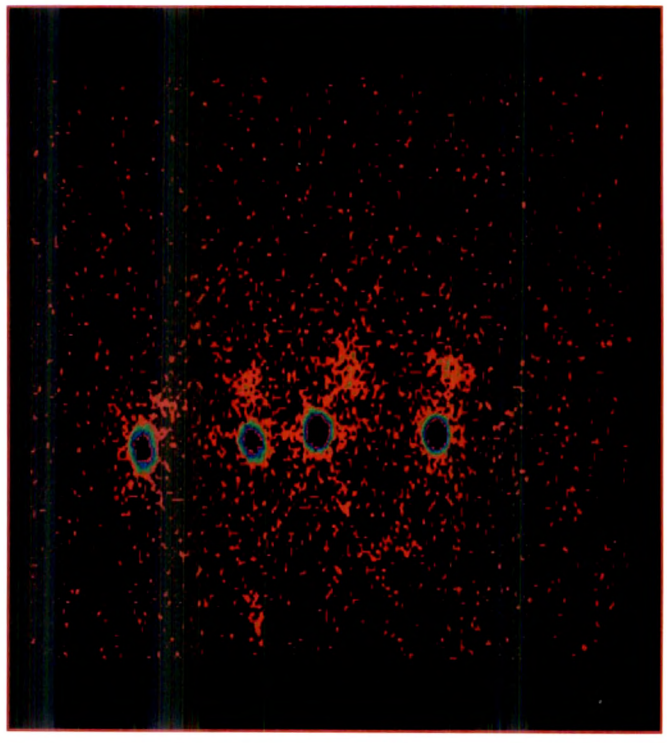


Fig. 3.4.8: Gamma camera image of rats after IA injection of **(5IV, 7aI-7bI, 7aIV)** after 24 h

From the obtained data and figures it is clear that all the synthesized CDS **(7aI-7bI, 7aIV)** showed high retention time in joints after IA administration as compared to the parent drug **(5IV)**. Further, radioactivity obtained for the synthesized CDS after 24 h was about 4 times higher in ROI compared to the parent drug. So it can be concluded that cationic molecules are retained for a longer period of time in joint cavity by ionic interaction and hence in the initial phase we can extend residence time of the drug by these CDS. Other representative derivatives from each CDS class have been evaluated and found to be similar in their retention behavior.

As per the proposed mechanism these CDS will deliver active drug slowly by hydrolysis of the ester or amide groups. Taking into consideration the rate of hydrolysis of the synthesized CDS in human serum it could be assumed that in the joint cavity the synthesized CDS will undergo rapid hydrolysis, as in inflammatory conditions level of various hydrolyzing enzymes is increased in joint cavity.

After hydrolysis the free drug can be slowly expelled from the joint and again reabsorbed in to joints. Further NSADs which have long half life coupled with joint affinity such as 6-MNA, BPA may have significant impact with respect to improved joint retention time when modified into quaternary derivatives.

4. Experimental

The experimental work has been divided into four parts:

4.1. Chemical studies

4.2. Hydrolyses kinetics

4.3. Cytotoxicity study (MTT assay)

4.4. Radiolabeling studies

4.1. Chemical studies

All the reagents and solvents required for syntheses were purified by general laboratory techniques before use. Melting points were determined using silicon oil bath type melting point apparatus (Veego) and are uncorrected. The completion of the reaction was monitored by thin layer chromatography (TLC) on silica gel pre-coated plates (60 F_{254}, Merck, 0.25 mm thickness), visualizing in ultraviolet light (254 nm) or iodine vapors. The yields reported here are un-optimized. Ultraviolet spectra (UV, λ_{max} in nm) were recorded on Shimadzu UV-1800 spectrophotometer. IR spectra (wave numbers in cm^{-1}) were recorded on a BRUKER ALPHA-T (Germany) FT-IR spectrophotometer using potassium bromide discs.

PMR spectra were recorded using Bruker Advance-II 400 MHz spectrometer in $CDCl_3$ or DMSO-d_6 solvents and expressed as δ ppm, coupling constant (*J*) has been expressed in Hz. Mass spectra were recorded using Thermo Fisher mass spectrometer using EI as ion source for all the compounds except pyridinium salts where ESI was used as ion source. HPLC analysis was performed using Shimadzu prominence system (Kyoto, Japan) consisting of LC-20AT Pump, and SPD 20 A detector. The chromatographic column used was phenomenex C-18, 250 mm length X 4.6 mm diameter with 0.5 μ particle size and Column temperature was maintained at 25-28 °C. Separation of analytes was performed under isocratic conditions at a flow-rate of 0.5-1.0 ml/min. The mobile phase consisted of phosphate buffer (PB, 15 mM) - acetonitrile.

Chromatographic separations were performed on columns using silica gel 100–200 mesh and neutral alumina, activity grade I. All reagents used were of analytical reagent grade obtained from S. d. fine chemicals, Spectrochem, Qualigens and Sigma-Aldrich.

4.1.1. Ethyl 3-pyridinecarboxylate (ethyl nicotinate) (2)

Nicotinic acid **(1)** (5 g, 40.65 mM) was refluxed with sulphuric acid (3.98 g, 40.65 mM) and absolute alcohol (3.73 g, 81.30 mM) for 10 h and the mixture was cooled to

room temperature and poured on to the crushed ice. The mixture was made alkaline by the addition of ammonia solution. The resulting mixture was extracted with ethyl acetate (3 x 20 ml). The solvent was dried and removed under vaccum to get the titled compound as an oil[1]. (5.5 g, 89.60 %).

Anal.:

TLC : R_f 0.74 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 217 nm, (log ε 3.99).

IR (Neat, cm^{-1}): 1717, 1588, 1276, 1107, 738 and 699

MS (m/z) : 151.07 (M^+)

4.1.2. *N*-(2-Hydroxyethyl)nicotinamide (4a)

A neat mixture of ethyl nicotinate **(2)** (5 g, 33.11 mM) and 2-aminoethanol **(3a)** (2.25 g 33.11 mM) was heated at 80-85 °C overnight. As the mixture was cooled, it solidified into a low melting white solid which was crystallized from isopropanol to get a hygroscopic white solid. (4.8 g, 87.32 %,). m.p. 88-90 °C (lit.[2] m.p. 88.5-89.5 °C)

Anal.:

TLC : R_f 0.69 (Methanol)

UV (MeOH) : 256.5 nm, (log ε 4.45).

IR (KBr, cm^{-1}): 3270, 1635, 1594, 1545, 1310, 1064, and 1027

PMR ($CDCl_3$): 9.07 (s, 1H, pyridine-*H*), 8.67-8.65 (d, 1H, pyridine-*H*), 8.45-8.43 (t, 1H, N*H*), 8.22-8.19 (d, 1H, pyridine-*H*), 7.41-7.37 (m, 1H, pyridine-*H*), 4.74 (s, 1H, O*H*), 3.70-3.68 (t, 2H, O-CH_2) and 3.52-3.47 (m, 2H, N-CH_2)

MS (m/z) : 167.10 (M+1)

4.1.3. *N*-(2-Hydroxypropyl)nicotinamide (4b)

The title compound was prepared from ethyl nicotinate **(2)** (5 g, 33.11 mM) and 1-amino-2-propanol **(3b)** (3.22 g, 43.0 mM). The neat mixture was heated and stirred at 80-85 °C for overnight. Excess of aminoalcohol was removed under vaccum to get a yellow oil of the product[2] (5.80 g, 97.31 %)

Anal.:

TLC : R_f 0.53 (Ethyl acetate)

UV (MeOH) : 240 nm, (log ε 3.56).

IR (Neat, cm^{-1}): 3285, 3074, 1639, 1593, 1301, 1122 and 702.

PMR ($CDCl_3$) : 9.0 (s, 1H, pyridine-*H*), 8.58-8.55 (d, 1H, pyridine-*H*), 8.18-8.12 (m, 2H, pyridine-*H*, N*H*), 7.33-7.29 (m, 1H, Pyridine-*H*), 4.05-3.99 (m, 1H, O-C*H*), 3.65 (s, 1H, O-*H*), 3.58-3.25 (m, 2H, N-CH_2), and 1.19-1.18 (d, 3H, CH_3)

MS (m/z) : 181.13 (M+1)

4.1.4. *N*-(1-Hydroxybutan-2-yl)nicotinamide (4c)

The title compound was prepared from ethyl nicotinate **(2)** (5.0 g, 33.11 mM) and 2-amino-1-butanol **(3c)** (3.83 g, 43.04 mM) following the method described for the synthesis of compound **(4b)** to give yellow oil of the desired product **(4c)** (6 g, 93.45 %).

Anal.:

TLC : R_f 0.48 (Methanol)

UV (MeOH) : 237 nm, (log ε 4.1).

IR (Neat, cm^{-1}): 3278, 1638, 1540, 1302 and 703

PMR ($CDCl_3$) : 9.07 (s, 1H, pyridine-*H*), 8.66-8.65 (d, 1H, pyridine-*H*), 8.22-8.19 (d, 1H, pyridine-*H*), 8.01-7.99 (d, 1H, N*H*), 7.40-7.36 (m, 1H, pyridine-*H*), 4.04 (bs, 1H, N-C*H*), 3.63-3.62 (d, 2H, O-CH_2), 3.55 (s, 1H, O-*H*), 1.74-1.53(m, 2H, -CH_2) and 0.97-0.94 (t, 3H, -CH_3)

MS (m/z) : 195.1 (M+1)

4.1.5. *N*-(1-Hydroxy-2-methylpropan-2-yl)nicotinamide (4d)

The title compound was prepared from ethyl nicotinate **(2)** (5.0 g, 33.11 mM) and 2-amino-2-methyl-1-propanol **(3d)** (3.83 g, 43.04 mM) following the method described for the synthesis of compound **(4b)** to give yellow oil of the desired product **(4g)** (5.90 g, 91.85 %).

Anal.:

TLC : R_f 0.55 (Methanol)

UV (MeOH) : 262 nm, (log ε 3.91)

IR (Neat, cm^{-1}): 3274, 3059, 1645, 1540, 1372 and 702

PMR ($CDCl_3$) : 8.90 (s, 1H, pyridine-*H*), 8.60-8.58 (d, 1H, pyridine-*H*), 8.07-8.04 (d, 1H, pyridine-*H*), 7.32-7.29 (m, 1H, Pyridine-*H*), 6.97(s,1H,N*H*), 4.62 (s, 1H, -OH), 3.66 (s, 2H, O-CH_2) and 1.41 (s, 6H,C(CH_3)$_2$)

MS (m/z) : 195.13 (M+1)

4.1.6. *N*-(3-Hydroxypropyl)nicotinamide (4e)

The title compound was prepared from ethyl nicotinate **(2)** (5.0 g, 33.11 mM) and 3-amino-1-propanol **(3e)** (2.73 g, 36.42 mM) following the method described for the synthesis of compound **(4b)** to give yellow oil of the desired product **(4e)** (5.60 g, 93.96 %).

Anal.:

TLC : R_f 0.39 (Methanol)

UV (MeOH) : 262 nm, (log ε 3.8)

IR (Neat, cm^{-1}): 3282, 1637, 1541, 1305 and 702

PMR ($CDCl_3$) : 9.0 (s, 1H, pyridine-*H*), 8.61-8.60 (d, 1H, pyridine-*H*), 8.45-8.43 (t, 1H, N*H*), 8.17-8.14 (d, 1H, pyridine-*H*),7.37-7.34 (m, 1H, pyridine-*H*), 4.44 (s, 1H, O-*H*), 3.72-3.69 (t, 2H, O-CH_2), 3.58-3.53 (m, 2H, N-CH_2) and 1.15-1.79 (m, 2H, -CH_2),

MS (m/z) : 180.1 (M+1)

4.1.7. *N*-(2-Aminoethyl)nicotinamide (4f)

In a 250 ml RBF fitted with dropping funnel, ethylenediamine **(3f)** was added and the contents were heated at 120 ^{0}C with vigorous stirring followed by slow addition of ethyl nicotinate **(2)** (5.0 g, 33.11 mM) in to the above solution over 30-45 min and the reaction continued for 10-12 h. Excess of ethylenediamine was removed under vaccum to give yellow oil of the desired product **(4f)** (4.1 g, 75.04 %) (Note: Product contained *N, N*-dinicotinoyl ethylenediamide (dimer) as an impurity in minor amounts and used further without purification).

Anal.:

TLC : R_f 0.47 (Ethyl acetate)

UV (MeOH) : 215 nm, (log ε 3.99)

IR (Neat, cm^{-1}): 3348, 3281, 3051, 1644, 1544, 1307, 825 and 706

PMR ($CDCl_3$) : 9.05 (s, 1H, pyridine-*H*), 8.65-8.63 (d, 1H, pyridine-*H*), 8.22 (bs, 1H, N*H*), 8.17-8.14 (m, 1H, pyridine-*H*), 7.36-7.32 (m, 1H, pyridine-*H*), 3.50-3.46 (m, 2H, N-CH_2), 2.93-2.90 (t, 2H, N-CH_2) and 2.70 (s, 2H, NH_2)

MS (m/z) : 166.0 (M+1)

4.1.8. *N*-(3-Aminopropyl)nicotinamide (4g)

The title compound was prepared from ethyl nicotinate **(2)** (5.0 g, 33.11 mM) and 1,3-diaminopropane **(3g)** (68.97 ml) following the method described for the synthesis of compound **(4f)** to give yellow oil of the desired product **(4g)** (5.1 g, 86.04 %).

Anal.:

TLC : R_f 0.41 (Ethyl acetate)

UV (MeOH) : 239 nm, (log ε 3.76)

IR (KBr, cm^{-1}): 3279, 3047, 1644, 1590, 1308 and 708

PMR ($CDCl_3$): 8.81 (s, 1H, pyridine-*H*), 8.35-8.33 (d, 1H, pyridine-*H*), 7.87-7.84 (d, 1H, pyridine-*H*), 7.05-7.02 (m, 1H, pyridine-*H*), 3.22-3.14 (m,1H, CON*H*), 2.52-2.49 (t, 2H, NH_2), 2.42-2.40 (t, 2H, N-CH_2), 1.54-1.39 (m, 2H, -CH_2) and 1.28-1.23 (m, 2H, -CH_2)

MS (m/z) : 180.02 (M+1)

4.1.9. 2-Nicotinamidoethyl 2-(4-isobutylphenyl)propanoate (6aI)

Ibuprofen **(5I)** (2.97 g, 14.45 mM) was dissolved in dichloromethane (DCM, 25 ml) and the reaction mixture was cooled to 0-3°C. Ethyl(dimethylaminopropyl) carobod-imide (EDC.HCl) (3.0 g, 15.66 mM) was added into the above reaction mixture and stirring continued for 10-15 min. Dimethylaminopyridine (DMAP) was added in catalytic amount (50 mg). In a separate conical flask the alcohol **(4a)** (2.0 g, 12.04 mM) was dissolved in anhydrous DCM (10 ml) and this solution was added dropwise with stirring to the previously chilled reaction mixture and stirring was continued till the reaction completed (12-18 h) at room temperature. Once the reaction was completed then the reaction mixture was diluted with dichloromethane and washed with chilled water (3 X 20 ml) followed by washing with sodium bicarbonate solution (10 %) (3 X 20 ml). Organic layer was separated, dried, and solvent removed under reduced pressure to afford the desired compound **(6aI)** as yellow oil. (3.1 g, 72.68 %)

Anal.:

TLC : R_f 0.65 (Ethyl acetate)

UV (MeOH) : 220 nm, (log ε 4.21)

IR (Neat, cm^{-1}): 3307, 1730, 1649, 1595, 1161 and 849

MS (m/z) : 354.18 (M^+)

4.1.10. 1-Nicotinamidopropan-2-yl 2-(4-isobutylphenyl)propanoate (6bI)

The title compound was prepared from ibuprofen **(5I)** (3.31 g, 16.01 mM), EDC. HCl (3.35 g, 17.55 mM) and the alcohol **(4b)** (2.63 g, 14.63 mM) following the method described for the synthesis of compound **(6aI)** to give yellow colored oil of the desired product **(6bI)** (3.50 g, 65.09 %).

Anal.:

TLC : R_f 0.68 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 219 nm, (log ε 3.34)

IR (Neat, cm^{-1}): 3308, 1728, 1651, 1536, 1164 and 733

MS (m/z) : 368.30 (M^+)

4.1.11. 2-Nicotinamidobutanyl 2-(4-isobutylphenyl)propanoate (6cI)

The title compound was prepared from ibuprofen **(5I)** (3.07 g, 13.55 mM), EDC. HCl (3.07 g, 14.91 mM) and the alcohol **(4c)** (2.630 g, 13.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellowish oil of the desired product **(6cI)** (3.80 g, 73.37 %).

Anal.:

TLC : R_f 0.65 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 220 nm, (log ε 3.20)

IR (Neat, cm^{-1}): 3303, 1730, 1647, 1535, 1161 and 733

MS (m/z) : 382.2 (M^+)

4.1.12. 2-Methyl-2-nicotinamidopropanyl 2-(4-isobutylphenyl)propanoate (6dI)

The title compound was prepared from ibuprofen **(5I)** (3.07 g, 13.55 mM), EDC. HCl (3.07 g, 14.91 mM) and the alcohol **(4d)** (2.63 g, 13.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil of the desired product **(6dI)** (3.70 g, 71.44 %).

Anal.:

TLC : R_f 0.63 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 220 nm, (log ε 3.90)

IR (Neat, cm^{-1}): 3307, 1730, 1658, 1522, 1162 and 734

MS (m/z) : 382.2 (M^+)

4.1.13. 3-Nicotinamidopropyl 2-(4-isobutylphenyl)propanoate (6eI)

The title compound was prepared from ibuprofen **(5I)** (3.61 g, 17.63 mM), EDC. HCl (3.62 g, 18.99 mM) and the alcohol **(4e)** (2.63 g, 14.61 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil of the desired product **(6eI)** (3.57 g, 66.39 %).

Anal.:

TLC : R_f 0.65 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 219 nm, (log ε 3.50)

IR (Neat, cm^{-1}): 3312, 1727, 1649, 1539, 1163 and 733

MS (m/z) : 368.2 (M^+)

4.1.14. *N*-[2-(2-(4-Isobutylphenyl)propanamido)ethyl]nicotinamide (6fI)

The title compound was prepared from ibuprofen **(5I)** (2.74 g, 13.33 mM), EDC. HCl (2.77 g, 14.54 mM) and the amine **(4f)** (2.0 g, 12.12 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil of the desired product **(6fI)** (3.10 g, 72.45 %).

Anal.:

TLC : R_f 0.70 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 220 nm, (log ε 4.01)

IR (Neat, cm^{-1}): 3327, 1627, 1534, 1346 and 1088

MS (m/z) : 353.20 (M^+)

4.1.15. *N*-[3-(2-(4-Isobutylphenyl)propanamido)propyl]nicotinamide (6gI)

The title compound was prepared from ibuprofen **(5I)** (2.53 g, 12.29 mM), EDC. HCl (2.56 g, 12.29 mM) and the amine **(4g)** (2.0 g, 11.17 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil of the desired product **(6gI)** (2.5 g, 60.96 %).

Anal.:

TLC : R_f 0.66 (Chloroform: Methanol, 1:0.3)

UV (MeOH) : 257, 262 nm, (log ε 4.10)

IR (Neat, cm^{-1}): 3306, 2956, 1640, 1594, 1550 and 709

MS (m/z) : 367.16 (M^+)

4.1.16. 2-Nicotinamidoethyl 2-(6-methoxy-2-naphthyl)propanoate (6aII)

The title compound was prepared from naproxen **(5II)** (1.52 g, 6.62 mM), EDC. HCl (1.38 g, 7.22 mM) and the alcohol **(4a)** (1.0 g, 6.02 mM) following the method described for the synthesis of compound **(6aI)** to give pink oil of the desired product[2] **(6aII)** (2.10 g, 92.22 %).

Anal.:

TLC : R_f 0.65 (Chloroform: Methanol 3 drop)

UV (MeOH) : 226 nm

IR (Neat, cm^{-1}): 3317.60, 3061, 2939, 1719.31, 1636.96, 1265 and 1168

4.1.17. 1-Nicotinamidopropan-2-yl 2-(6-methoxy-2-naphthyl)propanoate (6bII)

The title compound was prepared from naproxen **(5II)** (1.40 g, 6.11 mM), EDC. HCl (1.27 g, 6.66 mM) and the alcohol **(4b)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give oil of the desired product **(6bII)** (1.60 g, 73.46 %).

Anal.:

TLC : R_f 0.65 (Chloroform: Methanol 3 drop)

UV (MeOH) : 229 nm

IR (Neat, cm^{-1}): 3056, 1725, 1632, 1603, 1262 and 851

4.1.18. 2-Nicotinamidobutanyl 2-(6-methoxy-2-naphthyl)propanoate (6cII)

The title compound was prepared from naproxen **(5II)** (1.30 g, 5.67 mM), EDC. HCl (1.18 g, 6.18 mM) and the alcohol **(4c)** (1.0 g, 5.15 mM) following the method described for the synthesis of compound **(6aI)** to give white solid of the desired product **(6cII)** (1.30 g, 62.04 %). m.p. 98-100 °C

Anal.:

TLC : R_f 0.65 (Chloroform: Methanol 3 drop)

UV (MeOH) : 229 nm

IR (KBr, cm^{-1}): 3339, 1733, 1639, 1605, 1265 and 850

4.1.19. 2-Methyl-2-nicotinamidopropyl 2-(6-methoxy-2-naphthyl)propanoate (6dII)

The title compound was prepared from naproxen **(5II)** (1.30 g, 5.67 mM), EDC. HCl (1.18 g, 6.18 mM) and the alcohol **(4d)** (1.0 g, 5.15 mM) following the method

described for the synthesis of compound **(6aI)** to give white solid of the desired product **(6dII)** (1.50 g, 71.58 %). m.p. 102-104 °C

Anal.:

TLC	: R_f 0.65 (Chloroform: Methanol 3 drop)
UV (MeOH)	: 230 nm
IR (KBr, cm^{-1})	: 3310, 1634, 1262 and 1156

4.1.20. 3-Nicotinamidopropanyl 2-(6-methoxy-2-naphthyl)propanoate (6eII)

The title compound was prepared from naproxen **(6)** (1.40 g, 6.11 mM), EDC.HCl (1.27 g, 6.66 mM) and *N*-(3-hydroxypropyl)nicotinamide **(4e)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give white solid of the desired product[2] (1.30 g, 59.69 %) m.p. 108-111 °C (lit.[2] m.p. 100-102 °C)

Anal.:

TLC	: R_f 0.65 (Chloroform: Methanol 3 drop)
UV (MeOH)	: 230 nm
IR (KBr, cm^{-1})	: 3310, 1634, 1603, 1262 and 1174

4.1.21. *N*-[2-(2-(6-Methoxy-2-naphthyl)propanamido)ethyl]nicotinamide (6fII)

The title compound was prepared from naproxen **(6)** (1.53 g, 6.01 mM), EDC.HCl (1.38 g, 7.27 mM) and *N*-(2-aminoethyl)nicotinamide **(4f)** (1.0 g, 6.06 mM) following the method described for the synthesis of compound **(6aI)** to give yellow solid of the desired product (1.60 g, 69.94 %). m.p. 150-152 °C

Anal.:

TLC	: R_f 0.63 (Chloroform: Methanol 3 drop)
UV (MeOH)	: 230 nm
IR (KBr, cm^{-1})	: 3291, 1643, 1603, 1025 and 854

4.1.22. N-(3-Nicotinamidopropanyl)-2-(6-methoxy-2-naphthyl)propanamide (6gII)

The title compound was prepared from naproxen **(6)** (1.41 g, 6.14 mM), EDC.HCl (1.28 g, 6.70 mM) and *N*-(3-aminopropyl)nicotinamide **(4g)** (1.0 g, 5.58 mM) following the method described for the synthesis of compound **(6aI)** to give white solid of the desired product **(6gII)** (1.30 g, 59.44 %). m.p. 102-104 °C

Anal.:

TLC	: R_f 0.65 (Chloroform: Methanol 3 drop)

UV (MeOH) : 230 nm

IR (KBr, cm^{-1}) : 3310, 1638, 1213 and 851

4.1.23. 2-(Nicotinamido)ethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetate (6aIII)

The title compound was prepared from indomethacin **(5III)** (2.37 g, 6.62 mM), EDC.HCl (1.38 g, 7.22 mM) and the alcohol **(4a)** (1.0 g, 6.02 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil, which on crystallization by ethanol gave yellow solid and recrystallization from methanol to offered white solid of the desired product[2] **(6aIII)** (1.90 g, 62.33 %). m.p. 122-124 °C

Anal.:

TLC : R_f 0.72 (Chloroform: Methanol 3 drop)

UV (MeOH) : 222, 320 nm

IR (KBr, cm^{-1}): 3312, 1730, 1668, 1642, 1596 and 1024

4.1.24. 1-(Nicotinamido)propan-2-yl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methylindol)-3-acetate (6bIII)

The title compound was prepared from indomethacin **(5III)** (2.17 g, 6.11 mM), EDC. HCl (1.27 g, 7.22 mM) and the alcohol **(4b)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellow semisolid, which on crystallization by 2-propanol gave white solid of the desired product **(6bIII)** (1.70 g, 58.84 %). m.p. 112-114 °C

Anal.:

TLC : R_f 0.77 (Chloroform: Methanol 3 drop)

UV (MeOH) : 316 nm

IR (KBr, cm^{-1}): 3389, 1726, 1708, 1671, 1594, 753 and 705

4.1.25. 2-(Nicotinamido)butan-2-yl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methylindol)-3-acetate (6cIII)

The title compound was prepared from indomethacin **(5III)** (2.02 g, 5.67 mM), EDC. HCl (1.18 g, 6.18 mM) and the alcohol **(4c)** (1.0 g, 5.15 mM) following the method described for the synthesis of compound **(6aI)** to give yellow solid, which on crystallization by 2-propanol gave white solid of the desired product **(6cIII)** (0.90 g, 71.10 %). m.p. 157-159 °C

Anal.:

TLC : R_f 0.58 (Chloroform: Methanol 3 drop)

UV (MeOH) : 317 nm

IR (KBr, cm^{-1}): 3319, 1736, 1661, 1592, 1148, 853 and 753

4.1.26. 2-(Nicotinamido)-2-methylpropan-2-yl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methylindol)-3-acetate (6dIII)

The title compound was prepared from indomethacin **(5III)** (2.02 g, 5.67 mM), EDC. HCl (1.18 g, 6.18 mM) and the alcohol **(4d)** (1.0 g, 5.15 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil of the desired product **(6dIII)** (1.90 g, 69.02 %).

Anal.:

TLC : R_f 0.67 (Chloroform: Methanol 3 drop)

UV (MeOH) : 316 nm

IR (KBr, cm^{-1}): 3370, 1731, 1681, 1592 and 753

4.1.27. 3-Nicotinamidopropanyl 2-(1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetate (6eIII)

The title compound was prepared from indomethacin **(5III)** (2.18 g, 6.11 mM), EDC. HCl (1.27 g, 6.66 mM) and the alcohol **(4e)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil which on recrystallization by 2-propanol gave white solid of the desired product[2] **(6eIII)** (1.90 g, 65.77 %). m.p. 120-122 °C (lit.[2] m.p. 119-121 °C)

Anal.:

TLC : R_f 0.66 (Chloroform: Methanol 3 drop)

UV (MeOH) : 316 nm

IR (KBr, cm^{-1}) : 2967, 1731, 1677, 1591, 1311 and 753

4.1.28. N-(2-Nicotinamido)ethyl-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methylindol)-3-acetamide (6fIII)

The title compound was prepared from indomethacin **(5III)** (2.38 g, 6.66 mM), EDC.HCl (1.38 g, 7.27 mM) and amine **(4f)** (1.0 g, 6.06 mM) following the method described for the synthesis of compound **(6aI)** to offer yellow solid which on recrystall-

ization with 2-propanol-chloroform gave pure yellow solid of the desired product **(6fIII)** (1.70 g, 55.54 %). m.p. 195-196 °C

Anal.:

TLC : R_f 0.74 (Chloroform: Methanol 3 drop)

UV (MeOH) : 316 nm

IR (KBr, cm^{-1}) : 3286, 1673, 1593 and 753

4.1.29. 3-(Nicotinamido)propyl-2-(1-(4-chlorobenzoyl)-5-methoxy-2-methylindol)-3-acetamide (6gIII)

The title compound was prepared from indomethacin **(5III)** (2.19 g, 6.14 mM), EDC. HCl (1.28 g, 6.70 mM) and amine **(4g)** (1.0 g, 5.58 mM) following the method described for the synthesis of compound **(6aI)** to give yellow solid which was crystallized from methanol to yield pure yellow solid of the desired product **(6gIII)** (1.80 g, 62.08 %). m.p. 171-173 °C

Anal.:

TLC : R_f 0.75 (Chloroform: Methanol 3 drop)

UV (MeOH) : 316 nm

IR (KBr, cm^{-1}) : 3299, 1668, 1636, 1222 and 1151

4.1.30. 2-Nicotinamidoethyl 2-(6-methoxy-2-naphthyl)acetate (6aIV)

The title compound was prepared from 6-MNA **(5IV)** (1.43 g, 6.62 mM), EDC. HCl (1.38 g, 7.22 mM) and the alcohol **(4a)** (1.0 g, 6.02 mM) following the method described for the synthesis of compound **(6aI)** to yield yellow solid which on crystallization by ethyl acetate-methanol or chloroform-methanol offered white solid of the desired product **(6aIV)** (1.50 g, 68.40 %). m.p. 53-55 °C

Anal.:

TLC : R_f 0.53 (Chloroform: Methanol 3 drop)

UV (MeOH) : 229 nm

IR (KBr, cm^{-1}): 322, 1722, 1622, 1591, 1248 and 1029

4.1.31. 1-Nicotinamidopropan-2-yl 2-(6-methoxy-2-naphthyl)acetate (6bIV)

The title compound was prepared from 6-MNA **(5IV)** (1.31 g, 6.11 mM), EDC. HCl (1.27 g, 6.11 mM) and the alcohol **(4b)** (1.0 g, 5.55 mM) following the method

described for the synthesis of compound **(6aI)** to give yellow semisolid of the desired product **(6bIV)** (1.60 g, 76.10 %).

Anal.:

TLC : R_f 0.61 (Chloroform: Methanol 3 drop)

UV (MeOH) : 227 nm

IR (KBr, cm^{-1}): 3131, 1730, 1636, 1607, 1397 and 816

4.1.32. 2-Nicotinamidobutanyl 2-(6-methoxy-2-naphthyl)acetate (6cIV)

The title compound was prepared from 6-MNA **(5IV)** (1.31 g, 6.11 mM), EDC. HCl (1.27 g, 6.11 mM) and the alcohol **(4c)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellow semisolid which was further recrystallized from acetonitrile to get white solid of the desired product **(6cIV)** (1.70 g, 84.03 %). m.p. 69-71 ^{0}C

Anal.:

TLC : R_f 0.71 (Chloroform: Methanol 3 drop)

UV (MeOH) : 227 nm

IR (KBr, cm^{-1}): 3319, 1720, 1638, 1606, 1302, 852 and 745

4.1.33. 2-Methyl-2-nicotinamidopropanyl 2-(6-methoxy-2-naphthyl)acetate (6dIV)

The title compound was prepared from 6-MNA **(5IV)** (1.22 g, 5.67 mM), EDC. HCl (1.18 g, 6.18 mM) and the alcohol **(4d)** (1.0 g, 5.15 mM) following the method described for the synthesis of compound **(6aI)** to give yellow solid of the desired product **(6dIV)** (1.60 g, 79.18 %). m.p. 78-81 ^{0}C

Anal.:

TLC : R_f 0.68 (Chloroform: Methanol 3 drop)

UV (MeOH) : 227 nm

IR (KBr, cm^{-1}): 3341, 1735, 1637, 1606, 1316 and 853

4.1.34. 3-Nicotinamidopropanyl 2-(6-methoxy-2-naphthyl)acetate (6eIV)

The title compound was prepared from 6-MNA **(5IV)** (1.31 g, 6.11 mM), EDC. HCl (1.27 g, 6.11 mM) and the alcohol **(4e)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellow solid of the desired product **(6eIV)** (1.50 g, 71.42 %). m.p. 80-82 ^{0}C

Anal.:

TLC : R_f 0.68 (Chloroform: Methanol 3 drop)

UV (MeOH) : 227 nm

IR (KBr, cm^{-1}): 3285, 1729, 1630, 1607, 1268 and 853

4.1.35. 2-Nicotinamidoethyl 4-biphenylacetate (6aV)

The title compound was prepared from BPA **(5V)** (1.40 g, 6.62 mM), EDC (1.38 g, 7.22 mM) and the alcohol **(4a)** (1.0 g, 6.02 mM) following the method described for the synthesis of compound **(6aI)** to give yellow solid, which on crystallization by chloroform-methanol offered white solid of the desired product **(6aV)** (1.80 g, 82.50 %). m.p.111-113 ^oC

Anal.:

TLC : R_f 0.62 (Chloroform: Methanol 3 drop)

UV (MeOH) : 251 nm

IR (KBr, cm^{-1}): 3299, 1727, 1638, 1544, 1166 and 822

4.1.36. 1-Nicotinamidopropan-2-yl 4-biphenylacetate (6bV)

The title compound was prepared from BPA **(5V)** (1.29 g, 6.11 mM), EDC. HCl (1.27 g, 6.66 mM) and the alcohol **(4b)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give yellow oil of the desired product **(6bV)** (1.30 g, 62.49 %).

Anal.:

TLC : R_f 0.71 (Chloroform: Methanol 3 drop)

UV (MeOH) : 251 nm

IR (Neat, cm^{-1}): 3285, 1727, 1630, 1590 and 742

4.1.37. 2-Nicotinamidobutyl 4-biphenylacetate (6cV)

The title compound was prepared from BPA **(5V)** (1.20 g, 5.67 mM), EDC. HCl (1.18 g, 6.18 mM) and the alcohol **(4c)** (1.0 g, 5.15 mM) following the method described for the synthesis of compound **(6aI)** to give white solid of the desired product **(6cV)** (1.30 g, 64.93 %). m.p. 110-112 ^0C

Anal.:

TLC : R_f 0.67 (Chloroform: Methanol 3 drop)

UV (MeOH) : 251 nm

IR (KBr, cm^{-1}): 3305, 1717, 1638, 1590 and 748

4.1.38. 2-Methyl-2-nicotinamidopropyl 4-biphenylacetate (6dV)

The title compound was prepared from BPA **(5V)** (1.20 g, 5.67 mM), EDC (1.18 g, 6.18 mM) and the alcohol **(4d)** (1.0 g, 5.15 mM) following the method described for the synthesis of compound **(6aI)** to gave yellow oil of the desired product **(6dV)** (1.40 g, 69.92 %).

Anal.:

TLC : R_f 0.64 (Chloroform: Methanol 3 drop)

UV (MeOH) : 251 nm

IR (Neat, cm^{-1}): 3029, 1730, 1651, 1590 and 741

4.1.39. 3-Nicotinamidopropyl 4-biphenylacetate (6eV)

The title compound was prepared from BPA **(5V)** (1.29 g, 6.11 mM), EDC. HCl (1.27 g, 6.66 mM) and the alcohol **(4e)** (1.0 g, 5.55 mM) following the method described for the synthesis of compound **(6aI)** to give white solid of the desired product **(6eV)** (1.10 g, 52.87 %). m.p. 129-131 °C

Anal.:

TLC : R_f 0.53 (Chloroform: Methanol 3 drop)

UV (MeOH) : 251 nm

IR (KBr, cm^{-1}): 3285, 1727, 1630, 1590 and 742

4.1.40. 2-(1-Methylpyridinium-3-carboxamido)ethyl 2-(4-isobutylphenyl)propanoate iodide (7aI)

A reaction mixture containing ester **(6aI)** (2.5 g, 7.06 mM) and methyl iodide (2 g, 14.12 mM) in acetonitrile (20 ml) was refluxed for 2-3 h and the solvent and excess reagent removed under reduced pressure to afford yellow semisolid which on crystallization in ethyl acetate gave white solid of the desired product[17] **(7aI)** (3.1 g, 88.64 %). m.p. 148-150 °C (lit[17] m.p. 144-146 °C).

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop glacial acetic acid (GAA))

UV (MeOH) : 219 nm, (log ε 4.52)

IR (KBr, cm^{-1}): 3272, 1730, 1679, 1205 and 1174

PMR ($CDCl_3$): 9.80 (s, 1H, pyridinium-*H*), 8.89-8.87 (d, 2H, pyridinium-*H*), 8.00 -7.97 (m,1H, pyridinium-*H*), 8.72-8.69(m,1H, N*H*),7.15-7.13 (d, 2H , Ar-H_2), 6.99-6.97 (d, 2H,Ar-H_2), 4.50 (s, 3H, N-CH_3), 4.35-4.12

(m, 2H,-OCH_2), 3.79-3.74 (q, 1H,-CH), 3.68-3.60 (m, 2H,-CH_2), 2.33-2.31 (d, 2H,-CH_2), 1.77-1.70 (m, 1H, -CH), 1.43-1.41 (d, 3H -CH_3) and 0.81-0.79 (d, 6H, (CH_3)$_2$)

^{13}CMR ($CDCl_3$):175.0, 161.35, 146.82, 144.76, 144.76, 140.51, 137.45, 133.93, 129.29, 128.23, 127.32, 62.40, 49.42, 44.92, 44.89, 38.95, 30.15, 22.37 and 18.65

MS (m/z) : 369.2 (M^+)

HPLC : > 99.10 %

4.1.41. 1-(1-Methylpyridinium-3-carboxamido)propan-2yl 2-(4-isobutylphenyl) propanoate iodide (7bI)

The title compound was prepared from ester **(6bI)** (2.5 g, 6.79 mM) and methyl iodide (2.89 g, 20.35 mM) following the method described for the synthesis of compound **(7aI)** to give yellow oil of the desired product **(7bI)** (2.80 g, 80.82 %)

Anal.:

TLC : R_f 0.32 (Methanol: 1 drop GAA)

UV (MeOH) : 219 nm, (log ε 3.6)

IR (KBr, cm^{-1}): 3260, 1725, 1669, 1166 and 847

PMR ($CDCl_3$) :9.80 (s, 1H, pyridinium-H), 8.89-8.87 (d, 2H, pyridinium-H), 8.72-8.69 (m, 1H, NH), 8.00-7.97(m, 1H, pyridinium-H),7.15-7.13 (d,2H, Ar-CH_2), 6.99-6.97 (d, 2H, Ar-CH_2), 4.50 (s, 3H, N-CH_3),4.35-4.12 (m, 2H,-OCH_2), 3.79-3.74 (q, 1H,-CH), 3.68-3.60 (m,2H,-CH_2), 2.33-2.31 (d, 2H, -CH_2), 1.77-1.70 (m, 1H,-CH), 1.43-1.41 (d, 3H,-CH_3) and 0.81-0.79 (d, 6H, -(CH_3)$_2$).

MS (m/z) : 383.2(M^+)

HPLC : > 99.40 %

4.1.42. 2-(1-Methylpyridinium-3-carboxamido)butanyl 2-(4-isobutylphenyl) propanoate iodide (7cI)

The title compound was prepared from ester **(6cI)** (2.5 g, 6.54 mM) and methyl iodide (2.78 g, 19.57 mM) following the method described for the synthesis of compound **(7aI)** to give yellow oil of the desired product **(7cI)** (2.90 g, 84.56 %).

Anal.:

TLC : R_f 0.29 (Methanol: 1 drop GAA)

UV (MeOH) : 220 nm, (log ε 4.3)

IR (KBr, cm^{-1}): 3238, 1729, 1668, 1163 and 734

PMR ($CDCl_3$):9.80 (s, 1H, pyridinium-*H*), 9.06 (s, 1H, N*H*), 8.92-8.90 (d,1H,pyridinium-*H*, *J=8.12*), 8.45-8.43(d, 1H, pyridinium-*H*, *J=8.0*), 8.06 -8.03 (m, 1H, pyridinium-*H*, *J=6.96*), 7.20-7.01 (dd,4H, phenyl-*H*,*J=8.04,8.04*), 4.58 (s, 3H, N-CH_3), 4.34-4.29 (m,2H, -CH_2), 4.22-4.19(m, 1H, N-C*H*), 3.80-3.65 (q, 1H, Ar-C*H*),1.82-1.73 (m, 2H,-CH_2),1.72-1.67 (m, 1H,-C*H*), 1.49-1.44 (d, 3H,-CH_3), 0.96-0.93 (t, 3H,-CH_3) and 0.89-0.87 (d, 6H, (CH_3)$_2$)

MS (m/z) : 397.2 (M^+)

HPLC : > 98.89 %

4.1.43. 2-(1-Methylpyridinium-3-carboxamido)-2-methylpropanyl 2-(4-isobutyl phenyl)propanoate iodide (7dI)

The title compound was prepared from ester **(7dI)** (2.5 g, 6.54 mM) and methyl iodide (2.78 g, 19.57 mM) following the method described for the synthesis of compound **(7aI)** to give yellow oil of the desired product **(7dI)** (3.10 g, 90.40 %).

Anal.:

TLC : R_f 0.24 (Methanol: 1 drop GAA)

UV (MeOH) : 220 nm, (log ε 3.5)

IR (KBr, cm^{-1}): 3244, 1729, 1670, 1163, and 746

PMR ($CDCl_3$): 9.59 (s, 1H, pyridinium-*H*), 9.08-9.06 (d, 1H, pyridinium-*H*,*J=6.08*), 8.76-8.74 (d, 1H, pyridinium-*H*, *J=8.08*), 8.05-8.01 (t,1H, pyridinium-*H*, *J=6.12*), 7.97 (bs, 1H, N*H*), 7.21-7.03 (dd,4H, phenyl-*H*, *J=8.06*), 4.57 (s, 3H, N-CH_3), 4.33 (s, 2H, O-CH_2), 3.80-3.78 (q, 1H, Ar-C*H*), 2.40-2.38 (d, 2H, -CH_2), 1.82-1.78 (m, 1H, -C*H*), 1.50-1.48 (d, 3H, -CH_3), 1.46 (s, 6H, (CH_3)$_2$) and 0.90-0.85 (s, 6H, (-CH_3)$_2$)

^{13}CMR ($CDCl_3$):174.81, 61.22, 161.48-127.09, 68.53, 60.66, 55.46, 50.32, 49.45, 45.04, 30.14, 24.21, 22.36-18.31 and 14.10.

MS (m/z) : 397.25 (M^+)

HPLC : > 99.55 %

4.1.44. 3-(1-Methylpyridinium-3-carboxamido)propanyl 2-(4-isobutylphenyl)propa noate iodide (7eI)

The title compound was prepared from ester **(6eI)** (2.5 g, 6.54 mM) and methyl iodide (2.89 g, 19.62 mM) following the method described for the synthesis of compound **(7aI)** to give yellow oil of the desired product **(7eI)** (2.67 g, 77.06 %).

Anal.:

TLC : R_f 0.35 (Methanol: 1drop glacial acetic acid)

UV (MeOH) : 219 nm, (log ε 4.10)

IR (KBr, cm^{-1}): 3230, 2955, 1725, 1669, 1163 and 735

PMR ($CDCl_3$):10.10 (s, 1H, pyridinium-*H*), 9.02-9.0 (d, 1H, pyridinium-*H*,*J=8.08*), 8.90-8.87 (m, 1H, N*H*), 8.85-8.84 (d, 1H, pyridinium-*H*,*J=6.08*), 8.06-8.03 (t, 1H, pyridinium-*H*, *J=6.16*), 7.21-7.07 (dd,4H, phenyl-*H*, *J=8.08, 8.08*), 4.59 (s, 1H, N-C*H*), 4.25-4.13 (m, 2H, -CH_2), 3.76-3.70 (q, 1H, -C*H*), 3.54-3.49 (t, 2H, -CH_2), 3.22 (s, 1H, N*H*), 2.43-2.42 (d, 2H, Ar-CH_2), 2.08-2.02 (m, 2H, -CH_2),1.86-1.80 (m, 1H, C*H*), 1.53-1.44 (d, 3H, CH_3) and 0.90-0.87 (d, 6H, CH_3)$_2$)

MS (m/z) : 383.2 (M^+)

HPLC : > 99.55 %

4.1.45. *N*-[2-(2-(4-Isobutylphenyl)propanamido)ethyl]-1-methyl-3-carboxamido pyridinium iodide (7fI)

The title compound was prepared from amide intermediate **(6fI)** (2.0 g, 5.66 mM) and methyl iodide (2.41 g, 16.99 mM) following the method described for the synthesis of compound **(7aI)** to give yellow oil of the desired product **(7fI)** (2.30 g, 82.01 %).

Anal.:

TLC : R_f 0.32 (Methanol: 1drop glacial acetic acid)

UV (MeOH) : 219 nm, (log ε 3.15)

IR (KBr, cm^{-1}): 3337, 1645, 1548, 1236 and 671

PMR ($CDCl_3$) : 9.64 (s, 1H, Ar-*H*), 9.11-9.12 (d, 1H, Ar-*H*), 8.83-8.88 (t, 2H, Ar-*H*, N*H*), 8.02-8.05 (t, 1H, Ar-C*H*), 7.19-7.21(d, 2H, Ar-CH_2), 6.93-6.95 (d, 2H, Ar-CH_2), 4.50 (s, 3H,-CH_3), 3.64-3.66 (q, 1H,-CH_2), 3.43-3.50 (q, 3H,-CH_2, -N*H*), 3.33-3.36 (q, 1H,-CH_2), 2.29-2.31 (d,

2H,-C*H*$_2$), 1.71-1.74 (m, 1H, -C*H*), 1.37-1.38 (d, 3H,-C*H*$_3$) and 0.79-0.80 (d, 6H, (C*H*$_3$)$_2$)

^{13}CMR ($CDCl_3$): 174.32, 1160.97, 146.73, 145.25, 142.94, 139.13, 139.0, 133.61, 128.53, 127.34, 126.79, 48.37, 45.0, 44.32, 39.86, 39.02, 37.92, 29.65, 22.03 and 18.37

MS (m/z) : 368.1 (M^+)

HPLC : > 99.19 %

4.1.46. *N*-[3-(2-(4-Isobutylphenyl)propanamido)propyl]-1-methyl-3-carboxamido pyridinium iodide (7gI)

The title compound was prepared from amide intermediate **(6gI)** (2.0 g, ,5.44 mM) and methyl iodide (2.32 g, 16.34 mM) following the method described for the synthesis of compound **(7aI)** to give yellow semisolid of the desired product **(7gI)** (2.20 g, 79.31 %).

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 220 nm, (log ε 3.90)

IR (KBr, cm^{-1}): 3268, 3050, 1658, 1510 and 850

PMR ($CDCl_3$) : 9.84 (s, 1H, pyridinium-*H*), 9.14-9.12 (d, 1H, pyridinium-*H*, *J=6.08*), 9.03-9.00 (d, 1H, pyridinium -*H*, *J=8.12*), 8.81-8.78 (d,1H, pyridinium-*H*, *J=5.84*), 8.12-8.08 (t, 1H, N*H*), 7.27-7.06 (dd,4H, phenyl-*H*, *J=8.08*), 6.69-6.66 (t, 1H, N*H*), 4.60 (s, 3H, N-C*H*$_3$), 4.12-4.09 (q, 1H, Ar-C*H*), 3.75-3.67 (q, 2H, -C*H*$_2$), 3.46-3.34 (m, 2H, -C*H*$_2$), 2.43-2.40 (d, 3H, -C*H*$_3$), 1.85-1.77 (m, 2H,-C*H*$_2$), 1.49-1.44 (d, 2H, Ar-C*H*$_2$), 1.27-1.21 (m, 1H, C*H*) and 0.89-0.86 (d, 6H, (C*H*$_3$)$_2$).

^{13}CMR ($CDCl_3$):175.49, 161.17, 154.99, 146.85, 144.87, 140.98, 138.66, 137.65, 134.20, 130.01, 129.88, 128.19, 127.30, 53.77, 49.56, 46.32, 44.94, 37.12, 30.15, 28.64, 22.38, 18.56 and 14.67

MS (m/z) : 382.2 (M^+)

HPLC : > 99.19 %

4.1.47. 2-(1-Methylpyridinium-3-carboxamido)ethyl 2-(6-methoxy-2-naphthyl) propionate iodide (7aII)

A reaction mixture containing ester **(6aII)** (1.0 g, 2.64 mM) and methyl iodide (1.11 g, 7.92 mM) in acetonitrile/acetone (20 ml) were refluxed for 2-3 h. Solvent and the excess reagent were removed under reduced pressure to afford yellow semisolid which on crystallization in ethyl acetate gave light yellow solid of the desired product[2] which was found to be hygroscopic in nature **(7aII)** (1.1 g, 79.91 %) m.p. 168-170 °C. (lit.[2] m.p. 169-170)

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 226, 266 nm

IR (KBr, cm^{-1}): 3284, 1721, 1671, 1602 and 1175

PMR ($CDCl_3$): 9.34 (s, 1H, pyridinium-*H*), 9.15-9.13 (d, 1H, pyridinium-*H*, *J=8.0*), 9.04-9.02 (m, 1H, pyridinium-*H*, *J=8.0*), 8.74-8.72 (d,1H, pyridinium-*H*, *J=8.0*), 8.09-8.03 (m, 1H, N*H*), 7.66-7.07 (m,6H, naphthalene-*H*), 4.44 (s, 3H, N-C*H*$_3$), 4.28-4.25 (t, 2H,-C*H*$_2$),3.91-3.86 (q, 1H, C*H*), 3.67-3.56 (m, 2H, N-C*H*$_2$), 3.33 (s, 3H,O-C*H*$_3$) and 1.52-1.51 (d, 3H, -C*H*$_3$)

^{13}CMR ($CDCl_3$):172.73, 160.01, 155.85, 145.75, 143.94, 141.18, 134.28, 131.94, 127.82, 126.16, 124.97, 117.41, 104.33, 61.09, 54.14, 47.23, 43.29, 39.06, 38.85, 38.44, 38.02, 37.33 and 17.20

MS (m/z) : 393.40 (M^+)

HPLC : > 99.10 %

4.1.48. 1-(1-Methylpyridinium-3-carboxamido)-2-propanyl 2-(6-methoxy-2-naphthyl) propanoate iodide (7bII)

The title compound was prepared from ester **(6bII)** (1.0 g, 2.54 mM) and methyl iodide (1.07 g, 7.64 mM) following the method described for the synthesis of compound **(7aII)** to give yellow solid of the desired product **(7bII)** (0.90 g, 66.17 %). 188-190 °C

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (H_2O) : 228 nm

IR (KBr, cm^{-1}): 3349, 1721, 1666, 1602, 1187 and 892

PMR ($CDCl_3$): 9.08 (s, 1H, pyridinium-*H*), 9.02-9.01(d, 1H, pyridinium-*H*, *J=4.0*) 8.81 (bs, 1H, N*H*), 8.45-8.43 (d, 1H, pyridinium-*H*,*J=4.0*), 7.83-7.79 (t, 1H, pyridinium-*H*, *J*=4.0), 7.59-7.05 (m,6H, naphthalene-

H), 5.16-5.13 (m, 1H, OC*H*, *J*=2.92), 4.35 (s, 3H, N-CH_3), 3.92 (s, 3H, O-CH_3), 3.84-3.82 (q, 1H, Ar-C*H*,*J*=8.0), 3.37-3.33 (t, 2H, N-CH_2), 1.50-1.49 (d, 3H,-CH_3, *J*=7.2) and 1.33-1.32 (d, 3H, -CH_3, *J*=6.28)

MS (m/z) : 407.41(M^+)

HPLC : > 98.69 %

4.1.49. 2-(1-Methylpyridinium-3-carboxamido)butanyl 2-(6-methoxy-2-naphthyl) propanoate iodide (7cII)

The title compound was prepared from ester **(6cII)** (1.0 g, 2.46 mM) and methyl iodide (1.04 g, 7.38 mM) following the method described for the synthesis of compound **(7aII)** to give yellow oil of the desired product **(7cII)** (0.88 g, 65.23 %).

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (H_2O) : 228 nm

IR (KBr, cm^{-1}): 2971, 1727, 1668, 1604, 1239 and 854

PMR ($CDCl_3$) : 9.23 (s, 1H, pyridinium-*H*), 8.94-8.87 (dd, 1H, pyridinium-*H*, *J=8.0,8.0*), 8.61-8.57 (m, 1H, pyridinium-*H*, *J=8.0*), 8.18-8.13 (dd,1H, pyridinium-*H*, *J=8.0*), 7.85-7.75 (m, 1H, N*H*), 7.61-6.97 (m, 6H, naphthalene-*H*), 4.40 (s, 3H, N-CH_3), 4.34 (s, 3H, O-CH_3), 3.91-3.88 (d, 4H, (CH_2)$_2$, *J*=4.0), 1.74-1.65 (m, 1H, N-C*H*, *J=4.0*), 1.54-1.51 (d, 3H, -CH_3, *J*=1.76), 1.27-1.23 (q,1H,-C*H*) and 0.95-0.90 (t, 3H, -CH_3, *J= 4.0*)

^{13}CMR ($CDCl_3$): 174.95, 174.62, 171.21, 161.09, 157.52, 146.11, 145.94, 144.12-105.44, 65.78 60.40 55.71 51.62 49.12 45.07, 24.19 and 10.66

MS (m/z) : 421.2 (M^+)

HPLC : > 99.58 %

4.1.50. 2-Methyl-2-(1-methylpyridinium-3-carboxamido)propanyl 2-(6-methoxy-2-naphthyl)propanoate iodide (7dII)

The title compound was prepared from ester **(6dII)** (1.0 g, 2.46 mM) and methyl iodide (1.04 g, 7.38 mM) following the method described for the synthesis of compound **(7aII)** to give yellow colored hygroscopic solid of the desired product **(7dII)** (0.95 g, 70.42 %).

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (H_2O) : 228 nm

IR (KBr, cm^{-1}): 3448, 1730, 1658, 1605, 1372 and 854

PMR ($CDCl_3$): 9.29 (s, 1H, pyridinium-*H*), 9.11-9.10 (d, 1H, pyridinium-*H*,*J=8.0*), 8.65-8.63 (d, 1H, pyridinium-*H*, *J=8.0*), 8.28 (s, 1H,N*H*), 8.05-8.01 (m, 1H, pyridinium-*H*, *J=8.0*), 7.66-7.07 (m, 6H,naphthalene-*H*), 4.40 (s, 3H, N-CH_3), 3.90 (s, 3H, O-CH_3), 3.37-3.34(m, 1H, C*H*-CO), 3.02 (s, 2H, O-CH_2), 1.51-1.48 (d, 3H, C-CH_3, *J=4.0*) and 1.38-1.35 (d, 6H, C(CH_3)$_2$)

HPLC : > 99.58 %

4.1.51. 3-(1-Methylpyridinium-3-carboxamido)propanyl 2-(6-methoxy-2-naphthyl)propanoate iodide (7eII)

The title compound was prepared from ester **(6eII)** (0.5 g, 1.27 mM) and methyl iodide (0.53 g, 3.82 mM) following the method described for the synthesis of compound **(7aII)** to give a white solid of the desired product[2] **(7eII)** (0.55 g, 80.62 %). m.p. 98-101°C (lit.[2] m.p. 100-102°C)

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (H_2O) : 228, 264 nm

IR (KBr, cm^{-1}): 3470, 1716, 1667, 1599 and 1266

PMR ($CDCl_3$): 9.44 (s, 1H, Pyridinium-*H*), 9.15-9.13 (d, 1H, pyridinium-*H*,*J=8.0*), 9.06-9.03 (m, 1H, pyridinium-*H*, *J=8.0*), 8.91-8.89 (d, 1H,pyridinium-*H*, *J=8.0*), 8.23-8.19 (t, 1H, N*H*), 7.76-7.11 (m, 6H,naphthalene-*H*), 4.44 (s, 3H, N-CH_3), 4.15-4.11 (q, 2H, N-CH_2),3.91-3.89 (q, 1H, -COC*H*, *J*=7.12), 3.88 (s, 3H, O-CH_3), 3.40-3.35 (t, 2H, O-CH_2, *J*=6.6), 1.89-1.85 (m, 2H, -CH_2, *J*=6.6) and 1.52-1.50 (d, 3H, -CH_3, *J*=7.12)

^{13}CMR ($CDCl_3$):173.74, 160.65, 157.11-135.33, 133.58-105.19 (10C, Ar), 61.85, 54.90, 48.23, 44.65, 39.84, 27.87 and 18.28

MS (m/z) : 407.8 (M^+)

HPLC : > 99.58 %

4.1.52. *N*-[2-(2-(6-Methoxy-2-napthhyl)propanamido)ethyl]-1-methyl-3-carboxamido pyridinium iodide (7fII)

The title compound was prepared from amide intermediate **(6fII)** (1.0 g, 2.64 mM) and methyl iodide (1.83 g, 7.94 mM) following the method described for the synthesis of compound **(7aII)** to give yellow oil of the desired product **(7fII)** (0.78 g, 56.69 %).

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (H_2O) : 228 nm

IR (KBr, cm^{-1}) : 3393, 1665, 1603, 1210 and 857

PMR ($CDCl_3$): 9.17 (s, 1H, pyridinium-*H*), 9.01-8.99 (d, 1H, pyridinium-*H*,*J=8.0*), 8.88-8.85 (m, 1H, pyridinium-*H*, *J=8.0*), 8.61-8.59 (d,1H, pyridinium-*H*, *J=8.0*), 8.10-8.07 (m, 1H, N*H*), 7.99-7.94 (m,1H, N*H*), 7.77-7.07 (m, 6H, naphthaene-*H*), 4.36 (s, 3H, N-C*H*$_3$),3.89 (s, 3H, O-C*H*$_3$), 3.74-3.72 (q, 1H, -C*H*, *J=7.04*), 3.53-3.39(m, 2H, N-C*H*$_2$), 3.31-3.24 (t, 2H, N-C*H*$_2$) and 1.50-1.44 (d, 3H,-C*H*$_3$, *J=7.04*)

^{13}CMR ($CDCl_3$):174.11, 161.08, 157.20, 146.58, 144.97, 142.42, 137.08, 133.23, 128.98, 127.07, 126.38, 125.68, 118.77, 105.46, 55.12, 52.12, 48.19, 45.20, 39.78, 39.15, 37.84, 35.00 and 18.19

MS (m/z) : 391.8 (M^+)

HPLC : > 99.90 %

4.1.53. *N*-[3-(2-(6-Methoxy-2-naphthyl)propanamido)propanyl)-1-methyl-3-carboxamido pyridinium iodide (7gII)

The title compound was prepared from amide intermediate **(6gII)** (1.0 g, 2.55 mM) and methyl iodide (0.54 g, 7.92 mM) following the method described for the synthesis of compound **(7aII)** to give yellow oil which was further crystallized using ethyl acetate and methanol to give white solid of the desired product **(7gII)** (0.86 g, 62.99 %). m.p. 192-194 ^oC

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (H_2O) : 229 nm

IR (KBr, cm^{-1}): 3276, 1673, 1631, 1604, 1269 and 857

PMR ($CDCl_3$) :9.52 (s, 1H, pyridinium-*H*), 9.15-9.14 (d, 1H, pyridinium-*H*,*J=8.0*), 8.95-8.93 (d, 2H, pyridinium-*H*, N*H*, *J=8.0*), 8.14-8.10(m, 1H, pyridinium-*H*, *J=8.0*), 7.84 (m, 1H, N*H*), 7.70-7.65 (m,3H, Ar-*H*, 7,8, *J=8.0*),7.47-7.44 (d, 1H, Ar-*H*, *J=8.0*), 7.11-7.09 (d, 2H, Ar-*H*, *J=8.0*), 4.49 (s, 3H, N-CH_3), 3.90 (s, 3H, O-CH_3),3.79-3.77 (q, 1H, -C*H*, *J=6.96*), 3.38-3.37 (m, 2H, N-CH_2), 3.24-3.17 (m, 2H, N-CH_2), 1.78-1.75 (m, 2H, -*CH*$_2$, *J=6.36*) and 1.52-1.50(d, 3H,-CH_3, *J=6.96*)

^{13}CMR ($CDCl_3$): 173.78, 160.64, 156.89-137.17 (5C, pyridinium), 133.54-105.25 (10C, Ar), 54.92, 48.20, 45.27, 39.63, 37.29, 36.30 and 18.39

MS (m/z) : 406.6 (M^+)

HPLC : > 99.58 %

4.1.54. 2-(1-Methylpyridinium-3-carboxamido)ethyl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetate iodide (7aIII)

A reaction mixture containing ester intermediate **(6aIII)** (1.0 g, 1.97 mM) and methyl iodide (0.83 g, 5.92 mM) in acetonitrile/acetone (20 ml) was refluxed for 2-3 h. The solvent and the excess reagent were removed under reduced pressure to afford yellow solid which on crystallization in ethanol and small amount of ether gave light yellow solid of the desired product[2] **(7aIII)** (0.8 g, 62.48 %) m.p. 176-179 °C (lit.[2] m.p. 178-179 °C)

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 220, 320 nm

IR (KBr, cm^{-1}): 3276, 3040, 2949, 1728, 1683, 1662, 1597, 1228 and 1173

PMR ($CDCl_3$): 9.41 (s, 1H, pyridinium-*H*), 9.18-9.16 (d, 1H, pyridinium-*H*,*J=8.0*), 9.08 (m, 1H, pyridinium-*H*, *J=8.0*), 8.81-8.79 (d, 1H,pyridinium-*H*, *J=8.0*), 8.15-8.11 (t, 1H, N*H*), 7.69-7.55 (d, 2H,phenyl-*H*, *J=8.0*), 7.57-7.55 (d, 2H, phenyl-*H*, *J=8.0*), 6.95-6.56 (m, 3H, indol-*H*), 4.49 (s, 3H, N-CH_3), 4.31-4.28 (t, 2H, O-CH_2), 3.77-3.74 (m, 3H, O-CH_3), 3.71 (s, 2H, CO-CH_2), 3.67-3.63 (m, 2H, N-CH_2), 2.25 (s, 3H, Ar-CH_3)

^{13}CMR ($CDCl_3$): 170.17, 167.63, 160.93, 155.32, 146.84, 145.19, 142.58,138.16, 135.25, 133.61, 130.88, 130.07, 128.80, 127.30, 114.26, 112.21,

111.03, 101.27, 62.31, 55.33, 48.29, 40.25, 40.04, 39.83, 39.20, 38.55, 29.59 and 13.20

MS (m/z) : 520.60 (M^+)

HPLC : > 99.31 %

4.1.55. 1-(1-Methylpyridinium-3-carboxamido)propan-2-yl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetate iodide (7bIII)

The title compound was prepared from ester intermediate **(6bIII)** (1.0 g, 1.92 mM) and methyl iodide (0.81 g, 5.76 mM) following the method described for the synthesis of compound **(7aIII)** to give yellow solid. The product was purified by column chromatography using neutral alumina as stationary phase and ethyl acetate as eluent followed by 50 % methanol in ethyl acetate to get a yellow hygroscopic solid of the desired product **(7bIII)** (0.80 g, 62.83 %). m.p. 96-98 °C

Anal.:

TLC : R_f 0.39 (Methanol: 1 drop GAA)

UV (MeOH) : 315 nm

IR (KBr, cm^{-1}): 3439, 1729, 1675, 1590, 1323 and 754

PMR ($CDCl_3$):9.54 (s, 1H, Pyridinium-*H*) 8.95-8.93 (d, 1H, Pyridinium-*H*, *J=8.0,8.0*), 8.73-8.71 (d, 1H, Pyridinium-*H*, *J=8.0*) , 8.61-8.58 (m, 1H, Pyridinium-*H*, *J=8.0*), 7.92-7.88 (t, 1H, N*H*, *J*=6.08), 7.67-7.63 (d, 2H, Phenyl-*H*, *J*=8.0), 7.48- 7.45 (d, 2H, Phenyl-*H*,*J*=8.0), 6.97-6.96 (d,1H, Indo-*H*, *J*=2.48), 6.89 (5, 1H, Indol-*H*), 6.55-6.52 (dd, 1H, Indol-*H*, *J* =2.52), 5.26-5.21 (m, 1H, OC*H*), 4.47-4.44 (s, 3H, N-CH_3), 3.80 (s, 3H,-OCH_3), 3.58-3.54 (t, 2H, -CON*H*), 2.23 (s, 3H, Indol-CH_3), 2.13 (s, 2H, Indol-CH_2) and 1.36-1.30 (d, 3H, -CH_3, *J=8.0*).

HPLC : > 99.0%

4.1.56. 2-(1-Methylpyridinium-3-carboxamido)butanyl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetate iodide (7cIII)

The title compound was prepared from ester intermediate **(6cIII)** (1.0 g, 1.87 mM) and methyl iodide (0.79 g, 5.61 mM) following the method described for the synthesis of compound **(7aIII)** to afford a yellow semisolid which was recrystallized from ethyl

acetate-methanol to obtain yellow hygroscopic solid of the desired product **(7cIII)** (0.60 g, 47.40 %). m.p. 48-50 °C

Anal.:

TLC : R_f 0.41 (Methanol: 1 drop GAA)

UV (MeOH) : 228 nm

IR (KBr, cm^{-1}): 3436, 1733, 1672, 1590, 1322 and 754

PMR ($CDCl_3$) : 9.51 (s, 1H, Pyridinium-*H*) 8.97-8.95 (d, 1H, 1H, Pyridinium-*H*,*J=8.0,8.0*), 8.74-8.72 (d, 1H, Pyridinium-*H*, *J=8.0*), 8.28(bs,1H, N*H*), 7.93-7.89 (m, 1H, Pyridinium-*H*, *J*=8.0), 7.67-7.65 (d, 2H, Phenyl-*H*, *J=8.0*), 7.48- 7.46 (d, 2H, Phenyl-*H*, *J=8.0*), 6.94-6.93 (dd, 2H, Indol, *H*-6,7, *J=2.48*), 6.48-6.45 (dd,1H, Indol *H*-4, *J=2.48*), 4.48 (s, 3H, N-CH_3), 4.33 (s, 3H,-OCH_3), 3.83 (s, 3H, indol-CH_3), 3.69 (s, 2H, Indol-CH_2-), 2.20 (s, 2H, O-CH_2), 1.80-1.66 (m, 2H, C-CH_2-), 0.96-0.92 (t, 3H, C-CH_3) and 1.27-1.24 (m, 1H, N-C*H*).

HPLC : >98.6%

4.1.57. 2-Methyl-2-(1-methylpyridinium-3-carboxamido)propanyl 1-(4-chloro benzoyl)-5-methoxy-2-methylindol-3-acetate iodide (7dIII)

The title compound was prepared from ester intermediate **(6dIII)** (1.0 g, 1.87 mM) and methyl iodide (0.79 g, 5.61 mM) following the method described for the synthesis of compound **(7aIII)** to give yellow semisolid which was recrystallized from ethyl acetate-methanol to yield yellow hygroscopic solid of the desired product **(7dIII)** (0.80 g, 63.20 %). m.p. 86-88 °C

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 318 nm

IR (KBr, cm^{-1}): 3436, 1733, 1675, 1591, 1322 and 752

PMR ($CDCl_3$): 9.53 (s, 1H, Pyridinium-*H*) 9.0-8.98 (d, 1H, 1H, Pyridinium-*H*,*J*=8.0), 8.70-8.68 (d, 1H, Pyridinium-*H*, *J*=8.0), 7.69-7.93 (m,1H, Pyridinium-*H*, *J*=8.0), 7.87 (s, 1H, N*H*), 7.64- 7.62 (d, 2H,Phenyl-*H*, *J*=8.0), 7.47- 7.44 (d, 2H, Phenyl-*H*, *J*=8.0), 6.95 (s,1H, Indol *H*-4), 6.88- 6.86 (d,1H, Indol *H*-6, *J*=8.96), 6.56-6.54 (d, 1H, Indol *H*-7, *J*=8.96), 4.50 (s, 3H, N-CH_3), 4.38 (s, 2H,O-

CH_2-), 3.78 (s, 3H,-OCH_3), 3.76 (s, 2H, indol -CH_2), 2.28 (s,3H, Indol-CH_3), 1.49 (s, 6H, $(CH_3)_2$)

^{13}CMR ($CDCl_3$):170.93, 168.44, 160.97, 155.85, 148.11, 144.57 139.31, 135.79-129.18, 127.69, 114.79, 122.66, 111.54, 101.69, 68.92, 56.10, 55.38, 49.23, 30.52, 24.33, 13.75

MS (m/z) : 548.3 (M^+)

HPLC : >96.4 %

4.1.58. 3-(1-Methylpyridinium-3-carboxamido)propanyl 1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-acetate iodide (7eIII)

The title compound was prepared from ester intermediate **(6eIII)** (1.0 g, 1.91 mM) and methyl iodide (0.81 g, 5.76 mM) following the method described for the synthesis of compound **(7aIII)** to give yellow solid which on crystallization in ethanol and small amount of ether yielded light yellow solid of the desired product[2] **(7eIII)** (1.10 g, 86.40 %), m.p. 166-168 °C.

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 222, 320, 315 nm

IR (KBr, cm^{-1}): 3288, 1711, 1630, 1594, 1316, 827

PMR ($CDCl_3$): 9.53 (s, 1H, pyridinium-*H*), 9.20-9.18 (d, 1H, pyridinium-*H*, *J=8.0*), 9.07-9.04 (m, 1H, pyridinium-*H*, *J=8.0*), 8.98-8.96 (d,1H,pyridinium-*H*, *J=8.0*), 8.21-8.17 (t, 1H, CON*H*), 7.67-7.66 (d,2H, phenyl-*H*, *J=4.0*), 7.55-7.54 (d, 2H, *J=4.0*), 6.98-6.97 (s, 1H,indole-*H*), 6.92-6.89 (d, 1H, indole-*H*, *J*=9.04), 6.67-6.64 (d, 1H,indole-*H*, *J*=9.04), 4.50 (s, 3H, N-CH_3), 4.19-4.16 (t, 2H, O-CH_2,*J*=6.4), 3.80 (s, 3H, O-CH_3), 3.71 (s, 2H, indole-CH_2), 3.49-3.44 (q, 2H, N-CH_2, *J*=6.56), 2.32 (s, 3H, indole-CH_3) and 1.99-1.93 (m,2H, O-C-CH_2, *J*=6.56)

MS (m/z) : 535.8 (M^+)

^{13}CMR ($CDCl_3$):171.23, 168.43, 161.10, 155.99-101.63, 62.43, 56.06, 49.23, 36.73, 30.25 and 13.22

HPLC : >99.8 %

4.1.59. N-[2-(1-Methylpyridinium-3-carboxamido)ethyl-2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindole]-3-acetamide iodide (7fIII)

The title compound was prepared from amide intermediate **(6fIII)** (1.0 g, 1.98 mM) and methyl iodide (0.83 g, 5.94 mM) following the method described for the synthesis of compound **(7aIII)** to offer yellow semisolid of the desired product **(7fIII)** (0.8 g, 62.44 %).

Anal.:

TLC : R_f 0.38 (Methanol: 1 drop GAA)

UV (MeOH) : 258, 316 nm

IR (KBr, cm^{-1}): 3273, 1663, 1590, 1312 and 751

PMR ($CDCl_3$) : 9.34 (s, 1H, pyridinium-*H*), 9.14-9.13 (d, 1H, pyridinium-*H*, *J=6.04*), 8.83 (s, 1H, CO*NH*), 8.73-8.71 (d, 1H, pyridinium-*H*, *J=8.0*), 8.04-8.02 (m, 1H, pyridinium-*H*, *J=8.0*), 7.71-7.52 (d, 2H, phenyl-*H*, *J=8.0*), 7.54-7.52 (d, 2H, phenyl-*H*, *J=8.0*), 6.99 (s, 1H, indole-*H*), 6.92-6.90 (d, 1H, indole-*H*, *J=2.24*), 6.55-6.53 (d, 1H, indole-*H*, *J=2.24*), 4.45 (s, 3H, N-CH_3), 4.08-4.06 (t, 1H, CO*NH*), 3.77 (s, 3H, -OCH_3), 3.57-3.48 (m, 4H, N(CH_2)$_2$) and 3.44 (s, 2H, indole-CH_2), 2.26 (s, 3H, indole-CH_3)

^{13}CMR ($CDCl_3$): 171.15, 168.67, 161.39, 155.97-155.85, 146.08-144.24, 139.23, 56.25, 53.83, 49.12, 39.97-32.27 and 14.64

MS (m/z) : 519.0 (M^+)

HPLC : >95.2 %

4.1.60. N-[3-(1-Methylpyridinium-3-carboxamido)propyl]-2-[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol]-3-acetamide iodide (7gIII)

The title compound was prepared from amide intermediate **(6gIII)** (1.0 g, 1.92 mM) and methyl iodide (0.83 g, 5.78 mM) following the method described for the synthesis of compound **(7aIII)** to give yellow semisolid which was recrystallized from methanol: chloroform: ethyl acetate (1:1:1) to yield light yellow solid of the desired product **(7gIII)** (0.90 g, 70.67 %). m.p. 170-172 °C

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 255 nm

IR (KBr, cm^{-1}): 3299, 1668, 1591 and 754

PMR ($CDCl_3$): 9.5 (s, 1H, Pyridinium-*H*), 9.18-9.17 (d, 1H, Pyridinium-*H*, *J=4*), 9.0-8.98 (m, 1H, Pyridinium-*H*, *J=4.0*), 8.95-8.93 (d,1H, pyridinium-*H*, *J=8.0*), 8.19-8.15 (t, 1H, CONH, *J=6.16*), 7.97-7.95 (t, 1H, CON*H*, *J=5.76*), 7.70-7.52 (dd, 4H, phenyl-H), 7.09-6.62 (m, 3H, indol-*H*), 4.48 (s, 3H, N-CH_3), 3.79 (s, 3H, -OCH_3), 3.55 (s, 2H, indol-CH_2-), 3.41-3.38 (q, 2H, N-CH_2, *J=6.12*), 3.33 (s, 3H, Indol-CH_3), 3.26 (q, 2H,N-CH_2, *J=6.12*) and 1.81-1,75 (m, 2H, -CH_2-, *J=6.12*).

^{13}CMR ($CDCl_3$): 169.65, 167.64, 160.85, 155.45, 146.85, 145.29, 142.66, 137.92, 135.05, 133.86, 133.42, 130.94, 130.21, 127.32, 114.37, 114.02, 111.07, 101.53, 55.38, 48.26, 40.21, 40.01, 39.80, 39.17, 37.28, 36.39, 31.30, 28.71, 13.35

MS (m/z) : 533.3 (M^+)

HPLC : >99.8 %

4.1.61. 2-(1-Methylpyridinium-3-carboxamido)ethyl 2-(6-methoxy-2-naphthyl) acetate iodide (7aIV)

A reaction mixture containing ester intermediate **(6aIV)** (1.0 g, 2.74 mM) and methyl iodide (1.16 g, 8.23 mM) in acetonitrile (20 ml) were refluxed for 2-3 h. The solvent and excess reagent were removed under reduced pressure to afford yellow solid which on crystallization in chloroform-methanol yielded yellow solid of the desired product **(7aIV)** (1.2 g, 86.27 %). m.p. 132-134 °C

Anal.:

TLC : R_f 0.35 (Methanol: 1 drop GAA)

UV (MeOH) : 229 nm

IR (KBr, cm^{-1}): 3289, 2950, 1735, 1672, 1606 and 1187

PMR ($CDCl_3$): 9.38 (s, 1H, pyridinium-*H*), 9.11-9.08 (d, 2H, pyridinium-*H*, N*H*, *J=8.0*), 8.80-8.78 (d, 1H, pyridinium-*H*, *J=8.0*), 8.09-8.06 (m, 1H, pyridinium-*H*), 7.62-7.58 (m, 3H, naphthalene-*H*, *J=8.0*), 7.32-7.04 (m, 3H, naphthalene-*H*, *J=8.0*), 4.41 (s, 3H, N-CH_3), 4.26-4.23 (m,2H, N-CH_2, *J=4.0*), 3.85 (s, 3H, O-CH_3), 3.73 (s, 2H, Ar-CH_2) and 3.64-3.60 (m, 2H, O-CH_2)

^{13}CMR : 171.17, 161.22, 157.03, 146.94, 145.26, 142.51, 133.0, 129.11, 128.75, 127.99, 127.32, 126.56, 118.57, 105.44, 62.28, 55.11,

48.29, 40.35, 40.01, 39.80, 39.17 and 38.60

MS (m/z) : 379.0(M^+)

HPLC : >99.5 %

4.1.62. 1-(1-Methylpyridinium-3-carboxamido)propan-2-yl 2-(6-methoxy-2-naphthyl-2-yl)acetate iodide (7bIV)

The title compound was prepared from ester intermediate **(6bIV)** (0.5 g, 1.32 mM) and methyl iodide (0.55 g, 3.96 mM) following the method described for the synthesis of compound **(7aIV)** to yield a yellow oil but it contained some impurity. The crude oil was purified by column chromatography using neutral alumina as stationary phase and ethyl acetate as mobile phase followed by ethyl acetate: methanol (1:1) mixture to get pure yellow oil of the desired product **(7bIV)** (0.45 g, 64.44 %).

Anal.:

TLC : R_f 0.43 (Methanol: 1 drop GAA)

UV (MeOH) : 226 nm

IR (Neat, cm^{-1}): 3245, 1724, 1668, 1605, 1264 and 852

PMR ($CDCl_3$) : 9.26 (s, 1H, Pyridinium-*H*), 8.74-8.73 (d, 1H, Pyridinium-*H*, *J*=6.08), 8.61-8.59 (d,1H, Pyridinium-*H*, *J=8.0*), 8.55-8.53 (m, 1H, Pyridinium-*H*, *J=8.0*), 7.72-7.69 (m, 1H, N*H*, *J=4.0*), 7.58-7.00 (m, 3H, Ar-*H*, *J=4*), 7.35-7.32 (m, 1H, Ar-*H*, *J=4.0*) 7.06-7.0 (m, 2H, Ar-*H*, *J=4*), 5.26-5.22 (m,1H, O-C*H*), 4.27 (s, 3H, N-CH_3), 3.88 (s, 3H,-OCH_3,), 3.74 (s, 2H, Ar-CH_2), 3.64-3.53 (m,2H, N-CH_2, *J=4.0*), 1.32-1.30 (d, 3H, -CH_3, *J=4.0*)

^{13}CMR : δ172.18, 161.40, 157.57, 69.68, 55.60, 49.15, 44.45, 41.46 and 17.96

HPLC : >99.90 %

4.1.63. 2-(1-Methylpyridinium-3-carboxamido)butanyl 2-(6-methoxy-2-naphthyl) acetate iodide (7cIV)

The title compound was prepared from ester intermediate **(6cIV)** (0.5 g, 1.27 mM) and methyl iodide (0.53 g, 3.82 mM) following the method described for the synthesis of compound **(7aIV)** to give brown oil of the desired product **(7cIV)** (1.10 g, 80.70 %).

Anal.:

TLC : R_f 0.45 (Methanol: 1 drop GAA)

UV (H_2O) : 226 nm

IR (Neat, cm^{-1}): 3246, 1727, 1667, 1605, 1264, 850 and 742

PMR ($CDCl_3$) : δ 9.30 (s, 1H, Pyridinium-*H*), 8.76-8.74 (d, 1H, Pyridinium-*H*, *J=8.0*), 8.62-8.60 (d, 1H, Pyridinium-*H*, *J=8.0*), 8.27-8.25 (m, 1H, N*H*), 7.73-7.70 (m, 1H, Pyridinium-*H*, *J=8.0*), 7.58-7.53 (m, 3H, Ar-*H*, *J=8.0*), 7.37-7.27 (m, 1H, Ar-*H*, *J=8.0*), 7.14-7.0 (m, 2H, Ar-*H*, *J=8.0*), 4.41-4.31 (m, 2H, O-CH_2), 4.29 (s, 3H, N-CH_3), 3.90-3.89 (m, 1H, N-C*H*), 3.88 (s, 3H, O-CH_3) 3.77 (s, 2H, Ar-CH_2), 1.84-1.69 (m, 2H, -CH_2) and 0.99-0.95 (t, 3H, -CH_3, *J=7.44*)

^{13}CMR :172.14, 161.21, 157.57, 145.80, 144.26, 133.83, 129.38-105.50 (10C, Ar), 65.69, 55.58, 51.93, 49.06, 41.27, 23.97 and 10.78

MS (m/z) : 407.20 (M^+)

HPLC : >95.9 %

4.1.64. 2-Methyl-2-(1-methylpyridinium-3-carboxamido)propyl 2-(6-methoxy-2-naphthyl)acetate iodide (7dIV)

The title compound was prepared from ester intermediate **(6dIV)** (0.5 g, 1.32 mM) and methyl iodide (0.55 g, 3.96 mM) following the method described for the synthesis of compound **(7aIV)** to give light pink colored solid of the desired product **(7dIV)** (0.54 g, 79.32 %). m.p. 210-212 ^{0}C

Anal.:

TLC : R_f 0.48 (Methanol: 1 drop GAA)

UV (H_2O) : 226 nm

IR (KBr, cm^{-1}): 3267, 1737, 1660, 1607, 1329 and 857 and 751

PMR ($CDCl_3$): 9.36 (s, 1H, Pyridinium-*H*), 9.12-9.10 (d, 1H, Pyridinium-*H*,*J=8.0*), 8.75-8.73 (d, 1H, Pyridinium-*H*, *J=8.0*), 8.34 (s, 1H, N*H*), 8.10-8.05 (m, 1H, Pyridinium-*H*, *J*=8.0), 7.66-7.08 (d, 3H, Ar-*H* , *J=8.0*), 7.35-7.33 (d, 1H, Ar-*H*, *J=8.0*), 7.17-7.08 (m, 2H, Ar-*H*, *J=8.0*), 4.41 (s, 3H, N-CH_3), 4.33 (s, 2H, -CH_2), 3.90 (s, 3H, -OCH_3), 3.78 (s, 2H, O-CH_2) and 1.42 (s, 6H, C(CH_3)$_2$)

MS (m/z) : 407.2 (M^+)

HPLC : >99.2 %

4.1.65. 3-(1-Methylpyridinium-3-carboxamido)propanyl 2-(6-methoxy-2-naphthyl) acetate iodide (7eIV)

The title compound was prepared from ester intermediate **(6eIV)** (0.5 g, 1.32 mM) and methyl iodide (0.55 g, 3.96 mM) following the method described for the synthesis of compound **(7aIV)** to give yellowish brown oil of the desired product **(7eIV)** (0.50 g, 72.72 %).

Anal.:

TLC : R_f 0.45 (Methanol: 1 drop GAA)

UV (H_2O) : 226 nm

IR (Neat, cm^{-1}): 3248, 2950, 1724, 1666, 1605, 1265 and 850

PMR ($CDCl_3$): 9.45 (s, 1H, pyridinium-*H*), 9.15-9.13 (d, 1H, pyridinium-*H*, *J=8.0*), 9.13-9.10 (m, 1H, Pyridinium-*H*, *J=8.0*), 8.93-8.91 (d, 1H, pyridinium-*H*, *J=8.0*), 8.23-8.19 (t, 1H, CO*NH*), 7.75-7.73 (d, 2H, Ar-*H*, *J=8.0*), 7.68 (s, 1H, Ar-*H*), 7.38-7.36 (d, 1H, Ar-*H*, J=8.0), 7.23 (s, 1H, Ar-*H*), 7.14-7.12 (d, 1H, Ar-*H*, *J=8.0*), 4.44 (s, 3H, N-CH_3), 4.17-4.14 (t, 2H, O-CH_2, *J=8.0*), 3.88 (s, 3H, -OCH_3), 3.78 (s, 2H, Ar-CH_2-), 3.46-3.41 (q, 2H, N-CH_2, *J=8.0*) and 1.94-1.91 (p, 2H, -CH_2, *J=8.0*)

^{13}CMR : 171.05, 160.79, 157.08, 146.69-145.20 (2C), 142.81-133.39 (2C), 133.02-105.32 (10C, Ar), 61.99, 54.98, 48.31, 39.65, 36.43 and 27.90

MS (m/z) : 393.6 (M^+)

HPLC : >99.7%

4.1.66. 2-(1-Methylpyridinium-3-carboxamido)ethyl 4-biphenylacetate iodide (7aV)

A reaction mixture containing ester intermediate **(6aV)** (1.0 g, 2.74 mM) and methyl iodide (1.17 g, 8.32 mM) in acetonitrile (20 ml) were refluxed for 2-3 h. The solvent and excess reagent were removed under reduced pressure to afford a yellow solid which on crystallization in chloroform-methanol yielded yellow solid of the desired product **(7aV)** (1.10 g, 78.93 %). m.p. 140-142 °C

Anal.:

TLC : R_f 0.39 (Methanol: 1 drop GAA)

UV (MeOH) : 250 nm

IR (KBr, cm^{-1}) : 3271, 1721, 1672, 1543, and 1007

PMR ($CDCl_3$): 9.53 (s, 1H, pyridinium-*H*), 9.19-9.16 (m, 2H, pyridinium-*H*, N*H*, *J=8.0*), 8.96-8.94 (d, 1H, pyridinium-*H*), 8.19-8.16 (t, 1H,

pyridinium-*H*), 7.57-7.32 (m, 9H, biphenyl-*H*), 4.47 (s, 3H, N-CH_3), 4.30-4.28 (t, 2H, O-CH_2, *J=4.0*), 3.72 (s, 2H, Ar-CH_2) and 3.72-3.65 (q, 2H, N-CH_2, *J=4.0*)

^{13}CMR ($CDCl_3$):170.95, 160.84, 146.41, 145.03, 143.01, 139.71, 139.57, 138.57, 138.66, 133.27, 132.75, 129.54, 128.57, 127.26, 126.44, 126.18, 62.24, 48.41, 40.29, 39.87, 39.10, 38.89 and 38.45

MS (m/z) : 375.90 (M^+)

HPLC : >98.9 %

4.1.67. 1-(1-Methylpyridinium-3-carboxamido)propan-2-yl 4-biphenylacetate iodide (7bV)

The title compound was prepared from ester intermediate **(6bV)** (1.0 g, 2.67 mM) and methyl iodide (1.12 g, 8.01 mM) following the method described for the synthesis of compound **(7aV)** to give yellow semisolid. This semisolid mass was triturated with hexane first and then with hexane-ethyl acetate mixture. The solvent was decanted to leave pure product as a yellow oil of the desired product **(7bV)** (0.9 g, 65.27 %).

Anal.:

TLC : R_f 0.40 (Methanol)

UV (MeOH) : 252 nm

IR (KBr, cm^{-1}) : 3236, 1725, 1667, 1590 and 744

PMR ($CDCl_3$) : 9.46 (s, 1H, pyridinium-*H*), 8.79-8.73 (m, 2H, pyridinium-*H*, N*H*), 8.75-8.73 (d, 1H, CO-N*H*, *J=8.2*), 8.62-8.59 (d, 1H, pyridinium-*H*, *J=4*), 7.87-7.85 (m, 1H, pyridinium-*H*, *J=6.16*), 7.48-7.19 (m, 9H, biphenyl-*H*), 5.17-5.12 (m, 1H, CH-O, *J=4.0*), 4.30 (s, 3H, N-CH_3), 3.48-3.46 (t, 2H, N-CH_2, *J=6.4*), 1.99 (s, 2H, Ar-CH_2) and 1.24-1.23 (d, 3H, C-CH_3, *J=6.44*)

^{13}CMR :171.93, 161.47, 145.98-126.80 (17C, Ar), 69.75, 49.23, 44.59, 41.26 and 17.97

MS (m/z) : 389.2 (M^+)

HPLC : >97.8 %

4.1.68. 2-(1-Methylpyridinium-3-carboxamido)butanyl 4-biphenylacetate iodide (7cV)

The title compound was prepared from ester intermediate **(6cV)** (1.0 g, 2.57 mM) and methyl iodide (1.08 g, 7.72 mM) following the method described for the synthesis of

compound **(7aV)** to yield yellow semisolid. This semisolid mass was triturated with hexane and then with hexane-ethyl acetate mixture. The solvent was decanted to leave pure product as a yellow oil of the desired product **(7cV)** (0.80 g, 58.58 %).

Anal.:

TLC : R_f 0.42 (Methanol)

UV (MeOH) : 252 nm

IR (KBr, cm^{-1}) : 3247, 3058, 1727, 1665, 1590 and 743

PMR ($CDCl_3$) : 9.54 (s, 1H, pyridinium-*H*), 8.79-8.76 (m, 2H, pyridinium-*H*, N*H*,), 8.32-8.30 (d, 1H, pyridinium-*H*, *J=8.0*), 7.86-7.82 (m, 1H, pyridinium-*H*, *J=8.0*), 7.49-7.19 (m, 9H, biphenyl-*H*), 4.33 (s, 3H, N-CH_3), 4.29-4.18 (m, 2H, -CH_2), 3.65 (s, 2H, Ar-CH_2), 1.97 (d, 2H, O-CH_2), 1.79-1.64 (m, 1H, N-C*H*, *J=7.04*) and 0.92-0.89 (t, 3H, -CH_3).

^{13}CMR : 171.87, 161.19, 145.89-126.76, 65-67, 52.03, 46.16, 41.01, 23.99 and 10.81

MS (m/z) : 403.2 (M^+)

HPLC : >97.5 %

4.1.69. 2-Methyl-2-(1-methylpyridinium-3-carboxamido)propanyl 4-biphenylacetate iodide (7dV)

The title compound was prepared from ester intermediate **(6dV)** (1.0 g, 2.74 mM) and methyl iodide (1.17 g, 8.32 mM) following the method described for the synthesis of compound **(7aV)** to give yellow semisolid. This semisolid mass was triturated with hexane first and then with hexane-ethyl acetate mixture. The solvent was decanted to leave pure product as a yellow oil of the desired product **(7dV)** (0.85 g, 62.25 %).

Anal.:

TLC : R_f 0.39 (Methanol)

UV (MeOH) : 252 nm

IR (KBr, cm^{-1}) : 3436, 1728, 1668, 1590, 743

PMR ($CDCl_3$) : 9.52 (s, 1H, pyridinium-*H*), 8.84-8.82 (d, 1H, pyridinium-*H*, *J=8.0*), 8.70-8.68 (d, 1H, pyridinium-*H*, *J=8.0*), 7.93 (bs, 1H, N*H*), 7.89-7.86 (m, 1H, pyridinium-*H*, *J=8.0*), 7.53-7.31(m, 9H, biphenyl-*H*), 4.41 (s, 3H, N-CH_3), 4.37 (s, 2H, O-CH_2), 3.74 (s, 2H, Ar-CH_2) and 1.52 (s, 6H, (CH_3)$_2$)

HPLC : >98.7 %

4.1.70. 3-(1-Methylpyridinium-3-carboxamido)propanyl 4-biphenylacetate iodide (7eV)

The title compound was prepared from ester intermediate **(6eV)** (0.50 g, 1.33 mM) and methyl iodide (0.56 g, 4.0 mM) following the method described for the synthesis of compound **(7aV)** to give yellow semisolid. This semisolid mass purified by column chromatography using neutral alumina as stationary phase and ethyl acetate as eluent followed by ethyl acetate: methanol (1:1) to give pure yellow oil of the desired product **(7eV)** (0.49 g, 71.07 %).

Anal.:

TLC : R_f 0.39 (Methanol)

UV (MeOH) : 252 nm

IR (KBr, cm^{-1}): 3391, 2948, 1725, 1660, 1589 and 741

PMR ($CDCl_3$) : 9.93 (s, 1H, pyridinium-*H*), 8.98-8.96 (m, 2H, pyridinium-*H*, N*H*, *J=8.0*), 8.86-8.85 (d, 1H, pyridinium-*H*), 8.01-7.97 (m, 1H, pyridinium-*H*, *J=8.0*), 7.57-7.26 (m, 9H, biphenyl-*H*, 4.51 (s, 3H, N-CH_3), 4.23-4.20 (t, 2H, O-CH_2, *J=8.0*), 3.70 (s, 2H, Ar-CH_2), 3.58-3.53 (q, 2H, N-CH_2, *J=8.0*), 3.09 (bs, 1H, N*H*) and 2.08-2.05 (m, 2H, O-C-CH_2, *J=8.0*)

MS (m/z) : 389.2 (M^+)

HPLC : >96.3 %

4.2. Hydrolyses studies

All the synthesized CDS were evaluated for their stability in buffers (*p*H 6.0 and 7.4), which simulate the *p*H conditions existing in the synovial fluid and blood. The enzymatic susceptibility towards serum esterases of all the CDS was evaluated *in vitro* in pooled human serum (90 %). HPLC analysis was performed using shimadzu prominence system (Kyoto, Japan) consisting of LC-20AT Pump, and SPD 20 A detector.

The chromatographic column used was phenomenex C-18, 250 mm length X 4.6 mm diameter with 0.5 µ particle size and Column temperature was maintained at 25-28 °C. Separation of analyte was performed under isocratic conditions at a flow-rate of 0.5-1.5 ml/min. The mobile phase consisted of phosphate buffer (PB, *p*H 5-5.5, 15 mM) acetonitrile. 20 µl of sample was loaded using syringe through rheodyne injector. The solutions and the buffers used in the study were prepared in distilled water. The buffers used were prepared according to the procedure as given in USP. All the chemicals used

were of analytical reagent grade. Human Plasma was obtained from SSG Hospital and Indu Blood Bank, Vadodara, India. The absorbance measurements were made on a UV-visible spectrophotometer (UV-1800, Shimadzu Corporation, Japan).

Solutions and buffers

1. Sodium hydroxide (0.2 M): Sodium hydroxide (8.0 g) was dissolved in distilled water and volume made up to 1000 ml with distilled water.
2. Potassium dihydrogen phosphate (0.2 M): Potassium dihydrogen phosphate (27.22 g) was dissolved in distilled water and diluted to 1000 ml in a standard volumetric flask.
3. Phosphate buffer *p*H 6.0: Potassium dihydrogen phosphate (0.2 M, 50 ml) and sodium hydroxide solution (0.2 M, 5.6 ml) were taken in volumetric flask (200 ml) and the final volume was made up to 200 ml with distilled water.
4. Phosphate buffer *p*H 7.4: Potassium dihydrogen phosphate (0.2 M, 50 ml) and sodium hydroxide solution (0.2 M, 39.1 ml) were taken in volumetric flask (200 ml) and the final volume was made up to 200 ml with distilled water.
5. Mobile phase: The mobile phase was prepared from potassium dihydrogen phosphate solution (15 mM) by dissolving 2.041 g into 1000 ml water. The resulting solution was filtered through Whatman filter paper (0.22 μ) and appropriate amount of acetonitrile was added and the final solution sonicated for 10 min before use.

4.2.1. Analytical method validation

The method was validated for linearity. Linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample[5-6] Linearity in light absorption is examined to ensure that Beer's law operates over the range of interest.

For evaluation of the linearity of the HPLC method for the synthesized CDS, the standard solutions were prepared in the range of 1-100 μg/ml concentrations (n=3) and absorbance were recorded at respective λ max (nm). The method was said to be linear for estimation of CDS if its R^2 was near to 1. Least square regression method was used to determine the regression coefficient, r and the equation for the best fitting line.

4.2.2. General procedure for the preparation of calibration curve for estimating CDS in buffers and in human serum

A stock solution (1000 μg/ml) of CDS **(7aI-7gV)** was prepared in double distilled water and DMSO was used in small quantity if required followed by sonication for 5 min.

From this stock solution appropriate dilutions were prepared in double distilled water to give solutions of concentrations in the range of 1-200 µg/ml. Aliquots (0.1 ml) were withdrawn individually from these solutions, and transferred in to Eppendorf tube containing 0.9 ml of mobile phase. And from the above solution 20 µl of the sample was injected using 100 µl syringe and absorbance measured (mV) at the specified λ max (nm). Retention time of CDS was kept in between 3-6 min by adjusting phosphate buffer-acetonitrile ratio or controlling flow rate of mobile phase. Samples were analyzed in triplicate and calibration curve was plotted using concentration (µg) versus absrbance (mV) and the linearity was determined.

4.2.3. General procedure for the stability studies of CDS in phosphate buffer *p*H 6 and 7.4.

Phosphate buffer (*p*H 6 or 7.4) was equilibrated in a water bath at 37 °C. A stock solution was freshly prepared for each CDS by dissolving it in water and if required small amount of DMSO was used. 10 µl of the stock solution was added per milliliter of the buffer solution used in the study in each experiment. Aliquots of 100 µl were withdrawn at various time points and pipetted in to 400 µl portion of the mobile phase. The samples were centrifuged for 3 min at 4000 rpm and the supernatant was sampled to determine the rate of disappearance of the CDS using HPLC.

4.2.4. General procedure for the stability study of CDS in human serum

Human serum was incubated at 37±0.5 °C before use. A stock solution of CDS was freshly prepared by dissolving it in water and if required small amount of DMSO was used. In each experiment stock solution of CDS (100 µl) was added to serum (3ml) in the study. Aliquots of 100 µl were withdrawn at various time points and pipetted in to 400 µl portion of ice cold acetonitrile containing 5 % DMSO by volume. Samples were vortexed for 5 sec and centrifuged for 5 min at 4000 rpm and the supernatant was sampled to determine the half life of disappearance of CDS using HPLC. The first order equation ($t_{1/2}$ = 0.693/k) was used to calculate the half-lives.

4.3. Cytotoxicity study (MTT assay)

All the synthesized CDS were evaluated for their *in vitro* cytotoxicity on normal rat Liver cell line (BRL-3A) or mouse embryonic fibroblasts (NIH 3T3). These cell lines were procured from NCCS Pune and ACTRACT Mumbai. All the chemicals and media required for the study were purchased from Himedia Chemicals, Mumbai. Plates (96 well) and cultured flask were purchased from Tarson. All the compounds were perfectly dried

and UV treatment given before use. Stock solutions of all the derivatives were prepared in double distilled water and small amount of DMSO (20 µl) added if required with sonication for 3 min.

Reagents

1. 3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution: Prepared by dissolving appropriate quantities to get final concentration of 0.5 mg/ml of serum-free Dulbecco's Modified Eagle Medium (DMEM) medium.
2. Solubilization solution: DMSO 150 µl per well
3. Phosphate Buffered Saline (PBS; *p*H 7.4): Sodium phosphate monobasic (NaH_2PO_4) (0.63 g) and sodium phosphate dibasic (Na_2HPO_4) (0.17 g) and sodium chloride (NaCl) (4.5 g) were dissolved in sterile double distilled water (500 ml). The *p*H was then adjusted to 7.4 with sodium hydroxide solution and the solution filtered through Whatman filter paper (0.22 µ) under aseptic conditions and stored in refrigerator.

4.3.1. General Procedure

Cells are plated in 96 well plates at a concentration of 1 x 10^4 cells/well. Twenty four hours after plating, cells were washed twice with serum-free medium (100 µl) and starved by incubating the cells in serum-free medium for an hour at 37 °C. After starvation, cells were treated with synthesized CDS or standard drugs (NSAIDs) of different concentrations for 24 hours. At the end of treatment, the medium from all the wells was discarded and MTT (0.5 mg/ml) 100 µl containing DMEM was added to each well. The cells were then incubated for 4 h at 37 °C in the CO_2 incubator. The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of solubilization solution (DMSO) and mixed properly by pipeting up and down. Absorbance of the purple blue formazan dye was measured in microplate reader at 570 nm. The OD of each sample was then compared with the control OD and the graph was plotted.

4.4. Radiolabeling study

Silica gel coated fiber sheets (Gelman Sciences. Inc., Ann Arbor, MI) were used for performing the instant TLC (ITLC). A well type gamma ray spectrophotometer (Type GRS23C, Electronics Corporation of India ltd., Mumbai) was used for the gamma rays counting. The solutions were prepared in distilled water and all the chemicals and solvents used were of analytical grade. Sprague-Dawley rats (3 in each group) were used for the

studies. Carrageenan (1 % w/v in normal saline) was used for inducing inflammation. Imaging was performed using a Single Photon Emission Computerized Tomography (SPECT, LC 75-005, Diacam, Siemens, USA) gamma camera. Ketamine/diazepam in combination was used to induce anesthesia in animals. Radiolabeling study was performed at Institute of Nuclear Medicine and Allied Sciences (INMAS), New Delhi.

4.4.1. Chemicals and reagents

1. Sodium bicarbonate solution (1 %): Accurately weighed Sodium bicarbonate (1 g) was dissolved in sufficient quantity of water and volume was made up to 100 ml with water.
2. Stannous chloride solution (1 mg/ml.): Accurately weighed stannous chloride (100 mg) was dissolved in sufficient quantity of acetic acid (10 %) and volume was made up to 100 ml with acetic acid (10 %).
4. Acetic acid (10 %): Glacial acetic acid (11.4 ml) was dissolved in water and volume was made up to 100 ml with water.
5. Sodium chloride solution (0.9 %): Accurately weighed Sodium chloride (0.9 g) was dissolved in sufficient quantity of water and volume was made up to 100 ml with water.
6. Mobile Phase (PAW): Pyridine, acetic acid and water in 3:5:1.5 proportions was mixed.
7. Carrageenan (1 %): Carrageenan (10 mg) was dissolved in 1 ml of water.

4.4.2. Radiolabeling of compounds

The radiolabeling of the compounds with reduced ^{99m}Tc was carried out as per the direct labeling method.[15] ^{99m}Tc-$NaTcO_4$ (1.0 ml, 2 mCi/ml) was mixed well with stannous chloride solution (0.1 ml, 1 mg/ml, in acetic acid (10 %)). The *p*H was adjusted to 7.0 using sodium bicarbonate solution (0.5 M). To this mixture, solution of the compound (1.0 ml, 2 mg/ml) was added and the reaction mixture incubated for 15 minutes (37±1°C). The above experiment was repeated by varying the conditions one at a time i.e. using different moles of stannous chloride (50 µl-200 µl), changing the *p*H of the medium between 6 and 8 at various values and varying the incubation time period (15, 30 and 45 minutes) of the reaction mixture. The procedure was repeated exactly in the same manner for all of the CDS and the parent drugs.

4.4.3. Evaluation of the labeling efficiency

Ascending instant thin layer chromatography (ITLC) was performed using acetone (100 %) or saline (0.9 %) as the mobile phase. Radiolabeled complex (2-3 µl) was applied

at a point 1.0 cm away from one end of an ITLC-SG strip. The strip was developed in acetone or saline and the solvent front was allowed to rise upto 8 cm from the origin. The strip was cut one cm below the solvent front and the radioactivity in each segment was determined in the gamma ray counter. The free pertechnetate which moved with the solvent (R_f = 0.9) and the reduced/hydrolysed (R/H) technetium along with the labeled complex remaining at the point of application were determined. ITLC was also run in pyridine-acetic acid water (3: 5: 1.5 v/v) mobile phase to determine the amount of reduced/hydrolysed (R/H) ^{99m}Tc (radio-colloids). The R/H ^{99m}Tc remained at the point of application while both the free pertechnetate and the labeled complex moved away with the solvent front in this solvent system (PAW). The difference between the activity for the spots which moved along with the solvent front using either acetone or saline from that obtained in the PAW system (at the point of application) gave the net amount of ^{99m}Tc-labeled complex. This procedure was repeated for the radiolabeling of all the CDS and the parent NSAIDs and for all the experiments conducted for optimization of labeling parameters like *p*H, incubation time and quantity of the reducing agent used.

4.4.4. *In vitro* stability study of ^{99m}Tc- labeled complex in saline and human serum

The *in vitro* stability study[15] of radiolabeled complex was determined in sodium chloride (0.9 %) and in human serum separately by ascending thin layer chromatography. The ^{99m}Tc-labeled compound solution (0.1 ml) prepared in saline (0.9 %) as described above was mixed separately with human serum (1.9 ml) or normal saline (1.9 ml) and incubated (37±1 °C). ITLC was performed at different time intervals (0, 0.25, 0.5, 1.0, 2.0, 4.0 and 24 hours) as described above, in acetone to assess the stability of the complex. Any decrease in percentage of ^{99m}Tc-labeled complex was considered as its degree of degradation.

4.4.5. Gamma imaging studies

IA residence time of synthesized CDS and parent drugs were studied in normal and inflammatory conditions. The parent drug used for the study was 6-MNA **(5IV)**. Animals were divided into three groups **(A-C)** as follow:

Group A-Consisted of animals which were administered with 6-MNA by IA route.

Group B-Consisted of normal animals (without inducing inflammation) which were administered with individual CDS by IA route.

Group C-Consisted of animals (in which inflammation was induced) which were administered with individual CDS by IA route.

For group **C**, inflammation was induced 3 h prior to IA administration of parent drug or CDS. Three hour after injection the animals were anaesthetized, fixed on a board using the adhesive tapes and gamma imaging photographs were taken 1, 2, 6 and 24 h hour after injecting the complex (IA). Radioactivity counts were measured for region of interest (ROI) as well as for whole body and percent activities remaining after a particular time interval were calculated. The above described procedure was followed for studying the biodistribution of the complexes of the remaining CDS.

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