"DEVELOPMENT AND EVALUATION OF ASIATIC ACID NANOPARTICLES: FOR BRAIN SPECIFIC DRUG DELIVERY"

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BY

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DECLARATION

I hereby declare that the dissertation entitled "Development And Evaluation of Asiatic Acid Nanoparticles: For Brain Specific Drug Delivery", is based on the original work carried out by me under the guidance of Dr. Sanjeev R. Acharya, Associate professor, Department of Phytopharmaceuticals and Natural Products, Institute of Pharmacy, Nirma University. I also affirm that this work is original and has not been submitted in part or full for any other degree or diploma to this or any other university or institution.

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Tejas H.Mistry

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LIST OF ABBREVIATIONS

ml	Millilitre
µl	Microlitre
µg	Microgram
mg	Milligram
w/w	Weight by Weight
w/v	Weight by Volume
%	Percentage
H_2SO_4	Sulphuric acid
UV	Ultraviolet
Min	Minutes
h	Hour
S.ROBUSTA	*Shorea robusta*
CPR	Cumulative Percentage Release
AA	Asiatic acid
IR	Infrared Spectroscopy
HPTLC	High Performance Thin Liquid Chromatography
HPLC	High Performance Liquid Chromatography
EDAC	1-ethyl-3-(3-dimethylaminopropyl) Carbodimide Hydrochloride
PTD	P-toludine
NP	Nanoparticles
TLC	Thin Layer Chromatography
BSA	Bovine Serum Albumin
NHS	N-hydroxy Succinimide
DTNB	5,5'- dithio-bis(2-nitrobenzoic acid)
LOQ	Limit of Quantification
LOD	Limit of Detection
SD	Standard Deviation
%RSD	% Relative Standard Deviation

Abstract:

The least efficacy of most of the active pharmaceutical drugs in the brain is attributed to the blood–brain barrier (BBB), which represents insurmountable obstacle for the effective management of majority of CNS disorders. The present research is planned with the objective to develop novel polymeric bovine serum albumin nanoparticles coupled with natural tripeptide i.e. glutathione to enhance drug delivery to brain. To evaluate the brain targeting efficiency of the glutathione conjugated nanoparticles, Asiatic acid was explored as model compound. The developed particle of Asiatic acid were estimated by simple HPLC Method. Asiatic acid being a triterpene has great limitation for its bioavaibility in brain, further there is a great challenge in estimation of asiatic acid in a biological system, because of its weak chromophore. For estimation of Asiatic acid in blood plasma and biological system, a novel pre derivatization HPLC method was developed using EDAC as coupling agent & p-toludine as a derivatization agent.Biodistribution pattern and brain targeting potential of optimized glutathione conjugated BSA nanocarriers was determined using wistar rat as an animal model in comparison to non-conjugated BSA nanocarriers and asiatic acid solution. The results showed significant increase in Asiatic acid uptake in brain with glutathione conjugated BSA nanoparticles as compared to Asiatic acid solution. The present investigations demonstrated that glutathione can serve as a potential ligand for brain drug delivery, which was observed with glutathione coupled BSA nanoparticles resulted into enhanced delivery of Asiatic acid.

1. Introduction:

1.1 Introduction to Asiatic Acid:

Asiatic acid is a pentacyclic triterpene isolated from a variety of plants, including *Centella asiatica, Shorea robusta,* a herb that has been traditionally used in Indian and Chinese medicine and food science (Jeong *et al.* 2006; Kim *et al.*, 2004) & reported as one of the major active constituent responsible for the their various pharmacological activities. One of the major pharmacological activity reported for asiatic acid is neuroprotective, cognitive behavior. asiatic acid has been shown to exhibit neuro-protective properties both in cultured cells and *in vivo*. Furthermore, asiatic acid decreased cellular production of reactive oxygen species following C2-ceramide treatment. The most important pathological hallmark of Alzheimer's disease (AD) is deposition of senile plaques in the brain (Hyman and Tanzi, 1992). The senile plaque consists of diverse molecules but the major component is beta-amyloid (Ab) protein which is derived from proteolysis of a larger amyloid precursor protein and is concentrated in the plaque core. Asiaticoside and its derivatives were tested for potential protective effects against Ab-induced cell death but amongst all tested derivatives asiatic acid showed the strongest protection against Ab-induced cell death.

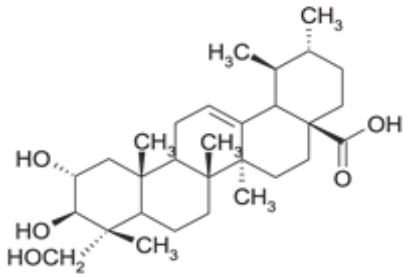


Fig 1.1. Structure of Asiatic Acid

1.2 Obstacles for Brain Drug Delivery:

Unlike most other organs, the cerebral blood compartment is not in free diffusional transposition with the interstitium of the brain. The brain is tightly dissevered from the circulating blood by a unique membranous barrier, the blood brain barrier (BBB).

Drug delivery to the brain is austerely haltered due to the exceptionally low permeability of the BBB. BBB composed of brain capillary endothelial cells (BECE) that are tightly connected and responsible for the extremely selective permeability attributes of the cerebrovasculature. (see figure 1.2.) The BBB is considered as homeostatic denial mechanism of the brain against pathogens and toxins. BBB also screens the biochemical, physicochemical and structural features of solutes at its periphery the BBB restrain solute ingress into the brain, by the transcellular route, due to an increased electrical resistance between the endothelial cells at the tight junctions (TJ).

In brain, capillaries are different from the capillaries of other parts of the body, intercellular cleft, pinocytosis, and fenestrate are virtually nonexistent and mitochondria present profusely; hence, diffusion occurs transcellularly. Therefore, only lipid soluble minuscule solutes that can freely diffuse through the capillary endothelial membrane i.e. may passively cross the BBB.

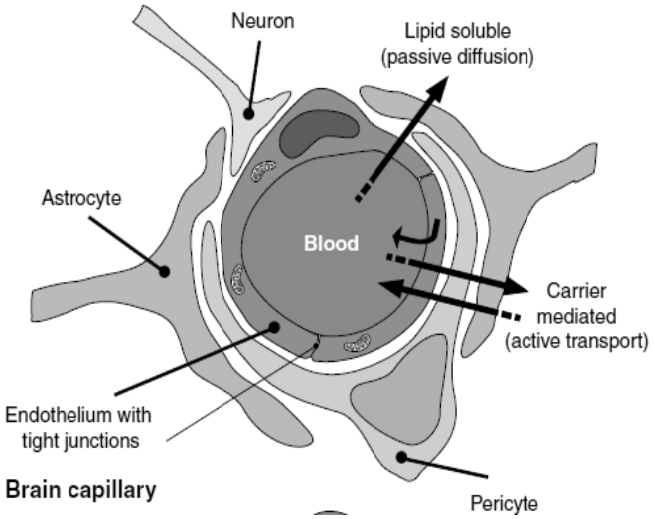


Fig. 1.2. Schematic presentation of Blood Brain Barrier

The BBB has additional, enzymatic aspects: solutes crossing the cell membrane are subsequently queered to degrading enzymes present in large numbers inside the endothelial cells that contain large densities of mitochondria, metabolically highly active organelles.

Enzymes and receptors ascertained in the BBB, among others, are adenylate cyclase, guanylate cyclase, Na+/K+ adenosine triphosphate (ATP)ase, alkaline phosphatase, catechol O-methyl transferase, monoamine oxidase, γ-aminobutyric acid (GABA) transaminase, and DOPA decarboxylase. BBB enzymes also recognize and rapidly degrade most peptides, including naturally occurring neuropeptides (Patel *et al.*, 2012).

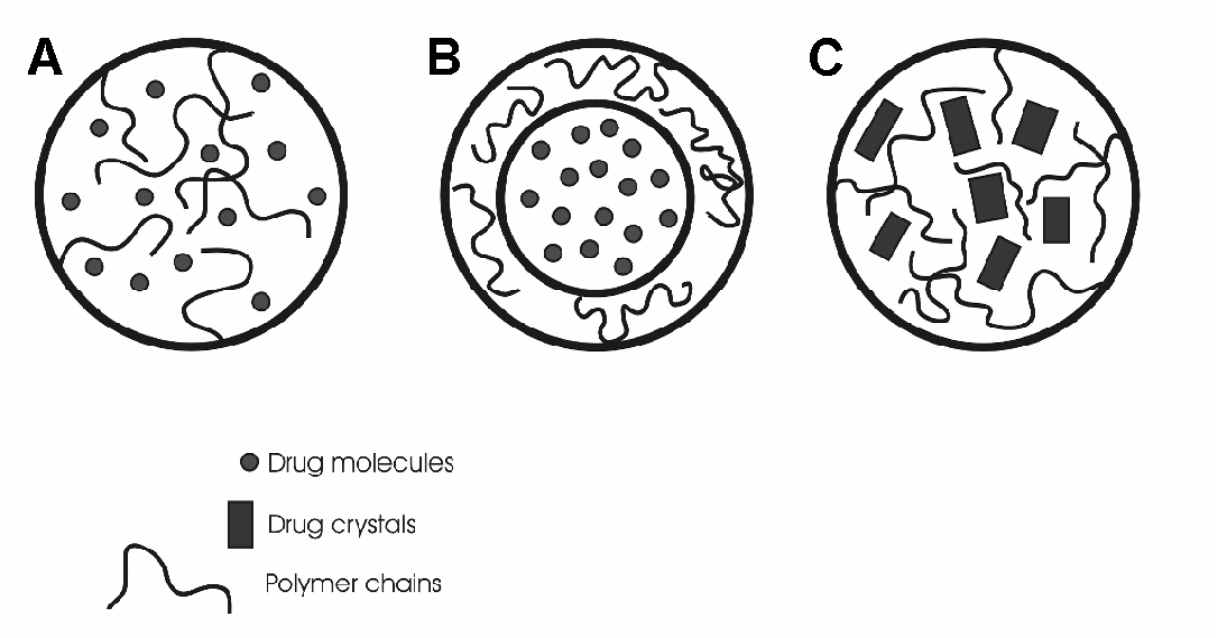
1.3 Role of Nanoparticles for Brain Drug Delivery:

Nanoparticles are solid, colloidal particles consisting of macromolecular substances that vary in size from 10 nm to 1000 nm.(Kreuter *et al.*, 2001) The drug of interest is dissolved, entrapped, adsorbed, attached or encapsulated into the nanoparticle matrix. Depending on the method of preparation, nanoparticles, nanospheres or nanocapsules can be obtained with different properties and release characteristics for the encapsulated therapeutic agent (Barrat *et al.*, 2000; Pitt *et al.*, 1983).

There are two types of nanoparticles depending on the preparation process:

1) Nanospheres
2) Nanocapsules

The term nanoparticle is a collective name for both nanospheres and nanocapsules. Nanospheres have a matrix type of structure. Drugs may be absorbed at the sphere surface or encapsulated within the particle. Nanocapsules are vesicular systems in which the drug is confined to a cavity consisting of an inner core surrounded by a polymeric membrane. In this case, the active substances are usually dissolved in the inner core but may also be adsorbed to the capsule surface.



(**Fig. 1.3. Schematics of exemplary types of drug nanoparticles. A. Matrix type nanosphere, drug molecules are evenly dispersed in the polymer matrix. B. Core shell nanocapsule, drug molecule is presented in a core covered with a polymer shell. C. Matrix type nanosphere where drug crystals are embedded in a polymer matrix)** (Vauthier *et al.*, 1991)

Polymeric nanoparticles prepared from natural and synthetic polymers have received the majority of attention due to higher stability as particulate carriers in the pharmaceutical and medical fields.(Couvreur *et al.*, 1995)

The biodegradable polymeric nanoparticles have an advantage over liposomes, niosomes and other vesicular drug delivery systems through their increased stability and their unique ability to provide an extended release of encapsulated drug. Polymeric nanoparticles are used most in medical applications and have considerable substantial attention as targeted drug-delivery carriers owing to their biocompatibility, physical stability, protection of incorporated labile drugs from degradation and controlled release.

For the brain drug delivery up to now most work were focused on nanoparticles of poly (alkylcyanoacrylate) (PACA) but PACA is not authorized to application in human. PACA nanoparticles will be rapidly degraded by esterases presented in biological fluid and its toxic product will stimulate damage to the central nervous system (CNS). Thus it is requisite to surrogate biodegradable polymer for preparation of nanoparticles for brain drug delivery.

These carriers have been investigated especially in drug-delivery systems for drug targeting because their particle size (ranging from 10 to 1000 nm) is acceptable for intravenous injection. The clearance mechanism of intravenous administered surface unaltered nanoparticles is the spontaneous adsorption of plasma protein (opsonization process), which are capable of interacting with the plasma membrane receptors on monocytes and macrophages, thus boosting particle recognition these carriers of nanoparticles have a further advantage over larger microparticles as they are better suited for intravenous (i.v.) delivery. The smallest capillaries in the body are 5–6 μm in diameter. The size of particles being distributed into the bloodstream must be significantly smaller than 5 μm, without forming aggregates to ensure that the particles do not form an embolism.

For therapeutic applications, the size of the nanoparticles must be accurately controlled to allow smooth passage through capillaries, while enabling targeting to specific body sites. Particle size, shape and number, together with surface charge and surface characteristics, all factors will modulate the biological fate of colloids upon injection (Patel *et al.*, 2012).

Generally, nanoparticles have relatively higher intracellular uptake compared to microparticles and are available to a much wider range of biological targets due to their small size and relative mobility.

Criteria for ideal polymeric carriers for nanoparticles & nanoparticle delivery systems are as followa:

Polymeric carriers:

- Easy to synthesize and characterize
- Inexpensive
- Biocompatible
- Biodegradable
- Non-immunogenic
- Non-toxic
- Water soluble

Nanoparticle delivery systems:

- Simple and inexpensive to manufacture and scale-up
- No heat, high shear forces or organic solvents involved in their preparation process
- Reproducible and stable
- Applicable to a broad category of drugs; small molecules, proteins and polynucleotides
- Ability to lyophilize
- Stable after administration
- Non-toxic

1.4 Role of Glutathione for Brain Drug Delivery:

In order to target potential therapeutic agent to the brain most efficient & safe way is to hijack the endogenous uptake-machinery of the BBB by associating the drug with compounds that are naturally transported into the brain. Glutathione is a natural anti-oxidant and found at high levels in the brain and its receptor is abundantly expressed at the blood-brain barrier. It also uniquely derogates common risks like adverse immunological reactions or interference with life-essential physiological pathways. From the experiments to-BBB technology has proven that as higher the amount of glutathione coating on the liposomes / Nanoparticles, the greater pool of free drug was actually delivered to the brain. to BBB technology has developed by G-technology (ITRI, Taiwan) which utilizes an endogenous receptor-targeted mechanism in combination with liposomes coated with glutathione-

conjugated PEG to mediate safe targeting and enhance the delivery of drugs to the brain. This approach is unique as it does not require drug modification and at the same time it gives rise to metabolic protection during transport and increased bioavailability at the target site (Patel *et al.*, 2012).

The concept of glutathione coupled nanoparticles emerged from the fact that the glutathione is most abundant antioxidant present in the human body, apart from that it also acts as an endogenous ligand for glutamate receptors i.e. N- methyl- D- aspartate (NMDA) and 2-amino-3-hydroxy -5- methyl-4- isoxazolepropionate (AMPA) receptors which are abundantly expressed in the brain. Thus, it was hypothesis that glutathione coupled polymeric nanoparticles constitute a promising drug delivery system for transport of variety of neurotherapeutics to the brain by surpassing BBB (Varga *et al.* 1997).

2. LITERATURE REVEIW:

2.1. Literature Review on *Shorea Robusta*:

Shorea is a genus of about 196 species of mainly rain forest tree in the family *Dipterocarpaceae. Dipterocarpaceous* plants are rich source of oligomeric stilbenes, some of which shows interesting biological properties. The oleoresin of the *Shorea robusta* is called as Shala niryasa, Kala, Sarja rasa which has the chemical constituents such as non-triterpene, dammarenolic acid, asiatic acid, dipterocarpol, triterpenic acid, tannic acid and phenolic content and possesses antibacterial, analgesic and wound healing effect. some of which show interesting biological properties, for example, as inhibitor of 5α-reductase (Sotheeswaran and Pasupathy, 1993), scavenger of superoxide (Hirano *et al.*, 2001) and cytotoxic agents (Tankara *et al.*, 2000). The *Shorea* species found in India are *S. assamica*, *S. robusta*, *S. roxburghii* and *S. tumbuggaia*. The last species is an endemic and globally endangered semi-evergreen tree species restricted to the southern Eastern Ghats up to 1000 m (Raju *et al.,* 2009). The medicinal property of the plant is highly influenced by the season in which it is cultivated and collected.

2.1.1. Chemical constituents of *S.robusta*:

Triterpenoids have been also reported from the resin of the southern indian sal tree (Saraswathy *et al.,* 1992). Several ursane derivatives have been isolated from resin of East Indian sal , ursolic acid, 2α,3β-dihydroxy-urs-12en-280ic acid, 2α, 3α-dihydroxy-urs-12-en-28-oic acid, 3 β, 23-dihydroxyolean 12-en-28-oic acid, 2α, 3β, 23-trihydroxy-urs-12-en-28-oic acid, 2a, 3β, 23-trihydroxy llβ methoxy-urs-12- en-28-oic acid are the isolated compounds. The resin, collected by tapping from the sal forest of Himalayan foot hills has yielded ursolic acid, α- and β- amyrin, magniferonic acid, benthamic acid, asiatic acid, α-amyrenone and uvaol as known compounds, of which only ursolic acid, asiatic acid have been previously reported from resin of *S.robusta.*

Triterpenoids like ursolic acid, α and β-amyrin, mangiferonic acid, benthamic acid, asiatic acid, α-amyrenone and uvaol have previously been reported. From the resin of *S.robusta* two

new triterpenoids, namely 3,25-epoxy-1,2,3,11-tetrahydroxyurs-12-en-28-oic acid and 3,25-epoxy 1,2,3-trihydroxyurs-l2-en-28-oic acid are isolated (Naznin and Hasan, 2009).

2.2. Literature Review of Pharmacology & Ethanobotanical of *S. Robusta*:

2.2.1. Pharmacology

1. Resin powder of *S.robosta* was tested in *Kohl-Chikni-Dawa* a compound ophthalmic formulation of Unani medicine on naphthalene induced cataracts in rats (Siddiqui *et al.,* 2002) and anticatarct activity of the *Kohl-Chikni-Dawa* a compound ophthalmic formulation of Unani medicine containing *S.robosta* in alloxan diabetic rats was tested (Siddiqui *et al.*,2003).
2. Ambrex a polyherbal formulation containing 10% *S.robusta* resin was found to be protective effect in ethanol induced gastric mucosal lesions in experimental rats (Narayan *et al.,* 2002).
3. Water extract of stem bark exhibits free radical scavenging activity in an antioxidant assay, the xanthine oxidase testing produced a 60% inhibition with *S.robusta* extract (Narayan *et al.,* 2002).
4. Gum resin of *Shorea robusta* had shown positive Brine shrimp (*Artemia salina*) Lethality Assay. LC_{50} value was found to be 100µg/ml after 24 h (Krishnaraju *et al.,* 2005).
5. Neem leaves along with Sal leaves are boiled in water and decoction is cooled and are taken once or thrice a day to cure diarrhoea and dysentery (Nayak *et al.,* 2004).The resin powder is used in Chicken pox (Nayak *et al.,* 2004). About 5gm of resin powder is taken along with warm milk to relieve chest pain and stomachache (Nayak., *et al.,* 2004).
6. *Shorea robusta* was reported as a fuel yielding, fuelwood, tree (Kataki and Konwer, 2002).
7. *Shorea robusta* Gaertn resin powder was reported to be used in the treatment of joint pain (Murugesa and Vaguppu, 1988).

8. Preparation of Carbonaceous Heavy Metal Adsorbent from Shorea robusta Leaf Litter Using Phosphoric Acid Impregnation (Surya *et al.*, 2011).

2.2.2. Ethanobotanical

1. Biodegradation of *Shorea robusta* Gaertn. leaf litter and the cycling of minerals in a tropical sal forest (shukla *et al.*,1984) chemosystematics and indigenous uses of *shorea* in south asia.
2. *Shorea robusta* – an excellent host tree for lichen growth in India (Singh *et al.*,2002).

2.3. Literature Review Role of Nanoparticles for Brain Delivery Systems:

(Patel *et al.*, 2012)

Particle size and surface characteristics of nanoparticles can be easily manipulated to achieve both passive and active drug targeting after parenteral administration. They control and sustain release of the drug during the transportation and at the site of localization, altering organ distribution of the drug and subsequent clearance of the drug so as to achieve increase in drug therapeutic efficacy and reduction in side effects. Controlled release and particle degradation characteristics can be readily modulated by the choice of matrix constituents. The use of biodegradable materials for nanoparticle preparation provides temporal as well as spatial control on drug release at the targeted site after injection over a period of days or even weeks. Drug loading is relatively high and drugs can be incorporated into the systems without any chemical reaction; this is an important factor for preserving the drug activity. Site-specific targeting can be achieved by attaching targeting ligands to surface of particles or use of magnetic steering. The system can be used for various routes of administration including oral, nasal, parenteral, intra-ocular etc. Due to their small size, nanoparticles can penetrate even into small capillaries and are taken up within cells, allowing an efficient drug accruement at the targeted sites in the body

2.3.1 Mechanism of Nanoparticle / Drug Transport Across BBB:

The BBB not only impedes the influx of intravascular substances from blood to brain, but also promotes transport of drug substance from blood to brain or from brain to blood through several transport systems such as carrier-mediated transport, active efflux transport etc.

Constructing nanoparticles with targeting ligands or coating them with agent can conduce to the development of carrier systems of radiated nature and target specific. The nanoparticles can gain access in the brain via, employment of a number of possible mechanisms. These mechanisms can explicate the movement of therapeutic bioactive agents across the BBB.

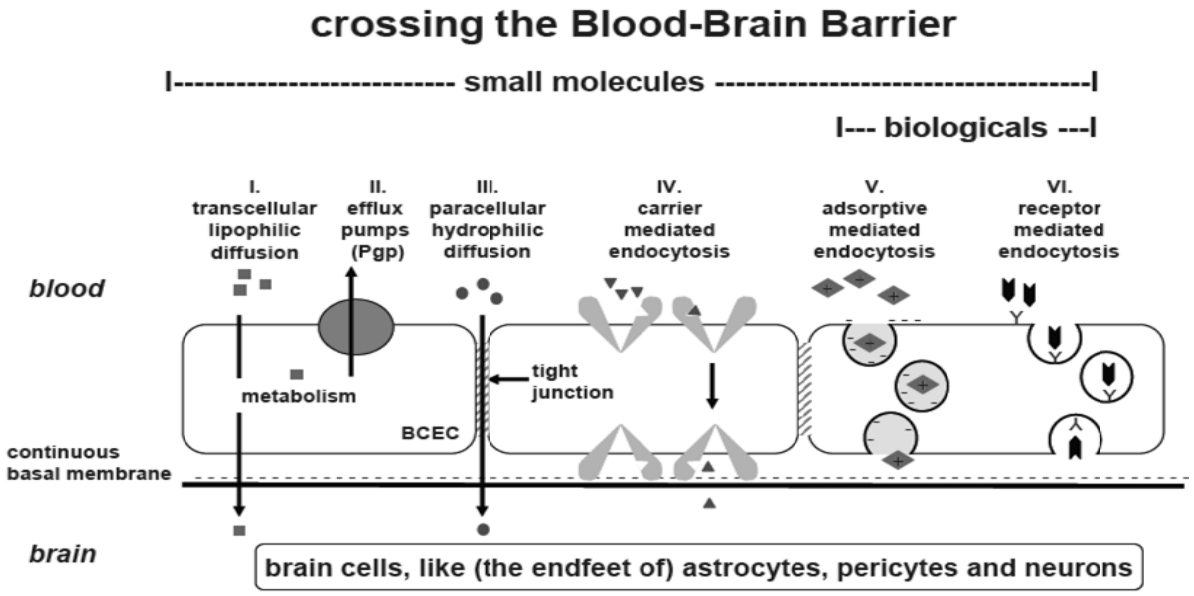


Fig. 2.1. Mechanism of Nanoparticle / Drug Transport Across BBB

Table 2.1. Polymeric Nanoparticulate Carrier to Shunt BBB

Formulation	Drug	Indication	Vector to Shunt BBB	Reference
PBCA Nanoparticles	Gemcetabine	Brain Tumor	Polysorbate 80	Chun X.W. et al
PBCA Nanoparticles	Rivastigmine	Alzhiemers Disease	Polysorbate 80	Barnsbas W. et al
Nanoparticles	---	---	Polysorbate 80	Kepan G. et al
PBCA Nanoparticles	Doxorubicin	Brain Tumor	Polysorbate 80 & Poloxamer 188	Petri B. et al
Polymeric Nanoparticles	---	---	PEG	Calvo et. Al
PEG-PLA Nanoparticles	---	---	Lactoferrin	Kaili Hu et al
PBCA Nanoparticles	---	---	Polysorbate 80	Alyautdin et al
Nanoparticle	Paclitaxel	Brain Tumor	Cetyl Alcohol / Polysorbate	Koziara et al
Nanoparticle	Penicillamine	Alzheimers Disease	---	Cui Z et al
PLGA Nanoparticles	---	---	Short Chain Synthetic Peptide	Costantino et al
Chitosan Nanoparticles	Peptide (Z-DEVD-FMK)	Capsase Inhibitor used in Cerebral Ischemia	PEG / OX 26	Aktas et al
PLGA Nanoparticles	Loperamide	Opioid Analgesic	Glycosylated Heptapeptide	Tosi et al
PBCA NPs	Loperamide, Dalargin	Opioid Analgesic	Apolipoprotein B & E	Kreuter et al.
PBCA NP	Kytophin	Analgesic	Polysorbate 80	Schroeder et al

2.4. Literature Review on Bovine Serum Albumin: (A. krishana *et al.*, 2011; Ji yeon *et al.,* 2011)

Albumin is an attractive macromolecular material and widely used to prepare nanoparticles, nanospheres and nanocapsules due to its availability in pure form and its biodegradability, nontoxicity and non immmunogenicity. Both Bovine Serum Albumin (BSA) and Human Serum Albumin (HSA) have been used to prepare drug delivery carriers. As a major plasma protein, albumin has a distinct edge over other materials for nanoparticle preparation. Albumin nanoparticles also carry reactive groups (thiol, amino, and carboxylic groups) on their surfaces that can be used for ligand binding and/or other surface modifications and also albumin nanoparticles offer the advantage that ligands can easily be attached by covalent linkage. Drugs entrapped in albumin nanoparticles can be digested by proteases or mechanical energy and drug loading can be quantified. A number of studies have shown that albumin accumulates in solid tumors making it a potential macromolecular carrier for the site-directed delivery of antitumor drugs.

Bovine serum albumin (BSA or "Fraction V") is a serum albumin protein derived from calfs. It is often used as a protein concentration standard. The "Fraction V" refers to albumin being the fifth fraction of the original Edwin Cohn purification methodology that made use of differential solubility characteristics of plasma proteins. By manipulating solvent concentrations, pH, salt levels, and temperature, Cohn was able to pull out successive "fractions" of blood plasma. The process was first commercialized with human albumin for medical use and later adopted for production of BSA.

2.4.1. Phase Seperation in Aqueous Medium:

BSA nanoparticles were prepared using a modified desolvation method. BSA powder was dissolved in distilled water. Desolvating agent was added continuously or intermittently into 1% BSA solution at pH 7 under stirring at 700 rpm at room temperature until the solution became just turbid. In continuous addition method desolvating agent was added continuously in the solution with rate addition about 1.0 to 2.0 ml per min and for intermittent method 2ml of desolvating agent was added for every 5 min interval (Merodio *et al.*, 2001).

2.4.2. Coacervation Technique:

Complex coacervation is a phase separation process that spontaneously occurs when two oppositely charged polyelectrolytes are mixed in an aqueous solution. Compared to other methods, this process can be performed entirely in an aqueous solution and at low temperature and thus has a better chance to preserve activity of the encapsulated substances. The colloidal particles produced are in the nanometer or micrometer scale depending on the substrates or the processing parameters used for example; pH, ionic strength and polyelectrolyte concentrations (Kaibara *et..al..*).

The major drawback of this technique is that complex coacervates have low drug loading efficiency and poor stability. Therefore, cross linking of the complex by chemical reagents such as toxic glutaraldehyde is necessary. It was implemented for preparation of BSA nanoparticles (Lazko *et al.*, 2004). Anhydrous ethyl alcohol was added to 150 ml BSA (5 mg/l in 10 mM Tris/HCl) contained 0.02% sodium azide, pH 7.5) till the solution became turbid then 150 µl of 25% glutaraldehyde was added for crosslinking. The reaction was continued at room temperature (24°C). Ethanolamine was added to block the non-reacted aldehyde functional group. Also Tween-20 was added at a final concentration of 0.01% (v/v) to stabilize the preparation. Large aggregates were eliminated by centrifuge (50,000 g, 30 min, 4°C). The supernatant was dialyzed and subsequently micro and ultrafiltrated through a 0.2 µm acetate membrane and polyvinylchloride copolymer membrane with cut off 300 kDa, respectively. The concentration of BSA determined with coomassie blue reagent. The size distribution and shape of BSA nanoparticles were determined by scanning electronic microscope.

2.4.3. Applications of BSA Nanoparticles:

Das & banerjee prepared aspirin loaded albumin nanoparticles are prepared by coacervation method. Albumin being both bioacceptable and biodegradable has a distinct advantage as a vehicle of drug delivery.

S. Segura & C. Gamazo studied the activity of IFN-γ, when it was either adsorbed or loaded in albumin nanoparticles.

2.5. Literature Review on Different Analytical Method for Asiatic Acid Determination:

Xiao-Chun Zheng *et al.*, 2009 reported High-performance liquid chromatography (HPLC) analyses were performed with a Jasco model equipped with a photodiode array detector (set at the wavelength of 248 nm), a 20 µl loop injection manual sampler and Empower software. For analysis, a reversed phase Water 100 RP-18 (250 cm × 4.6 mm; 5µ) end capped. Column was eluted with Methanol : Water (85:15) in gradient mode. The flow rate of 1 ml/min was maintained and the column eluent was monitored continuously at 248 nm. Quantitation of the compounds was carried out by measuring the peak areas in relation to those of standards chromatographed under the same conditions. Asiatic acid was eluted with a retention time of 12.00 min and monitored at 248 nm (Xiao-Chun Zheng *et..al..*).

R. grimaldi *et al.*, 1990 reported A new HPLC assay method was used to investigate the pharmacokinetics of asiatic acid after oral administration of the total triterpenic fraction of *Centella asiatica* in single doses (30 or 60 mg) and after a 7-day treatment (30 or 60 mg twice daily). Twelve healthy volunteers received each treatment following a randomized cross-over design with trials separated by a 3 week interval. The time of peak plasma concentration was not affected by dosage difference or by treatment scheme. Differences in peak plasma concentration and area under the concentration vs. time curve from 0 to 24 h. & calculated after 30 or 60 mg administration (single dose) were accounted for by the different dose regimen. However, after chronic treatment with both 30 and 60 mg, peak plasma concentrations, AUC & plasma concmetration at 24 h and half life were significantly higher than those observed after the corresponding single dose administration. This phenomenon could be explained by a metabolic interaction between asiatic acid and asiaticoside, which is transformed into asiatic acid in vivo.

For quantification of triterpenoids from plant extracts, the procedure used by Shiau *et al.*, 2009 was employed. To 0.25 mL of plant extract, 0.25 mL vanillin solution (10%) and 2.5 mL of sulphuric acid (72% w/v) were added and thoroughly mixed in an ice water bath. The mixture was then warmed in a water bath at 65ºC for 10 min and then cooled in ice-cold

water bath. Absorbance at 535 nm was recorded against the blank using a Shimadzu UV/Visible (160) spectrophotometer.

Phensri Thongnopnua *et al.*, 2008 repoted asiatic acid, an active metabolite of asiaticoside from the plant *Centella asiatica.* A single step liquid-liquid extraction and high-performance liquid chromatographic (HPLC) method have been developed and validated for the quantification of asiatic acid in human plasma. A reversed-phase C18 column was used for the separation of asiatic acid and acenaphthrene (internal standard) with a mobile phase composed of methanol and acetonitrile in ultra pure water (5:57:38, v/v), operated at the flow rate of 1.0 ml/min. The eluent was quantitated at 217 nm. The accuracy and precision of the method was confirmed for both intra-day and inter-day with the % bias and %RSD less than 8%. Recovery of asiatic acid from human plasma was 95.0%. The method would possibly be applicable for pharmacokinetic study of asiatic acid in human plasma following oral administration of *Centella asiatica* alcoholic extract or asiaticoside

2.6. Literature Review on Ligand Conjugated Loaded BSA Nanoparticles:

Patel *et al.*, 2012 reported that the least efficacy of most of the active pharmaceutical ingredients in the brain is attributed to the blood–brain barrier (BBB), which represents insurmountable obstacle for the effective management of majority of CNS disorders. The present research is planned with the objective to design novel poly (d, l) lactide (PLA) nanoparticles coupled with natural tripeptide i.e. glutathione to enhance drug delivery to brain. To evaluate the brain targeting efficiency of the glutathione conjugated nanoparticles, fluorescein sodium was explored as model compound due to its polar nature and least possibility to cross BBB. The entrapment efficiency of fluorescein sodium was improved by screening several formulation variables like drug: polymer ratio, solvent selection, electrolyte addition and pH alteration. Scanning Electron Micrograph (SEM) and dynamic light scattering results of optimized formulation showed that prepared nanoparticles have a round and regular shape with a mean diameter of 257.8 ± 3.78 nm with narrow size distribution. Biodistribution pattern and brain targeting potential of optimized glutathione conjugated PLA nanocarriers was determined using wistar rat as an animal model in comparison to non-conjugated PLA nanocarriers and fluorescein sodium solution. The results showed significant increase in fluorescein sodium uptake in brain with glutathione conjugated PLA nanoparticles as compared to fluorescein sodium solution. The present investigations demonstrated that glutathione can serve as a potential ligand for brain drug delivery, which was observed with glutathione coupled PLA nanoparticles resulted into enhanced delivery of drug nearly 5 folds in brain.

2.7. Literature Review on Coupling Agent for UV Method:

There is a great challenge in estimation of asiatic acid in a biological system, because of it weak chromophore. Hence, in present study we have developed the estimation method for asiatic acid in biological systems for its biodistribution study based on coupling reaction. Firstly carboxylic acid groups of asiatic acid was activated using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), which leads to formation of O-isoacylurea, followed by, nucleophilic attack of amine group of P-toludine on above intermediate resulted into the formation of an amide bond.

2.7.1. Carbodiimides: (Castro *et al.*, 1975)

For estimation of asiatic acid in biological system based on two step carbodiimide conjugation using EDAC as coupling agents. Dicyclohexylcarbodiimide (DCC) and diisopropylcarbodiimide (DIC) are commonly used to prepare amides, esters and acid anhydrides from carboxylic acids. These reagents can also convert primary amides to nitriles, which can be useful in organic synthesis but is a trouble some side reaction of asparagine and glutamine residues in peptide synthesis. Dicyclohexylurea, the byproduct formed from DCC, is nearly insoluble in most organic solvents and precipitates from the reaction mixture as the reaction progresses. Hence DCC is very useful in solution phase reactions, but is not appropriate for reactions on resin. DIC is used instead in solid phase synthesis since the urea byproduct is more soluble and will remain in solution. In certain applications, such as modifying proteins, ethyl-(N',N'-dimethylamino)propylcarbodiimide hydrochloride (EDC) is used. This carbodiimide reagent and its urea by-product are water soluble, so the byproduct and any excess reagent are removed by aqueous extraction. Carbodiimide activation of amino acid derivatives often causes a partial racemization of the amino acid. In peptide synthesis, adding an equivalent of 1-hydroxybenzotriazole (HOBt) minimizes this problem. The OBt esters that form as intermediates couple with primary amines with little racemization, although certain residues such as histidine may be troublesome. Coupling an amino acid derivative to a hydroxy-functionalized resin requires a catalytic amount of 4-(N,Ndimethylamino) pyridine (DMAP). The basic DMAP can produce undesirable levels of racemization, so no more than 0.15 equivalents should be used.

2.7.2. Phosphonium-Based Reagents:

To avoid the racemization and side reactions that can occur with carbodiimide reagents, many alternative reagents were developed to generate esters in situ. (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) is one of the first reagents developed. BOP does not generate asparagine and glutamine dehydration byproducts and racemization is minimal. BOP is also useful for preparing esters under mild conditions. It must be handled with caution as highly carcinogenic hexamethylphosphoramide is formed as a byproduct in coupling reactions. (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate couples amino acids as efficiently as BOP, but the by-products are less hazardous. Coupling reactions are rapid, being nearly complete within a few minutes. (Benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate may be used in place of BOP in peptide synthesis without loss of coupling efficiency (Kim *et al.*, 1994).

2.7.3. Aminium-Based Reagents:

Two other popular coupling reagents are O-(Benzotriazol-1-yl)-N,N,N’,N’-tetramethyluronium hexafluorophosphate (HBTU) and O-(Benzotriazol-1-yl)- N,N,N’,N’-tetramethyluronium tetrafluoroborate (TBTU) As their names reflect, these reagents were believed to have a uranium structure, but crystal and solution structure studies revealed that these reagents actually have aminium structure. Both are very efficient peptide coupling reagents with little racemization. Coupling reactions are complete in as little as six minutes and when HOBt is added, racemization can be reduced to insignificant levels.4 This makes these the reagents of choice in critical applications. TBTU was very effective, for instance, in key macrocyclization and coupling steps in the total synthesis of the macrocyclic peptide cyclotheonamide B.These reagents should in equal molar amounts relative to the carboxylic acid component of the coupling reaction. Excess HBTU and TBTU can react with the unprotected N-terminal of the peptide and form a guanylidine moiety that blocks further elongation of the peptide (Knorr *et al.*, 1989).

3.1. Aim:

Asiatic acid is a pentacyclic triterpene isolated from a variety of plants, including *Centella asiatica*, *Shorea robusta*, and reported as one of the major active constituent responsible for the their various pharmacological activities. One of the major pharmacological activity reported for asiatic acid is neuroprotective, cognitive behaviour. Delivery of drugs to the brain is one of the most challenging issues for the pharmaceutical research, as most of the neurotherapeutics exhibit limited transport across the blood brain barrier. asiatic acid being a triterpene has great limitation for its bioavaibility in brain, further there is a great challenge in estimation of asiatic acid in a biological system, because of it weak chromophore. Hence, in present study, an attempts have been made to develop asiatic acid loaded nanopaerticles based formulation to improves pharmacokinetic profile of the drug. We have also developed the estimation method for asiatic acid in biological systems for its biodistribution study. The present study is the first of its kind for a development of formulation of asiatic acid specifically design for targeting brain.

3.2. Objective:

To isolate asiatic acid from *shorea robusta* by different methods to increase yield of asiatic acid & optimization of isolation methods

To develop UV, HPTLC & HPLC methods for estimation of asiatic acid from different formulations based on derivatization and direct methods.

To prepare & evaluate asiatic acid loaded bovine serum albumin (BSA) nanoparticles

To prepare and evaluate glutathione conjugated asiatic acid loaded bovine serum albumin nanoparticles for brain trageting delivery

To perform the bio distribution study of asiatic acid, glutathione conjugated & unconjugated asiatic acid loaded BSA nanoparticles

To develop & perform HPLC method for estimation of asiatic acid in biological systems.

4. Materials & Method:

4.1. List of Equipments:

Table: 4.1. List of Equipments

Equipments	Manufacturing
High Performance Thin Layer Chromatography	Camag Distributor, Switzerland Anchrom Enterprise Pvt. Ltd. Mumbai, India.
UV/Visible spectrophotometer UV 2450	Shimadzu Scientific Instrument, Japan
Sonicator Bath	Trans-o-Sonic D-Compact, Mumbai, India
Hot air oven	EIE Instruments Pvt. Ltd., Ahmedabad, India
Digital pH meter	Elico LI 612 and ANALAB, Ahemdabad, India
Electronic Balance BL-220H	Shimadzu Analytical Pvt. Ltd., Japan
High Pefromance Liquid Chromatography	JASCO, Japan

4.2. Isolation & Characterization of Asiatic Acid:

4.2.1. Selection of Plant Material for Isolation of Asiatic Acid:

Asiatic acid is pentacylic triterpenoid generally found in many indigenous specie like *Centella asiatica* & *Shorea robusta*. Amongst them *Shorea robusta* was found with almost 11% w/w of asiatic acid and hence we have explored this species for further studies.

4.2.2. Isolation of Crude Asiatic Acid from *Shorea Robusta*:

Isolation of crude asiatic acid was done by three different steps:

Step 1: Extraction with petroleum ether (Defatting)

Step 2: Extraction of powder with 90% methanol

Step 3: Isolation of crude asiatic acid from methanolic extract

Step 1: Extraction with petroleum ether:

1. 1 kg of dry powder of was packed in to a clean soxhlet extraction unit.
2. Seven liters of petroleum ether (60-80^{o}C) was added and extracted for 24-36 h till all the components are soluble in petroleum ether.
3. Petroleum extract was collected and distilled in a distillation unit**,** then a net weight of 250 gm of ether extracts was obtained.
4. Petroleum ether extraction was used for defatted dried resin powder.

Step 2: Extraction of powder with 90% methanol:

1. The above dried powder was then extracted with 90% methanol and the extraction was carried out for 24-36 h till the total methanol extract was obtained.
2. The methanol soluble extract was distilled and finally 175 gm of the thick paste was obtained**.**

Step 3: Isolation of crude asiatic acid from methanolic extract:

1. 175 gm of thick paste of methanol extract was dissolved in 1% alcoholic potassium hydroxide solution on continuously stirring for 45 min to 1 h.

2. The solution was then filtered through filter paper to separate the undissolved particles diluted hydrochloric acid was added slowly under constant stirring, during which the asiatic acid was precipitated.
3. Precipitated solution was filtered under suction and precipitates were dried, the crude asiatic acid was obtained.
4. The crude asiatic acid was estimated by high performance thin layer chromatography.

4.2.3. Selection of Extraction Method:

Extraction method of asiatic acid from *Shorea robusta* was selected after comparing three different methods i.e conventional extraction using soxhlet apparatus, ultrasonication method and microwave assisted method to obtained highest yield of asiatic acid from powder. The extraction of asiatic acid by all three above mentioned methods were done by as per 4.2.2. The step 1 and step 3 were carried out same as by conventional method, while yield of asiatic acid in step 2 was compared between all three methods. After completion of step 3, estimation of crude asiatic acid was done using high performance thin layer chromatography as per method and comparison was made to select the suitable method with minimum time and maximum yield.

a. Extraction using conventional soxhlet method:

b. Extraction assisted through ultrasonication:

c. Extraction assisted through micro wave:

4.2.3. (b). Isolation of Asiatic Acid from *Shorea Robusta* by Ultrasonicator Probe Extraction Method:

The extraction of powder with 90% methanol was done by ultrasonicator probe

1. The above dried powder was then extracted with 90% methanol and the extraction was carried out for 1 h. at different time interval, the sample were collected for estimation of crude asiatic acid by High Performance Thin Layer Chromatography (HPTLC) to determine at which time the maximum amount of asiatic acid was obtained from the powder.
2. The methanol soluble extract was distilled and finally, the thick paste was obtained & further step 3 was carried out as per 4.2.2.

4.2.3. (c). Isolation of Asiatic Acid from *Shorea Robusta* by Microwave Assisted Extraction Method:

The extraction of powder with 90% methanol was done by microwave assisted extraction.

1. The above dried powder was then extracted with 90% methanol and the extraction was carried out for 10 minutes, at different time interval the sample were collected for estimation of asiatic acid by High performance Thin Layer Chromatography (HPTLC) to determine at which time the maximum amount of asiatic acid was obtained from the powder.
2. The methanol soluble extract was distilled and finally, the thick paste was obtained & further process was carried out as per 4.2.2.

4.2.4. Purification of Asiatic Acid by Column Chromatography

Vacuum Assisted Column Chromatography:

Silica gel (260 gm, 200-400 # size) was mixed with n-hexane to form a homogenous suspension and stirred using a glass-stirring rod to remove bubbles. The silica gel slurry was poured into a glass column (4.0cm × 75.0cm) to result in a packed silica column of height 45 cm and diameter 4 cm. The powder was carefully layered on the column gel bed. There was vacuum pump (operated at 5 psi pressure) was attached to column to suck mobile phase from column. Column was eluted grading from 100% Hexane, Ethyl acetate, Chloroform: Methanol (5:5). Fractions were collected in aliquots of 100ml. Collected fractions were concentrated on water bath at 70-80ºC. The column fractions were analyzed by thin layer chromatography (TLC, Merck, silica gel F254) / by high performance liquid chromatography.

4.2.5. Identification of Asiatic Acid by Thin Layer Chromatography:

Stationary phase - Pre-coated silica gel aluminum Plate 60F-254

Mobile phase – Chloroform: Methanol (90:10)

Standard preparation - Different concentration of Standard asiatic acid dissolved in methanol.

Sample preparation - Different extracted sample of asiatic acid dissolved in methanol.

Spraying Reagent - Anisaldehyde Sulphuric Acid

Mode of detection- Derivatization method

4.3. Analytical Method Development for Estimation of Asiatic Acid:

4.3.1. HPTLC Method for Estimation of Asiatic Acid from Sample:

4.3.1.1. Apparatus and Instruments:

- Pre-coated silica gel aluminum Plate 60F-254 (20 x 20 cm with 250 µm thickness) (E. Merck)
- Camag – 100 µl Applicator syringe (Hamilton, Bonaduz, Schweiz)
- Camag Applicator – Linomat V
- Camag – Twin trough chamber (10 x 10) with stainless steel lid
- Camag TLC scanner3 (UV-Densitometric scanning)
- UV cabinet with dual wavelength UV lamp
- Balance model: KEROY, Keroy (Balance) Pvt. Ltd.

4.3.1.2. Reagents and Materials:

- Sample of asiatic acid collected by Extraction method
- Methanol, AR grade, S. D. fine chemicals, Mumbai
- Standard asiatic acid, sigma aldrich
- Ethyl acetate
- Chloroform

4.3.1.3. Chromatographic Condition:

- **Stationary phase:** Pre-coated silica gel aluminum Plate 60F-254 (250µm thickness) (E. Merck) pre washed with methanol then dried for 20 min at 110°C.
- **Mobile phase:** Methanol : Chloroform (90:10)

- **Chamber saturation:** 20 min
- **Distance run:** 90mm
- **Band width:** 5 mm
- **Distance between two spots:** 4mm
- **Distance at the edges of the plate:** 10mm
- **Scanning wavelength:** 615 nm
- **Slit dimension:** 4 X 0.20 mm
- **Evaluation Mode:** Absorbance
- **Lamp:** Deuterium/Tungsten

4.3.1.4. Preparation of Standard Solution:

For Ultrasonicator Probe Extraction Method:

10 mg standard asiatic acid were accurately weighed into a 10 ml volumetric flask, dissolved in 5 ml methanol and the solution was made up to 10 ml. From the above stock solution 1ml is taken and diluted up to 10ml to prepare final concentration of 0.1mg/ml.

For Microwave Assisted Extraction Method:

10 mg standard asiatic acid were accurately weighed into a 10 ml volumetric flask, dissolved in 5 ml methanol and the solution was made up to 10 ml. From the above stock solution 1ml is taken and diluted up to 10ml to prepare final concentration of 0.1mg/ml, from stock solution, 1ml is taken and diluted up to 10 ml to prepare final concentration of 0.01mg/ml.

4.3.1.5. Preparation of Sample Solution:

For Ultrasonicator Probe Extraction Method:

The sample was collected at 10, 20, 30, 40 & 50 mins to determine at which time highest yield of crude asiatic acid was obtained from sample. The collected samples were accurately weighed into a 10 ml volumetric flask, dissolved in 10ml methanol. From the prepared solution 1 ml is taken and diluted up to 10 ml & spotted on the TLC plate under nitrogen stream using Linomat V to obtain final concentration of asiatic acid from sample at different time interval.

For Microwave Extraction Method:

The sample was collected at 1, 2, 5 & 10 mins at highest frequency to determine at which time highest yield of crude asiatic acid was obtained from sample. The collected samples were accurately weighed into a 10 ml volumetric flask, dissolved in 10ml methanol. From the prepared solution 1 ml is taken and diluted up to 10 ml & spotted on the TLC plate under nitrogen stream using Linomat V to obtain final concentration of asiatic acid from sample at different time interval.

4.3.1.6. Method Validation Parameter for HPTLC Method:

4.3.1.6. A. Preparation of Linearity Curve:

Linearity curve of asiatic acid was obtained using standard solution of 500-3000 µg/spot from the stock solution 500 ppm. Calibration curve of Average area v/s concentration was plotted.

4.3.1.6. B. Precision

Intraday and Interday Precision

Intraday and Interday precision of the asiatic acid were measured in terms of %RSD. The experiment was repeated three times in a day using different concentration for intraday precision and different concentrations on three different days for Interday precision. The concentration selected for intraday & interday precision was 1500µg/spot, 2000µg/spot & 2500µg/spot, respectively.

4.3.1.6. C. LOD and LOQ

Limit of Detection (LOD)

It is a quantitative parameter. LOD is the lowest concentration of the analyte in sample that can be detected, but not necessarily quantities precisely and accurately. LOD was calculated using following equations:

$$LOD = 3.3\ \frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

Limit of Quantification (LOQ)

It is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals.The LOQ also varies with the type of method employed and nature of samples. LOQ was calculated using following equations;

$$LOQ = 10\ \frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

4.3.2. UV- Visible Spectroscopy Method for Estimation of Asiatic Acid from Sample on Basis of Triterpenoids (Vaniline – Sulphuric acid Assay) (Nair *et al.*, 2009)**:**

4.3.2.1. Apparatus and Instruments:

- Shimazdu – UV Cabinet
- Balance model: KEROY, Keroy (Balance) Pvt. Ltd.

4.3.2.2. Reagents and Materials:

- asiatic acid – Sigma Aldrich
- Methanol, AR grade, S. D. fine chemicals, Mumbai
- Vaniline AR grade, CDH Laboratory
- Sulphuric acid 98% - Merck

4.3.2.3. Preparation of Standard Solution:

10 mg standard asiatic acid were accurately weighed into a 10 ml volumetric flask, dissolved in 5 ml methanol and the solution was made up to 10 ml to made final concentration 1 mg/ml. from stock solution, different concentration range from 10 to 300 µg/ml were prepared.

4.3.2.4. Procedure:

0.25 ml of sample was added in to the clean & dry test tube. 0.25 ml of 10% w/v of vaniline solution (1mg of vaniline AR grade dissolved in 98% ethanol) & 72%w/v 98% sulphuric acid AR grade were added in to the above test tube in ice cold condition. The resulting solution was placed on water bath for 10 min at 65°C to develop color. After 10 min the final solution was placed in ice cold water for 5 min & the resulting solution was determined at particular wavelength (535nm) by spectroscopic method.

4.3.2.5. Method Validation Parameter for Vaniline Sulphuric Assay:

4.3.2.5. A. Preparation of Linearity Curve:

Linearity curve of asiatic acid was obtained using standard solution of 10-300 µg/ml from the stock solution 1mg/ml. Calibration curve of Average absorbance v/s concentration was plotted.

4.3.2.5. B. Precision

Intraday and Interday Precision

Intraday and Interday precision of the asiatic acid were measured in terms of %RSD. The experiment was repeated three times in a day using different concentration for intraday precision and different concentrations on three different days for Interday precision. The concentration selected for intraday & interday precision was 20µg/ml, 30µg/ml & 40µg/ml, respectively.

4.3.2.5. C. LOD and LOQ

Limit of Detection (LOD)

It is a quantitative parameter. LOD is the lowest concentration of the analyte in sample that can be detected, but not necessarily quantities precisely and accurately. LOD was calculated using following equations:

$$LOD = 3.3\ \frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

Limit of Quantification (LOQ)

It is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals.The LOQ also varies with the type of method employed and nature of samples. LOQ was calculated using following equations;

$$LOQ = 10\ \frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

4.3.3. U.V. Method for Estimation of Asiatic Acid from Sample on Basis of Amide Bond Formation:

Asiatic acid has absorption at low UV wavelength range. But if UV wavelength is set at 200 nm, interferences from substances may be significant and may affect the specificity. A novel derivatization UV method was developed, with detection at 267 nm for the investigation of total concentration of asiatic acid in plasma after i.v. administration/ in formulation. P-toluidine (PTD) as the derivatizing reagent can be coupled with the free carboxylic acid group of asiatic acid. After derivatization, the maximum absorption wavelength of the derivative was 267nm at which interferences became insignificant and the response was greatly enhanced compared with unmodified asiatic acid detected at 200 nm.

4.3.3.1. Apparatus and Instruments:

- Shimazdu – UV Cabinet
- Balance model: KEROY, Keroy (Balance) Pvt. Ltd.

4.3.3.2. Reagents and Materials:

- asiatic acid – sigma Aldrich
- Methanol, AR grade, S. D. fine chemicals, Mumbai
- 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC or EDAC)
- N- hydroxy Succinimide
- P-toludine

4.3.3.3. Introduction to Carbodimides Reaction:

1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, usually known as water soluble carbodiimide, EDC, EDAC or WSC is a versatile modern coupling agent for the synthesis of pharmaceuticals. It is an easily handled solid which is now replacing (and is often superior to) N,N'-dicyclohexylcarbodiimide (DCC). EDC has a high solubility in water (> 200g/l) and is also soluble in a variety of organic solvents e.g. dichloromethane, tetrahydrofuran and dimethylformamide.

EDC reacts with carboxylic acid groups to form an active *O*-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture. The primary amine forms an amide bond with the original carboxyl group, and an EDC by-product is released as a soluble urea derivative. The *O*-acylisourea intermediate is unstable in aqueous solutions; failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyls, and the release of an N-unsubstituted urea.

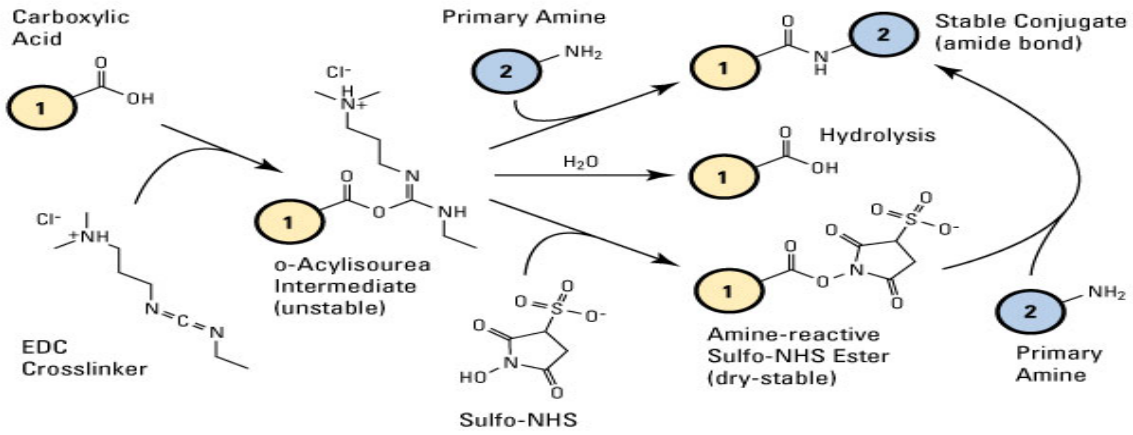


Fig. 4.1. Reaction Mechanisms of Carbodimides Reaction (Pancaud *et al.*, 2008)

EDC crosslinking is most efficient in acidic (pH 4.5) conditions and must be performed in buffers devoid of extraneous carboxyls and amines. MES buffer (4-morpholinoethanesulfonic acid) is a suitable carbodiimide reaction buffer. Phosphate buffers and neutral pH (up to 7.2) conditions are compatible with the reaction chemistry, albeit with lower efficiency; increasing the amount of EDC in a reaction solution can compensate for the reduced efficiency.

N-hydroxysuccinimide (NHS) or its water-soluble analog (Sulfo-NHS) is often included in EDC coupling protocols to improve efficiency or create dry-stable (amine-reactive) intermediates. EDC couples NHS to carboxyls, forming an NHS ester that is considerably

more stable than the *O*-acylisourea intermediate while allowing for efficient conjugation to primary amines at physiologic pH.

4.3.3.4. Preparation of Standard Solution:

10 mg standard asiatic acid were accurately weighed into a 10 ml volumetric flask, dissolved in 5 ml methanol and the solution was made up to 10 ml. from above solution, 1 ml is taken & diluted to 10 ml with methanol in volumetric flask to made final concentration of solution is 100µg/ml. from the stock solution, the calibration curve were prepared by different concentration range from 0.05µg/ml to 2.0 µg/ml of standard asiatic acid.

4.3.3.5. Preparation of Sample Solution:

0.25 ml of sample solution was added in to clean & dry test tube. 350 µl of EDC solution (freshly prepared, 2.5 mg/ml in dicholoromethane) and 150 µl of p-toludine solution (freshly prepared, 2.5 mg/ml dichloromethane) were added in above test tube & the resulting solution was kept for 3 hours at room temperature for complete reaction for formation of amide bond. The final solution was determined at particular wavelength by UV Spectroscopic Method.

4.3.3.6. Method Validation Parameter for Derivatization Method:

4.3.3.6. A. Preparation of Linearity Curve:

Linearity curve of asiatic acid was obtained using standard solution of 0.05-2.0 µg/ml from the stock solution 100 µg/ml. Calibration curve of Average absorbance v/s concentration was plotted.

4.3.3.6. B. Precision

Intraday and Interday Precision

Intraday and Interday precision of the asiatic acid were measured in terms of %RSD. The experiment was repeated three times in a day using different concentration for intraday precision and different concentrations on three different days for Interday precision. The concentration selected for intraday & interday precision was 0.2µg/ml, 0.1µg/ml & 0.8µg/ml, respectively.

4.3.3.6. C. LOD and LOQ

Limit of Detection (LOD)

It is a quantitative parameter. LOD is the lowest concentration of the analyte in sample that can be detected, but not necessarily quantities precisely and accurately. LOD was calculated using following equations:

$$LOD = 3.3\ \frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

Limit of Quantification (LOQ)

It is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals.The LOQ also varies with the type of method employed and nature of samples. LOQ was calculated using following equations;

$$LOQ = 10\ \frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

4.3.4. HPLC Method for Estimation of Asiatic Acid

4.3.4.1. Apparatus and Instruments:

HPLC

JASCO 200 Series HPLC model (JASCO, Inc JAPAN) with PU-2080 plus pump, MX-2080-31 mixer, Rheodyne model 7125 with 20µl fixed loop injector and Photo Diode Array detector was used. The software used was borwin version 1.50

pH Meter

pH meter 111E/101E (Analabs Scientific Instruments Ltd,) having resolution of ±0.01 pH and accuracy of ±0.01pH was used.

Analytical Balance

Model CX 220 analytical balance (CITIZEN, India) having capacity of 10 mg to 220 mg was used.

Sonicator

Model Trans-O-Sonic, D compact having capacity of 2 liter was used.

4.3.4.2. Reagents and Materials:

- Sample of asiatic acid collected by Extraction method
- Methanol, HPLC grade, S. D. fine chemicals, Mumbai
- Standard asiatic acid, Sigma Aldrich
- Millipore Water, Institute of science, Nirma University

4.3.4.3. Chromatographic Condition:

- **Column:** Water C18 column (250mm×4.6mm,i.d., 5µm, Dikma, USA)
- **Mobile phase:** Methanol HPLC grade: Water (75:25)
- **Column saturation:** 20 min
- **Run time:** 15 min
- **Flow rate:** 1 ml/min
- **Temperature:** Room temperature
- **Injection Volume:** 20 µl
- **Wavelength:** 220 nm at UV Wavelength
- **Blank:** Methanol
- **Evaluation Mode:** Area
- **Detector:** UV/PDA
- **Injection Mode:** Manual

4.3.4.4. Preparation of Standard Solution:

The stock solution of asiatic acid (2000 ppm) was prepared in high performance liquid chromatography grade methanol & stored at 4°C before use. The solutions stored at 4°C were found to be stable for at least 3 months.

100 mg of standard asiatic acid was dissolved in 50 ml volumetric flask with methanol. From asiatic acid stock solution, the different concentration range from 200 to 1800 ppm were diluted to 10 ml with methanol and appropriate volumes of these Asiatic acid solutions were injected into high performance liquid chromatography, respectively.

4.3.4.5. Preparation of Solution:

The prepared nanoparticle formulation sample was dissolved / dispersed in HPLC grade methanol & sonicate for 60 min. on water type sonicator. The resulting solution was filtered by 0.45 µm filter paper by vacuum pump to remove any particulate matter present in the sample solution if any particulate matter present in the sample solution causes blockage of column. The clear solution was injected in to the HPLC system at wavelength of 220 nm.

4.3.4.6. Method Validation Parameter for HPLC Method:

4.3.4.6. A. Preparation of Linearity Curve:

Linearity curve of asiatic acid was obtained using standard solution of 200-1800 µg/ml from the stock solution 2000 µg/ml. Calibration curve of Average area v/s concentration was plotted.

4.3.4.6. B. Precision

Intraday and Interday Precision

Intraday and Interday precision of the asiatic acid were measured in terms of %RSD. The experiment was repeated three times in a day using different concentration for intraday precision and different concentrations on three different days for Interday precision. The concentration selected for intraday & interday precision was 200µg/ml, 400µg/ml & 600µg/ml, respectively.

4.3.4.6. C. LOD and LOQ

Limit of Detection (LOD)

It is a quantitative parameter. LOD is the lowest concentration of the analyte in sample that can be detected, but not necessarily quantities precisely and accurately. LOD was calculated using following equations:

$$LOD = 3.3\,\frac{\sigma}{S}$$

Where, σ = Standard deviation of intercepts

S= Slope of straight line

Limit of Quantification (LOQ)

It is the lowest concentration of analyte in a sample that may be measured in a sample matrix such as impurities in bulk drug substances and degradation products in finished pharmaceuticals.The LOQ also varies with the type of method employed and nature of samples. LOQ was calculated using following equations;

$$LOQ = 10\,\frac{\sigma}{S}$$

Where, $\sigma = \text{Standard deviation of intercepts}$

S= Slope of straight line

4.3.5. HPLC Method for Estimation of Asiatic Acid in Plasma & Organs:

(Xiao chung *et al.,* 2009)

Asiatic acid has absorption at low UV wavelength range. But if UV wavelength is set at 200 nm, interferences from endogenous substances may be significant and may affect the specificity of HPLC. A novel precolumn derivatization reversed-phase high-performance liquid chromatography (RP-HPLC) method with UV–visible detection for the quantitative determination of total concentration of asiatic acid (AA) in plasma is described. Asiatic acid was extracted with n-hexane-dichloromethane-2-propanol (20:10:1, v/v/v) from plasma, which had been hydrolyzed by acid and derivatized with p-Toluidine. Chromatographic separation was achieved on a C18 column using gradient elution in a water–methanol system. Detection was set at UV wavelength of 255 nm.

4.3.5.1. Apparatus and Instruments:

HPLC

JASCO 200 Series HPLC model (JASCO, Inc JAPAN) with PU-2080 plus pump, MX-2080-31 mixer, Rheodyne model 7125 with 20µl fixed loop injector and Photo Diode Array detector was used. The software used was Borwin version 1.50.

pH Meter

pH meter 111E/101E (Analabs Scientific Instruments Ltd,) having resolution of ±0.01 pH and accuracy of ±0.01pH was used.

Analytical Balance

Model CX 220 analytical balance (CITIZEN, India) having capacity of 10 mg to 220 mg was used.

Sonicator

Model Trans-O-Sonic, D compact having capacity of 2 liter was used.

4.3.5.2. Reagents and Materials:

- Methanol, HPLC grade, S. D. fine chemicals, Mumbai
- Standard asiatic acid, Sigma Aldrich
- Millipore Water, Institute of science, Nirma University
- n-hexane-dichloromethane-2-propanol – CDH Laboratory
- 1-Ethyl-3-(3-dimethylaminopropyl)-Carbodiimides hydrochloride (EDC·HCl)
- P- Toludine
- Ursolic Acid – Internal Standard

4.3.5.3. Chromatographic Condition:

- **Column:** Water C18 column (250mm×4.6mm,i.d., 5µm, Dikma, USA)
- **Mobile phase:** The mobile phase solvent A was HPLC-grade water and the solvent B was methanol. A gradient elution was used to elute AA and UA derivatives from the column (0–8 min, 15% A, 85% B; 8–36 min, 15% A, 85% B→8% A, 92% B; 36–37min, 8% A, 92% B→0% A, 100% B; 37–43 min, 0% A, 100% B).
- **Column saturation:** 20 min
- **Run time:** 40 min
- **Flow rate:** 1 ml/min
- **Temperature:** 25°C
- **Injection Volume:** 20 µl
- **Wavelength:** 255 nm at UV Wavelength
- **Blank:** Methanol
- **Evaluation Mode:** Area
- **Detector:** UV/PDA
- **Injection Mode:** Manual

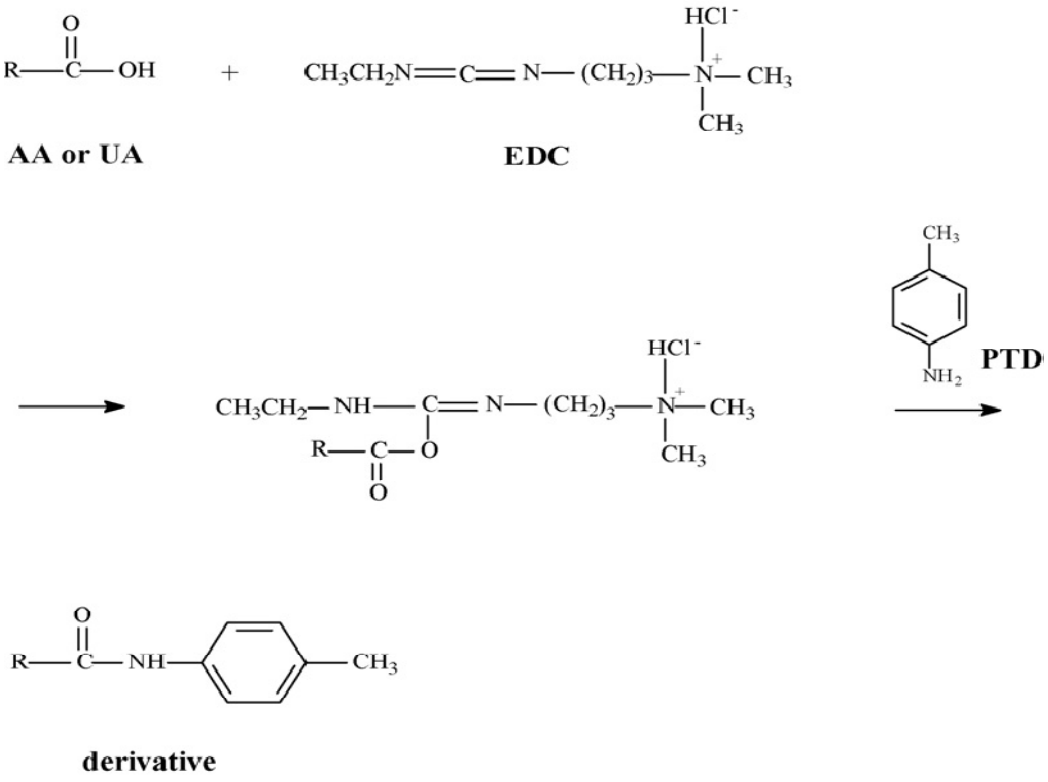


Fig. 4.2. Derivatization Scheme of Asiatic Acid/ Ursolic Acid with P-toludine. (Xiao chung *et al.*, 2009)

4.3.5.4. Preparation of Standard Solution:

The known standard concentration of asiatic acid (1000µg/ml) was spiked to blank plasma, 40 µl of internal standard solution was added and then extracted with 6 ml n-hexane-dichloromethane-2-propanol (20:10:1, v/v/v). After vortexing for 10min, sample was centrifuged at 5500rpm for 10min. The organic layer was transferred to another tube and evaporated to dryness in a water bath at 35^0C under a stream of nitrogen.

To the residue, 150 µl of PTD solution (2.5 mg/ml, prepared freshly in methylene dichloride) and 350 µl of EDC solution (2.5 mg/ml, prepared freshly in methylene dichloride) were added. The mixture was vortexed gently for 5min and then kept at 30°C for 3 h. The solution was evaporated to dryness under nitrogen. The residue was reconstituted with 200 µl of

methanol–water (85:15) and centrifuged at 12,000rpm for 5 min. An aliquot of 50 µl of the resulting solution was injected into the HPLC system for analysis.

4.3.5.5. Preparation of Solution:

To 1.0 ml of sample/plasma, 40 µl of internal standard solution was added and then extracted with 6 ml n-hexane-dichloromethane-2-propanol (20:10:1, v/v/v). After vortexing for 10min, sample was centrifuged at 5500rpm for 10min. The organic layer was transferred to another tube and evaporated to dryness in a water bath at 35°C under a stream of nitrogen.

To the residue, 150 µl of PTD solution (2.5 mg/ml, prepared freshly in methylene dichloride) and 350 µl of EDC solution (2.5 mg/ml, prepared freshly in methylene dichloride) were added. The mixture was vortexed gently for 5min and then kept at 30ºC for 3 h. The solution was evaporated to dryness under nitrogen. The residue was reconstituted with 200 µl of methanol–water (85:15) and centrifuged at 12,000rpm for 5 min. An aliquot of 50 µl of the resulting solution was injected into the HPLC system for analysis.

4.4. Preparation of Asiatic Acid Loaded BSA Nanoparticles for Brain Specific Delivery

4.4.1. Method of Preparation for Asiatic Acid Loaded BSA Nanoparticles (Patel *et al.*, 2012):

The asiatic acid loaded BSA nanoparticles were prepared modified desolvation method.

4.4.1.1. Asiatic Acid in Organic Phase:

Asiatic acid loaded BSA Nanoparticles were prepared by modified desolvation technique. 160 mg of Poloxamar and Brij-35 were dissolved in 20 ml water on magnetic stirring followed by solubilization of 100 mg bovine serum albumin in surfactant/stabilizer solution. asiatic acid solution was prepared in ethanol i.e. desolvating agent for BSA (Different ratio of asiatic acid dissolved in 20 ml of anhydrous 98% ethanol) and added dropwise to the polymeric solution aided with magnetic stirring to precipitate dissolved BSA as a nanoparticles encapsulated with asiatic acid. The nanoparticle matrix was rigidized by addition of cross linker i.e. glutaradehyde at a concentration 0.25 % v/v. The resulting nanoparticulate dispersion was stirred for 6 h at room temprature to complete the cross linking reaction. After rigidization, asiatic acid encapsulated BSA nanoparticles were seperated by centrifugation for 15 min. at 15000 g from the dispersion followed by washing with distilled water to remove excess of glutaraldehyde and unentrapped asiatic acid.

4.4.1.2. Asiatic Acid in Aqueous Phase:

160 mg of Poloxamar and Brij-35 was dissolved in 20 ml water on magnetic stirring, then followed by solubilization of 100 mg bovine serum albumin in surfactant/stabilizer solution. The sufficient quantity of asiatic acid in powder foam was added in to polymeric solution & the solution containing different ratio of asiatic acid were sonicate for 2 h on water bath sonicator . The 20 ml of anhydrous ethanol 98% solution added drop wise to the polymeric solution aided with magnetic stirring to precipitate dissolved BSA as a nanoparticles

encapsulated with asiatic acid. the nanoparticle matrix was rigidized by addition of cross linker i.e. glutaraldehyde at a concentration 0.25 % v/v. The resulting nanoparticulate dispersion was stirred for 6 h at room temperature to complete the cross linking reaction. After rigidization, asiatic acid encapsulated BSA nanoparticles were separated by centrifugation for 15 min at 15000 g from the dispersion followed by washing with distilled water to remove excess of glutaraldehyde and unentrapped asiatic acid.

4.4.1.3. Preparation of Glutathione Conjugated Asiatic Acid Loaded BSA Nanoparticles:

Glutathione was explored as a vector for enhancing drug delivery to brain. Glutathione was coupled to the surface of asiatic acid loaded BSA nanoparticles using two step Carbodiimides chemistry. First step involved activation of free carboxylic groups of the surface BSA nanoparticles (60 mg) by addition of equal volume of EDAC (1-ethyl-3-(3-dimethyl aminopropyl) Carbodiimides hydrochloride) solution (60 mg/ml) in 2 ml of phosphate buffer (pH 4.0). the mixture was incubated at room temperature for 45 minutes, the unreacted EDAC was removed by centrifugation and the nanoparticles were resuspended in 2 ml phosphate buffer (pH 7.4). After activation of carboxylic acid group of BSA nanoparticles, same volume of glutathione solution (60mg/ml) was added to the dispersion containing and mixture was shaken gently at room temperature for 3-6 h. (Leila *et al.*, 2003) the conjugated nanoparticles were centrifuged for 15 min. at 15000 g to remove excess of glutathione. Finally, glutathione conjugated nanoparticles were lyophilized for 24 h. (EIE Instruments Pvt. Ltd. Ahmadabad) and stored at 4°C till further use.

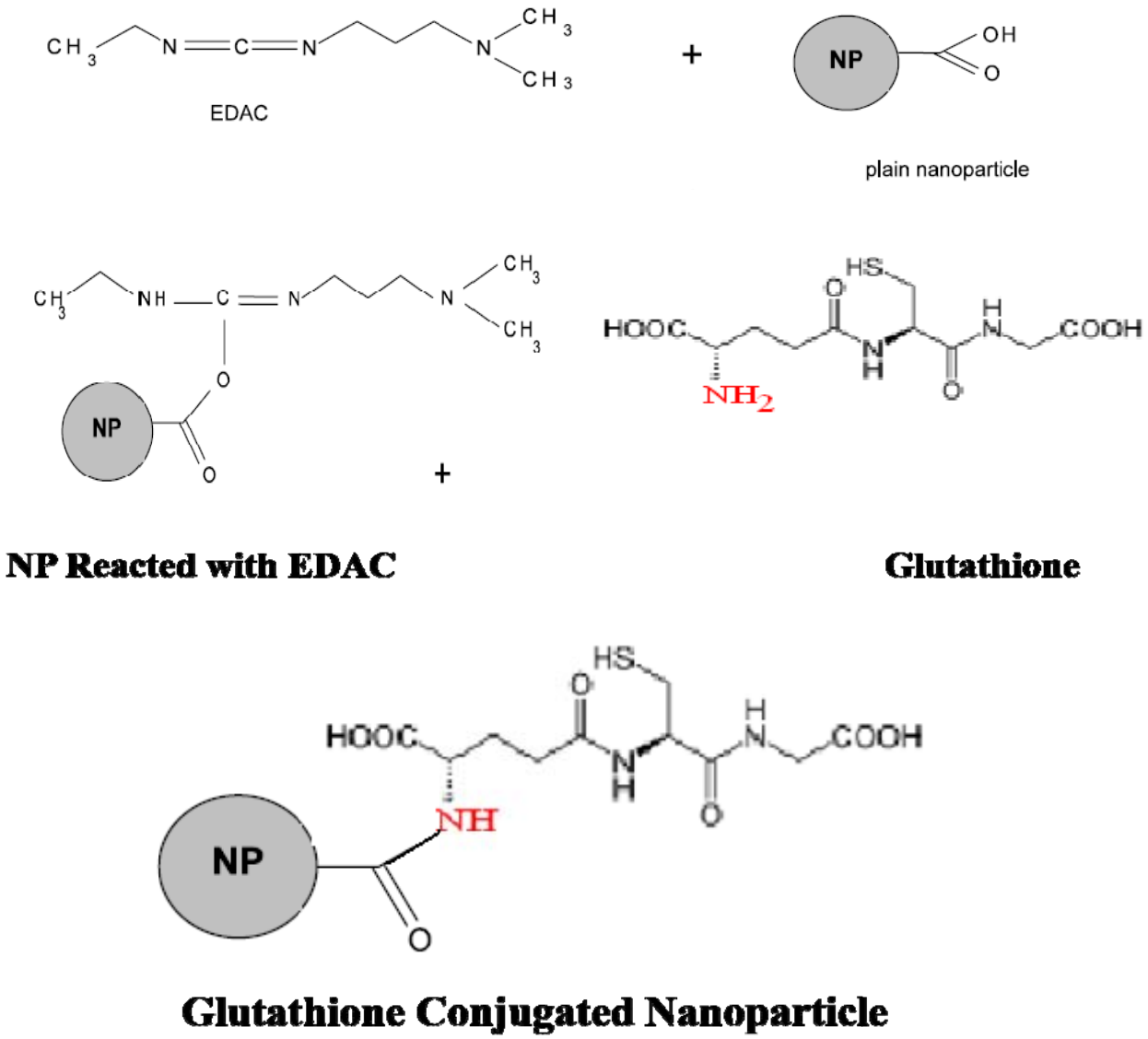


Fig. 4.3. Conjugation Mechanism of Glutathione with Nanoparticles (Patel *et al.*, 2012)

4.5. Characterization of Asiatic Acid Loaded BSA Nanoparticles:

4.5.1. Determination of Process Yield

The process yield of BSA nanoparticles containing asiatic acid was determined as weight percentage of final product after with respect to the intial total amount of drug, polymer and other solid materials used for the preparation.

$$\%\ Process\ yield\ = \frac{Practical\ yield}{Theoretical\ yield} \times 100$$

4.5.2. Particle size

The particle size was determined by dynamic light scattering, using a Malvern system 4700 instrument, with vertically polarized light supplied by an argon-ion laser (Cryonics) operated at 40 mW. All experiments were performed at a temperature of 25.0±10°C at a measuring angle of 900 to the incident beam.

4.5.3. Zeta Potential Analysis:

Zeta potential of BSA nanoparticles containing asiatic acid with and without conjugated glutathione were separately measured in deionized water using Malvern instruments.

4.5.4. Determination of Drug Loading Capacity: (Genta *et al.*, 1997)

The 20 mg of nanoparticles containing asiatic acid were digested with 50 ml Methanol for 6 h on water type sonicator bath. After complete digestion, samples were centrifuged at 15000 g for 15 min for removal of polymeric fragments and content of asiatic acid measured by spectrophotometrically/ HPLC.

\

$$\% \text{ Entrapment Efficiency} = \frac{\text{Amount of drug present in sample}}{\text{Total amount of drug taken}} \times 100$$

$$\% \text{ Drug Loading } = \frac{\text{Amount of drug present in sample}}{\text{Total amount of Nanoparticles}} \times 100$$

4.5.5. Quantitative Estimation of Glutathione with Ellman's Reagent (Leila *et al.*,2003)

The approximate numbers of glutathione units conjugated to the nanoparticle surface were determine using Ellman's assay. Briefly 30 mg of glutathione conjugated nanoparticles dispersed in 2.5 ml of phosphate buffer pH 7.2 containing 1mM EDTA and to this suspension 0.25 ml of (5,5'- dithio-bis(2-nitrobenzoic acid) DTNB solution (Ellman's reagent) was added. The mixture was incubated for 20 min at room temperature under mild stirring. Then, the nanoparticulate suspension was centrifuged at 15000g for 15 min and supernant layer was collected. To the collected supernatant layer 2 ml of 0.3M sodium hydrogen phosphate solution was added and concentration of glutathione was measured spectrophotometrically at 412 nm.

4.5.6. *In Vitro* Release Studies:

The *in vitro* release study was done by dialysis bag technique. The dialysis bag was soaked in respective solution for 24 h. The release of the asiatic acid from optimized nanoparticles formulations were studied by dialysis bag diffusion technique in phosphate buffer with pH 4.0 and 7.4, which represents the endo- lysosomal compartment pH and physiological pH. Nanoparticles equivalents to 1 mg of asiatic acid was placed in a dialysis bag and sealed at both the ends with clips. The dialysis bag then immersed into the receptor compartment containing 30 ml of phosphate buffer and stirred continuously at 75± 5 rpm and maintained at 37± 0.5°C. The receptor compartment was closed with lid to prevent evaporation of the dissolution medium. Samples were withdrawn at regular time interval & same volume was replaced with fresh dissolution medium. The samples were measured by spectrophotometrically / HPLC method against dummy nanoparticles, prepared as reagent

blanks and treated similarly to the asiatic acid BSA loaded nanoparticles (Barnadas *et al.,* 2008).

4.5.7. Biodistribution & Brain Targeting Study:

The brain targeting efficiency and biodistribution study of the glutathione coupled nanoparticles formulation was carried out on healthy wistar rats. The animals were divided into three groups, each group containing six animals. All animals received a dose equivalent to 75mg/kg (Lee *et al.,* 2012) of asiatic acid in phosphate buffer saline through lateral tail vein. Group 1 received Asiatic acid alone solution in PBS, group 2 received a BSA loaded asiatic acid nanoparticles suspended in PBS and group 3 received Glutathione conjugated BSA loaded NPs suspended in PBS. After 2 hr, 5 hr and 24 hr. of post injection, the animals were euthanized and the brain, spleen, kidney, liver and lungs were removed, washed, weighed and stored at -20ºC till further analysis. The collected organs were rinsed, whipped and homogenized with 10 volume of ice cold PBS with pH 7.4 followed by a 3-6 h. Extraction at room temperature in the dark. The mixtures were then centrifuged at 15000g for 15 min. The concentration of asiatic acid in the supernatant was measured by HPLC method against blank organ homogenate (Morten *et al.,* 2004).

4.5.8. Differential Scanning Colorimetry (DSC):

DSC studies were performed to established compatibility behavior between drug and selected excipients in solid state. Briefly, drug, BSA, its physical mixture (1:1) and optimized asiatic acid loaded BSA nanoparticles (drug to polymer ratio 1:5) was subjected to thermal analysis and observed for its melting event.

5. Results & Discussions:

5.1. TLC Profile of Asiatic Acid:

TLC plate was developed in the mobile system, chloroform: methanol (90:10). The spots were derivatized by derivatization agent i.e. using anisaldehyde sulphuric acid agent, showing 0.61±0.02 Rf value for asiatic acid in sample.

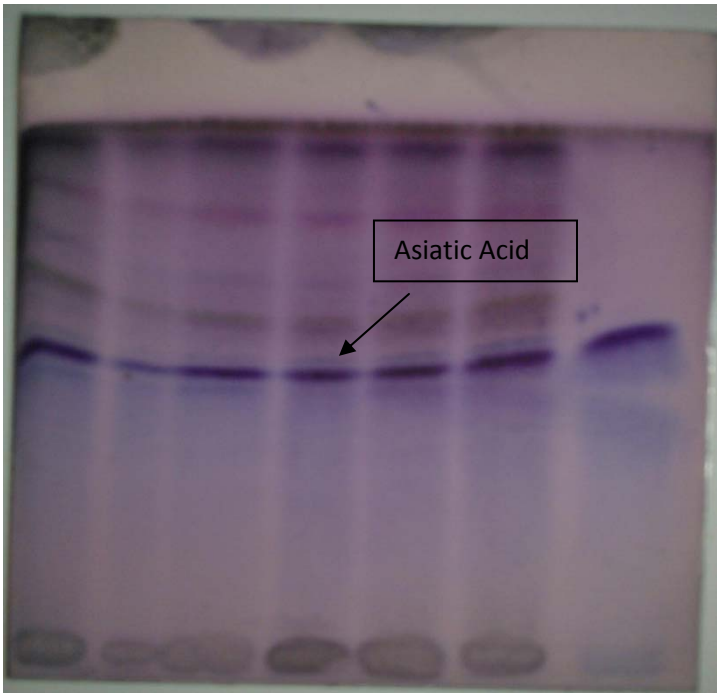


Fig.5.1. TLC Profile of Asiatic Acid

Rf Value of Asiatic acid =

$$\frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

From the TLC profile, Rf value of asiatic acid was found to be 0.61±0.02 with reference to standard asiatic acid.

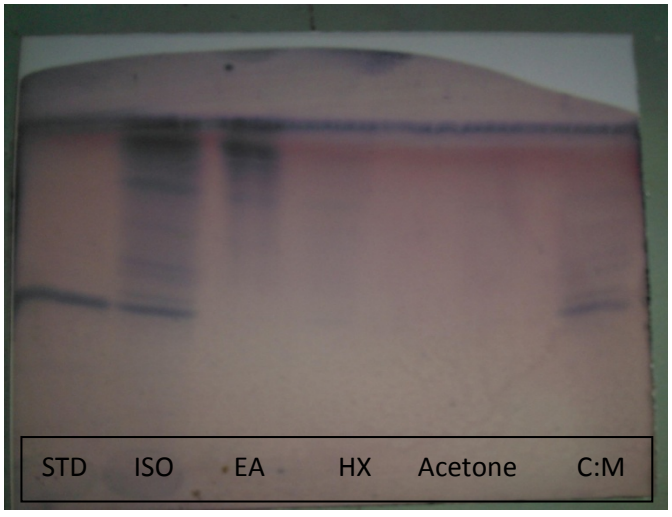
5.2. % Yield of Crude Asiatic Acid & Purification of Asiatic Acid by Column Chromatography:

The highest yield of crude asiatic acid from powder was found to be 0.712 gm obtained by ultrasonicator probe process. The % yields of crude asiatic acid obtained by different extraction method are shown in following table.

Sr No.	Extraction Method	Yield of Crude Asiatic Acid (5 gm)
1	Conventional Method	0.538 gm
2	Microwave Assisted	0.61 gm
3	Ultrasonicator Probe	0.712 gm

Table: 5.1. Yield of Asiatic Acid by Different Methods of Extraction

The % w/w yield of crude asiatic acid were found to be 10.76%w/w, 12.2%w/w & 14.24% w/w by Conventional method, Microwave assisted extraction & Ultrasonicator probe extraction, respectively.



STD.: Standard Asiatic Acid, **ISO:** Isolated Asiatic Acid, **EA:** Ethyl Acetate Fraction, **HX:** Hexane Fraction, **C: M (5:5):** Cholorofoam: Methanol Fraction

Fig. 5.2. TLC Profile of Asiatic Acid by Column Chromatography

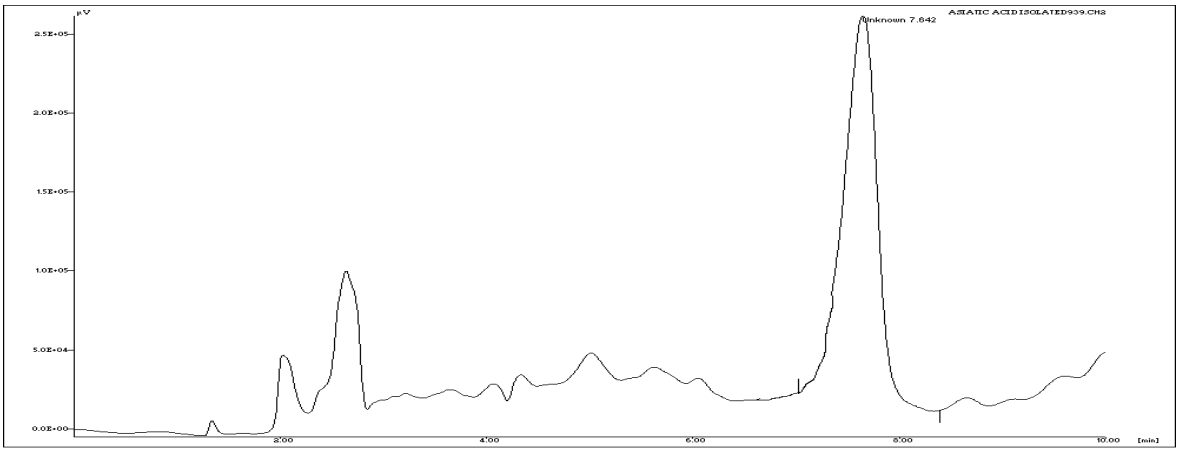


Fig.5.3. HPLC Chromatogram of Asiatic Acid by Column Chromatography

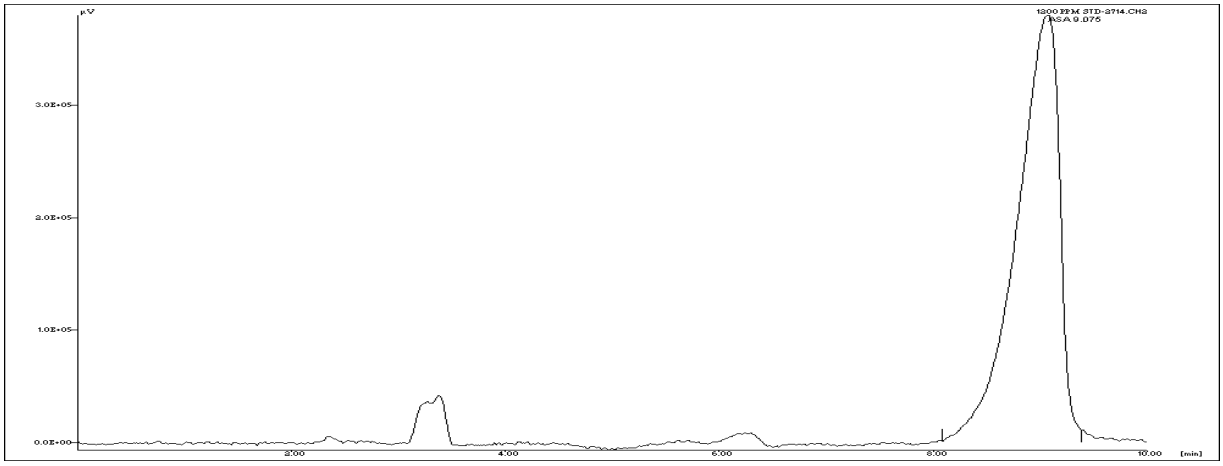


Fig.5.4. HPLC Chromatogram of Standard Asiatic Acid

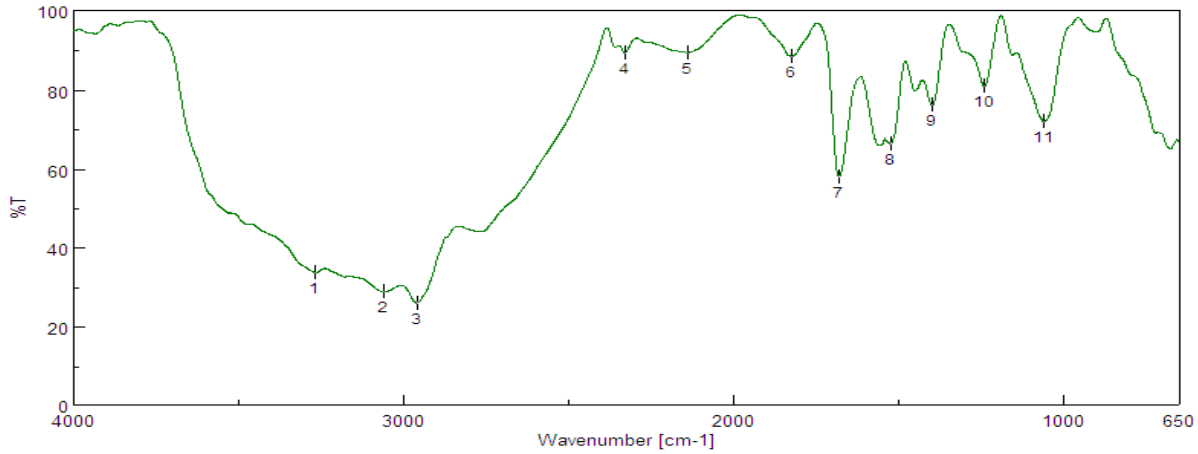


Fig.5.5. IR Spectra of Asiatic Acid by Column Chromatography

From the result, the retention time of asiatic acid purified by column & standard have a same retention time. The IR Spectra showed that IR pattern of asiatic acid was same as the standard. The HPLC & IR showed that major amount of other constituents were removed by column chromatography.

5.3. HPTLC Method Development for Estimation of Asiatic Acid:

The sample extracts were collected at different time interval during ultrasonication & microwave assisted extraction process to determine at which time the highest yield of crude asiatic acid was achieved.

TLC plate was developed in the mobile system, chloroform: methanol (9:1). The spots were detected in UV 615 nm, showing 0.62±0.02 Rf value for crude asiatic acid in sample. The plate was scanned at the 615 nm in Camag (TLC) Scanner.

5.3.1. HPTLC Fingerprint Results for Ultrasonicator Probe Extractor:

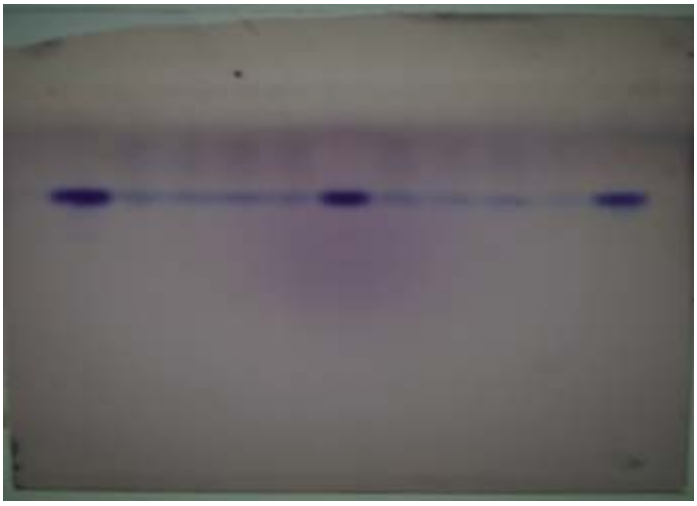


Fig.5.6. HPTLC Method Development for Estimation of Asiatic Acid in Sample at Different Time Interval by Ultrasonicator Probe Extraction

The HPTLC fingerprinting result showed that maximum amount of crude asiatic acid was extracted from the sample at **40 min** by Ultrasonicator probe extractor.

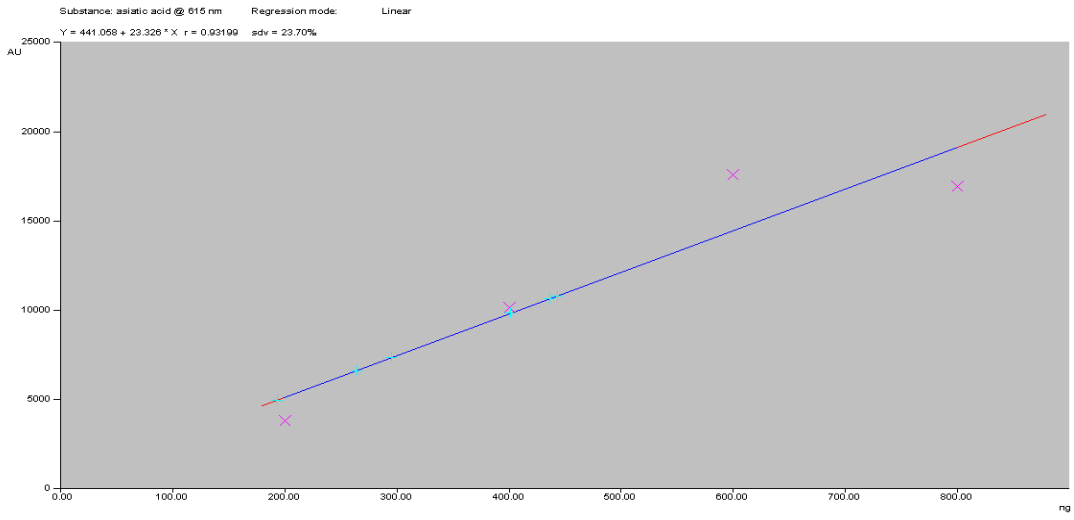
The HPTLC fingerprinting result showed that maximum amount of crude asiatic acid was extracted from the sample at **1 min** at higher frequency by Microwave Assisted Extraction.

Table: 5.2. HPTLC Fingerprinting Showing Respective Rf and Amount in All Tracks When Scanned at 615nm for Sample at Different Time Interval by Ultrasonicator Probe.

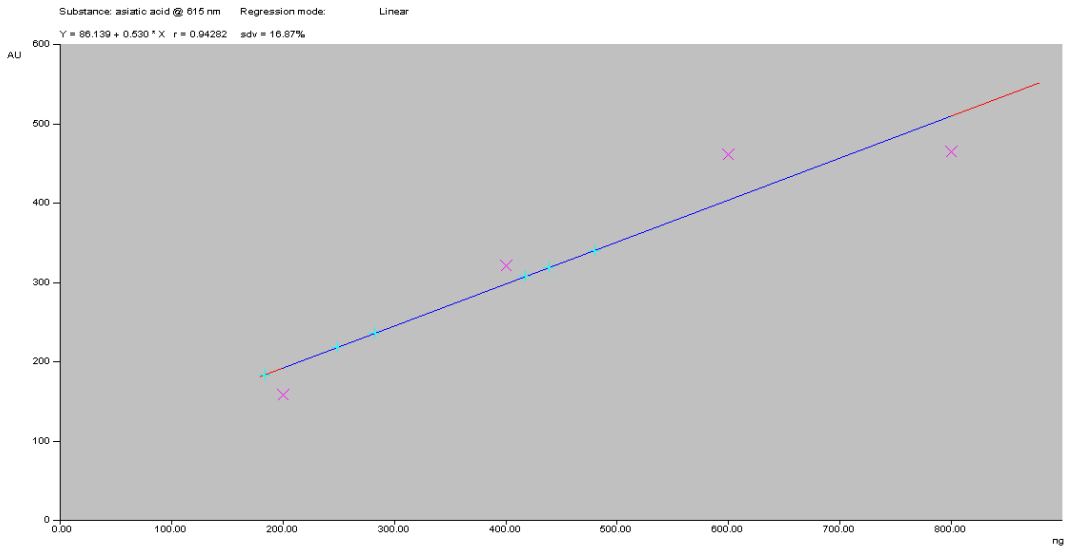
Track	Vial	Rf	Amount Present In Sample at Dift. time
200 ng	1	0.62	-
10 min	2	0.62	183.90 ng
20 min	3	0.62	248.53 ng
400 ng	1	0.62	
30 min	4	0.62	282.01 ng
40 min	**5**	**0.62**	**439.23 ng**
600 ng	1	0.64	-
50 min	6	0.64	417.49 ng
800 ng	1	0.64	-

5.3.2. Graph Area & Graph Height of Ultrasonicator Probe Extractor:

The r^2 value for area & height were obtained when scanned at 615 nm for sample at different time interval:



Y= 86.13 + 0.53*X **R^2: 0.9319**



Y=441.05 + 23.32*X **R^2: 0.9428**

5.3.3. HPTLC Fingerprint Results for Microwave Assisted Extractor:

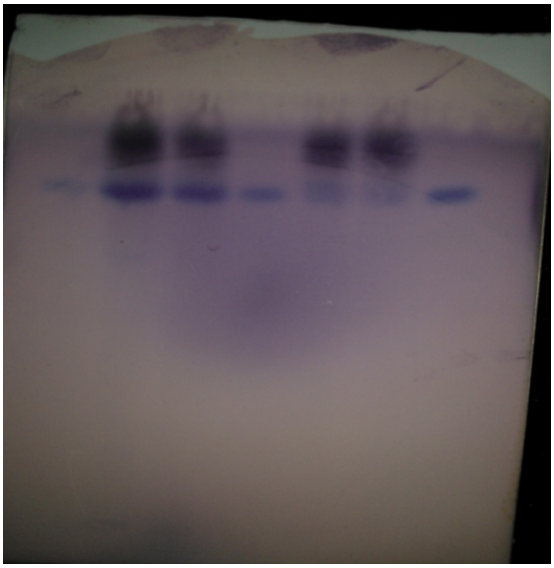


Fig.5.7. HPTLC Method Development for Estimation of Asiatic Acid in Sample at Different Time Interval by Microwave Assisted Extraction

TLC plate was developed in the mobile system, chloroform: methanol (9:1). The spots were detected in UV 615 nm, showing 0.68±0.02 Rf value for crude asiatic acid and in sample. The plate was scanned at the 615 nm in Camag (TLC) Scanner.

Table: 5.3. HPTLC Fingerprinting Showing Respective Rf and Amount in All Tracks When Scanned at 615nm for Sample at Different Time Interval by Ultrasonicator.

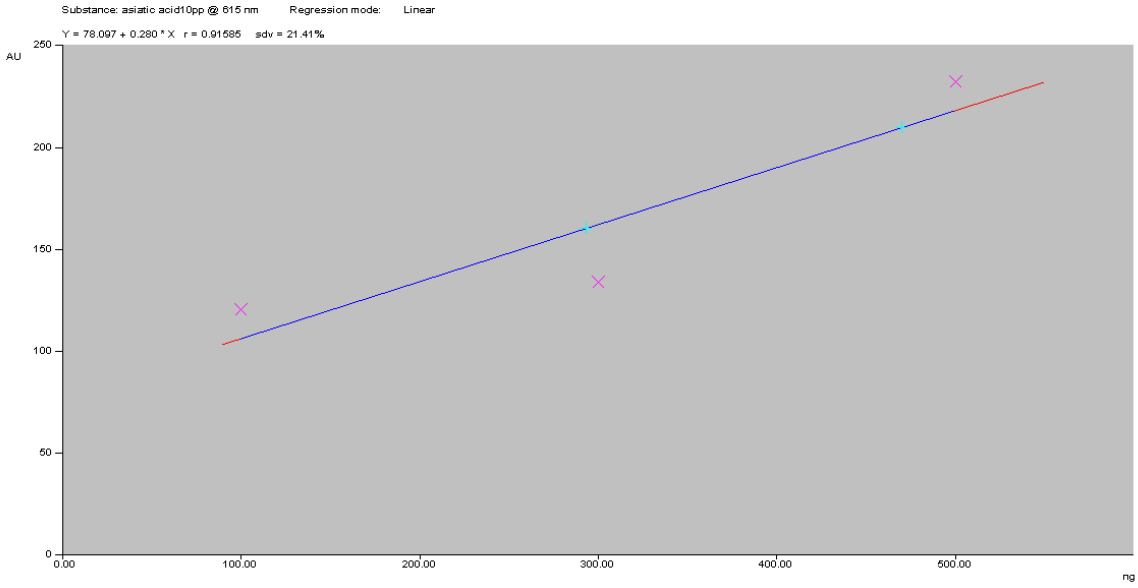
Track	Vial	Rf	Amount Present In Sample at Dift. time
100 ng	1	0.68	-
1 min	**2**	**0.68**	**469.79 ng**
2 min	3	0.68	292.95 ng
300 ng	1	0.68	
5 min	4	0.68	89.8 ng
10 min	5	0.68	87.5 ng
500 ng	1	0.68	-

5.3.4. Graph Area & Graph Height of Ultrasonicator Probe Extractor:

The r^2 value for area & height were obtained when scanned at 615 nm for sample at different time interval:

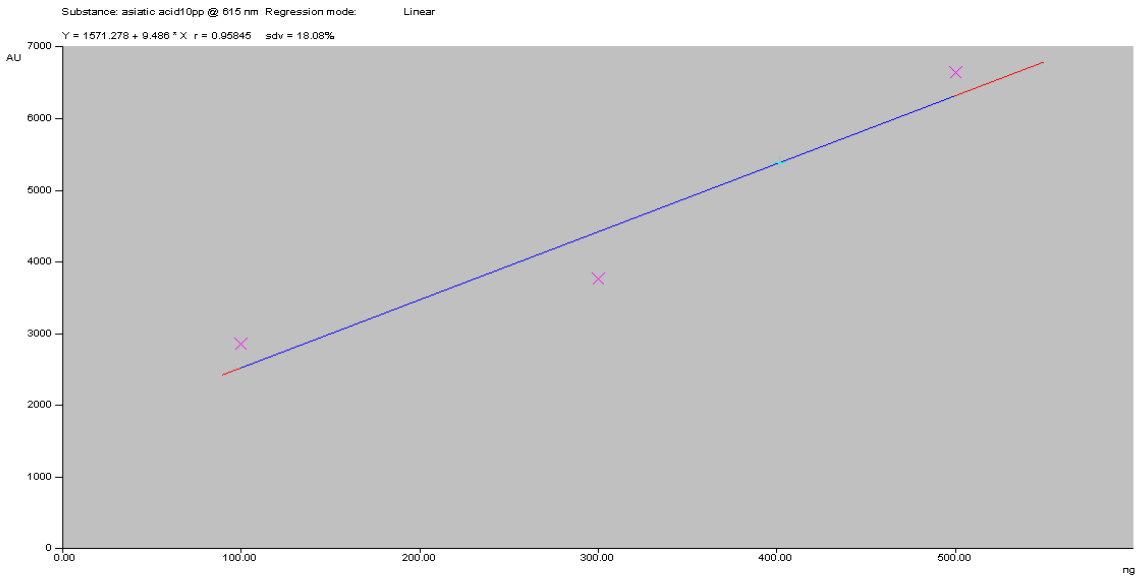
Y= 78.09 + 0.28*X

R^2: 0.915



Y= 1571.27 + 0.48*X

R^2: 0.958



From HPTLC result, it can be concluded that highest yield of crude asiatic acid from sample were extracted at 40 min by ultrasonicator probe & at 1 min by microwave assisted extractor.

5.3.5. Validation Parameter for HPTLC Method:

5.3.5. A. Linearity:

Asiatic acid showed linear response in range 500-3000 µg/spot in methanol with correlation co-efficient of 0.990 at 615 nm.

Table: 5.4. Linearity of Asiatic Acid (500-3000 µg/spot) at 615 nm

Conc. (µg/mL)	Mean Absorbance ± S.D.*	
500	1428±12.10	
1000	2487±10.25	
1500	3307±24.26	
2000	4258±09.25	
2500	4925±1.25	
3000	5507±0.25	
Linearity Equation		Y=1.637x + 786
Correlation Coefficient		0.990
Slope		1.637
Intercept		786

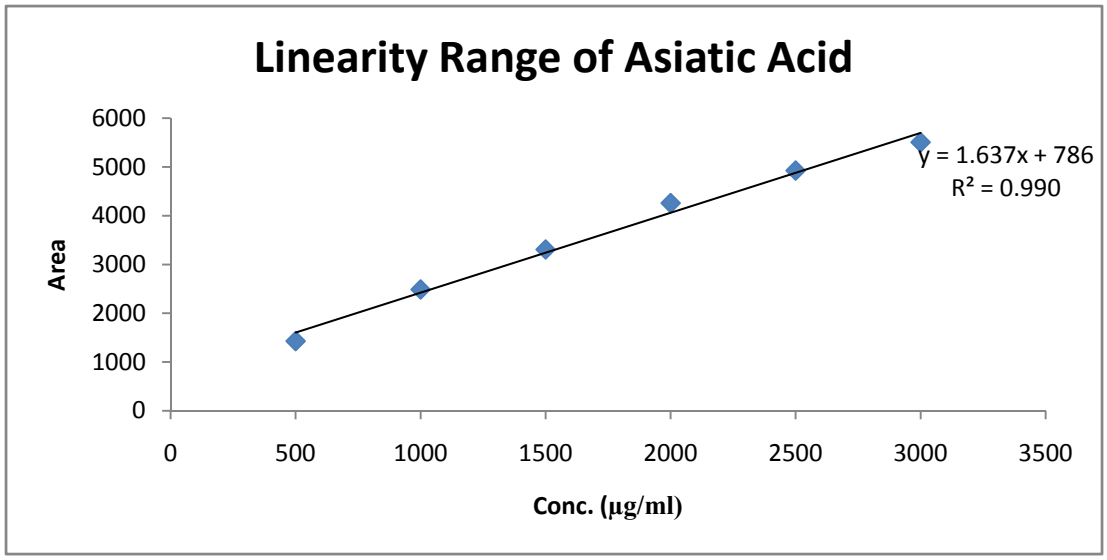


Fig. 5.8. Linearity Graph of Asiatic Acid by HPTLC Method

5.3.5. B. Precision:

A) Intraday Precision:

Table: 5.5. Intraday Precision for Asiatic Acid (615 nm)

Sr. No	Concenration (µg /spot)	Peak area		Rf value
		Mean ± SD	% RSD	
1	1500	3307±4.28	1.25%	0.62
2	2000	4260.6±6.46	1.5%	0.61
3	2500	4927.6±8.27	1.7%	0.61

n=3*

Intraday precision was determined by measuring the area at three different concentrations levels for three times in a day. The concentrations selected were 1500, 2000 & 2500 µg/spot for asiatic acid. The % RSD was found to be 0.125%, 0.15% & 0.17%, respectively.

B) Interday Precision:

It was determined by measuring the area at three different concentrations levels for three days. The concentrations selected were 1500, 2000 & 2500 µg/spot for asiatic acid. The % RSD was found to be 0.16%, 0.105% & 0.147%, respectively.

Table: 5.6. Interday Precision for Asiatic Acid (615 nm)

Sr. No	Concenration (mg /spot)	Peak area		Rf value
		Mean± SD	% RSD	
1	1500	3287±5.28	1.6	0.62
2	2000	4239.6±4.46	1.05	0.60
3	2500	4935±7.27	1.47	0.63

n=3*

5.3.5. C. LOD & LOQ:

Table: 5.7. LOD & LOQ of Asiatic Acid by HPTLC Method

Drug	LOD (ng/spot)	LOQ (ng/spot)
Asiatic Acid	200	600

5.4. HPLC Result for Estimation of Asiatic Acid from Sample:

The calibration curve of asiatic acid was prepared in methanol from concentration range from 200 to 1800 µg/ml. The retention time of asiatic acid were found to be 7.2 to 8.1 minutes depending up on the temperature conditions. In conclusion, a simple, sensitive and selective HPLC method with UV detection for the determination of total concentration of asiatic acid in sample was developed. The assay had been successfully applied to study the asiatic acid in different kind of formulation.

Table: 5.8. Calibration Curve of Asiatic Acid in Methanol at Wavelength of 220 nm

Sr No.	Concentration of Asiatic Acid (µg/ml)	Retention Time (min.)	Area (µv. sec)
1	200	7.23	2087398
2	400	7.2	3807403
3	600	7.5	5419179
4	800	7.83	6959949
5	1000	7.9	7932712
6	1200	7.5	9837312
7	1400	8.1	11678711
8	1600	8.1	12116558
9	1800	8.2	13725132

5.4.1. Determination of Retention Time of Asiatic Acid:

The different concentration of standard solution of asiatic acid was injected individually into HPLC and analysed as per optimized HPLC condition to determine Rt of asiatic acid. Peak of asiatic acid in methanol chromatogram was confirmed by comparison of Rt of asiatic acid in methanol and Rt of isolated asiatic acid in powder. The retention time of asiatic acid was found to be 7.5 min.

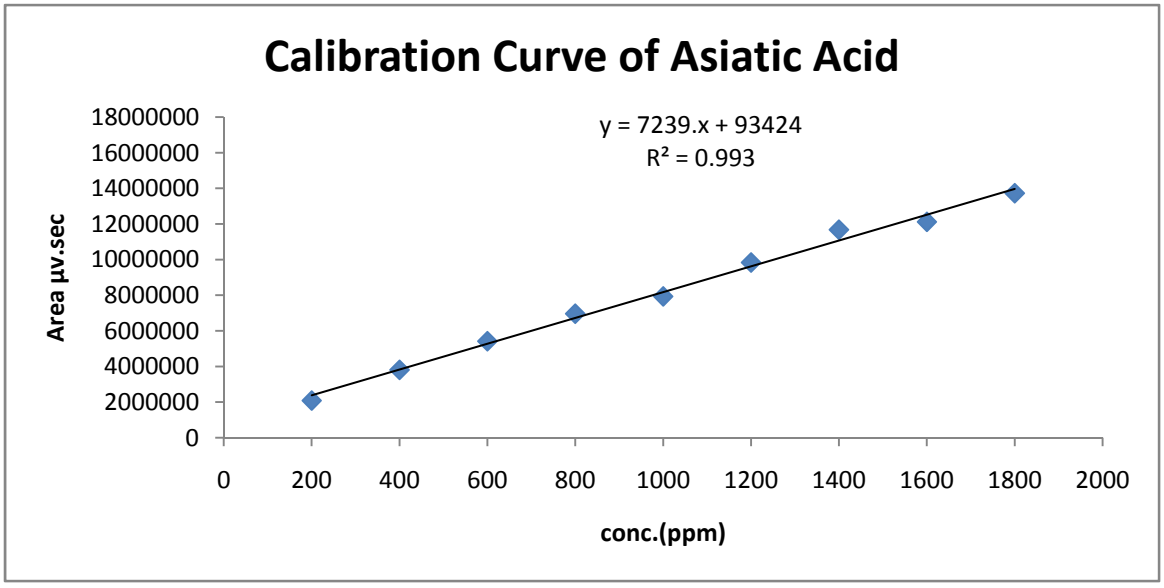


Fig.5.9. Calibration Curve of Asiatic Acid at 220 nm

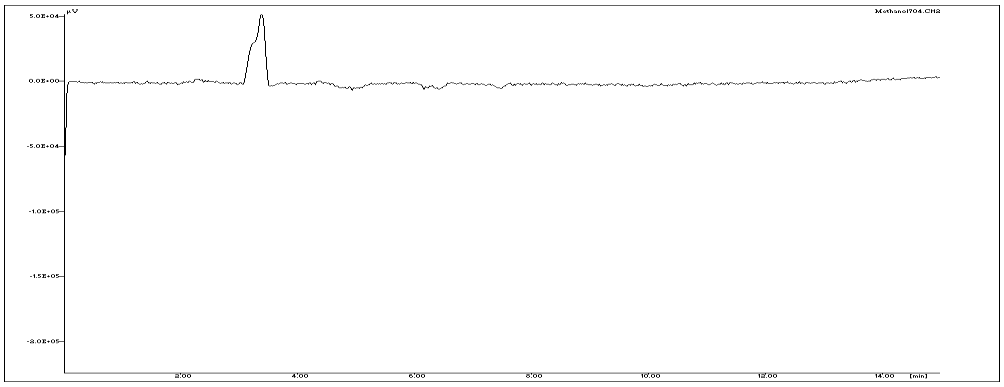


Fig.5.10. HPLC Chromatogram of Blank Methanol at 220 nm

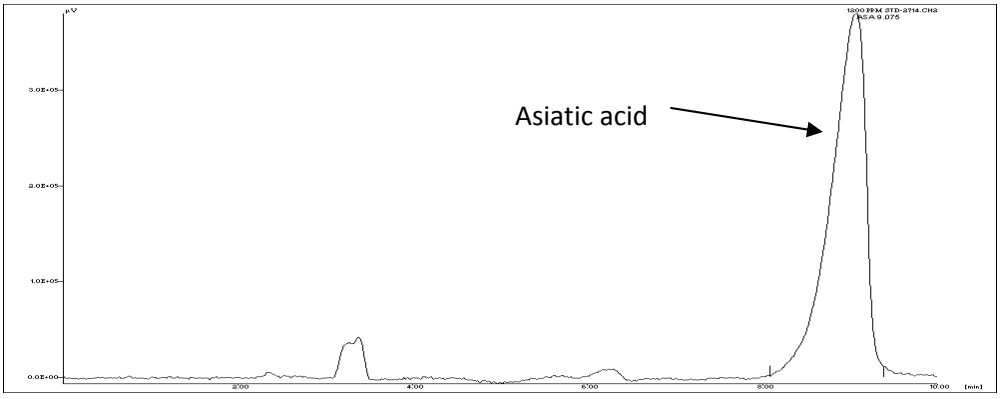


Fig.5.11. HPLC Chromatogram of 1200 µg/ml Standard Asiatic Acid

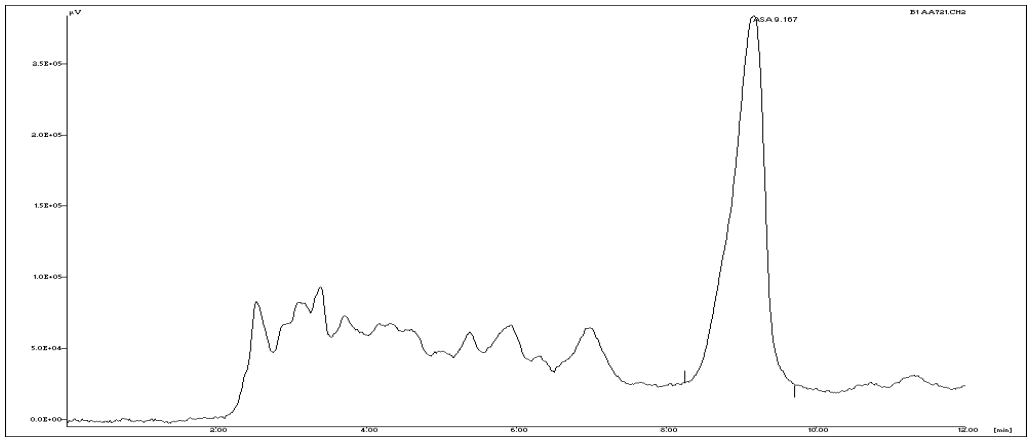


Fig.5.12. HPLC Chromatogram of Isolated Asiatic Acid at 220 nm

5.4.2. Validation Parameter for HPLC Method:

5.4.2. A. Linearity:

Asiatic acid showed linear response in range 200-1800 µg/ml in methanol with correlation co-efficient of 0.993 at 220 nm.

Table: 5.9. Linearity of Asiatic Acid (200-1800 µg/ml) at 220 nm

Linearity Equation	Y=7239x + 93424
Correlation Coefficient	0.993
Slope	7239
Intercept	93424

5.4.2. B. Precision:

A) Intraday Precision:

Intraday precision was determined by measuring the area at three different concentrations levels for three times in a day. The concentrations selected were 200, 400 & 600 µg/ml for asaitic acid. The % RSD was found to be 0.15%, 0.17% & 0.04%, respectively.

Table: 5.10. Intraday Precision for Asiatic Acid (220 nm)

Conc. (ppm)	Peak Area	Conc. (ppm)	Mean Peak Area ± S.D.*	Mean Conc. (ppm) ± S.D.*	% R.S.D.
200	2087398	275	2087396 ± 6.80	275.4 ± 0.44	0.15
	2087401	275.88			
	2087388	275.44			
400	3807403	513.05	3807314 ± 103.8	513.03 ± 0.9	0.17
	3807201	513.02			
	3807338	513.04			
600	4419178	597.56	4419178 ± 2	597.6 ± 0.24	0.04
	4419180	597.98			
	4419176	597.55			

n=3*

B) Interday Precision:

It was determined by measuring the area at three different concentrations levels for three days. The concentrations selected were 200, 400 & 600 µg/ml for asaitic acid. The % RSD was found to be 0.11%, 0.16% & 0.11%, respectively.

Table: 5.11. Interday Precision for Asiatic Acid (220 nm)

Conc. (ppm)	Peak Area	Conc. (ppm)	Mean Peak Area ± S.D.*	Mean Conc. (ppm) ± S.D.*	% R.S.D.
200	2087391	275.44	2087385 ± 23.5	275.5 ± 0.31	0.11
	2087405	275.90			
	2087359	275.30			
400	3807399	513.05	3807332 ± 70.19	513.4 ± 0.87	0.16
	3807259	513.03			
	3807338	513.04			
600	4419158	597.55	4419167 ± 12.6	597.6 ± 0.6	0.11
	4419182	597.56			
	4419162	597.561			

n=3*

5.4.2. C. LOD & LOQ:

Table: 5.12. LOD & LOQ of Asiatic Acid by HPTLC Method

Drug	LOD (µg/ml)	LOQ (µg/ml)
Asiatic Acid	0.0006	0.00209

5.5. UV - Visible Spectroscopy Result for Estimation of Asiatic acid from Sample:

The estimation of asiatic acid was done by vaniline sulphuric acid at 535 nm based on Triterpenoids. The calibration curves of asiatic acid were prepared by different concentration from range of 10 to 300 µg/ml. From the graph, the r^2 value was found to be 0.990. A simple, precise and accurate UV- Visible spectrophotometric method was developed for estimation of asiatic acid based on Triterpenoids.

Table: 5.13. Calibration Curve of Asiatic Acid by Vaniline Sulphuric Assay at 535 nm

Conc. (µg/mL)	Mean Absorbance ± S.D.*	% R.S.D.
10	0.067 ± 0.0005	0.74
20	0.078 ± 0.0005	0.65
30	0.087 ± 0.0002	0.22
40	0.092 ± 0.0001	0.11
50	0.122 ± 0.015	0.12
100	0.204 ± 0.0011	0.54
150	0.25 ± 0.001	0.40
200	0.351 ± 0.0005	0.14
250	0.379 ± 0.0015	0.39
300	0.440 ± 0.0015	0.34
Linearity Equation		y = 0.001x +0.054
Correlation Coefficient		0.990
Slope		0.001
Intercept		0.0.054

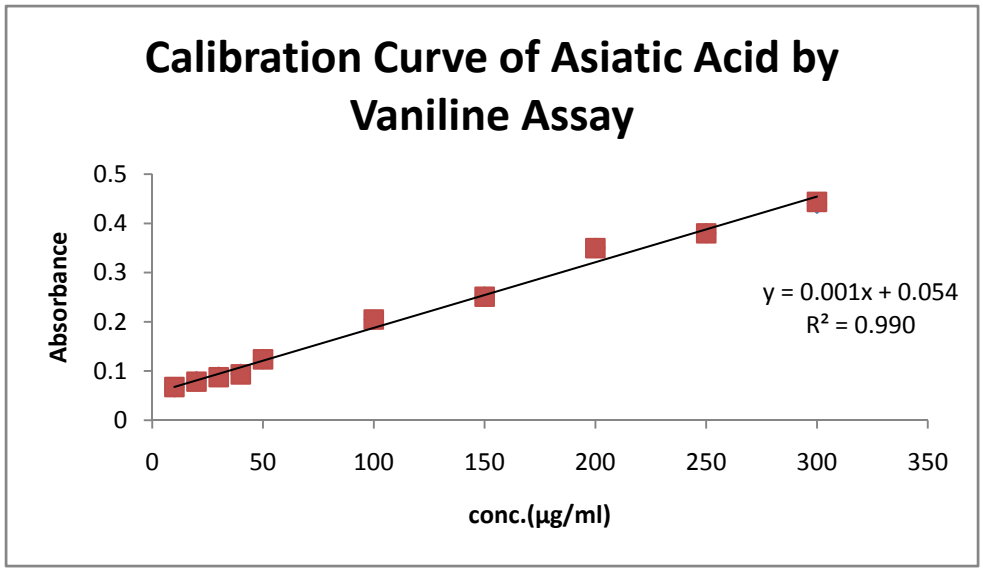


Fig.5.13. Calibration Curve of Asiatic Acid at 535 nm

5.5.1. Validation Parameter for UV-Visible Method:

5.5.1. A. Linearity:

Asiatic acid showed linear response in range 10-300 µg/ml in methanol with correlation co-efficient of 0.990 at 535 nm.

5.5.1. B. Precision:

A) Intraday Precision:

Intraday precision was determined by measuring the area at three different concentrations levels for three times in a day. The concentrations selected were 20, 30 & 40 µg/ml for asaitic acid. The % RSD was found to be 2.66%, 4.6% & 2.5%, respectively.

Table: 5.14. Intraday Precision of Asiatic Acid at 535 nm

Conc. (µg/mL)	Absorbance	Conc. (µg/mL)	Mean absorbance ± S.D.*	Mean Conc. (µg/mL) ± S.D.*	% R.S.D.
20	0.078	22	0.0773 ± 0.001	21.66 ± 0.57	2.66
	0.076	21			
	0.078	22			
30	0.087	30	0.0866 ± 0.0015	29.66 ± 1.52	4.6
	0.085	28			
	0.088	31			
40	0.092	38	0.093± 0.001	39 ± 1.0	2.5
	0.093	39			
	0.094	40			

n=3*

B) Interday Precision:

The concentrations selected were 20, 30 & 40 µg/ml for asaitic acid. The % RSD was found to be 0.66%, 0.4% & 2.7%, respectively.

Table: 5.15. Interday Precision of Asiatic Acid at 535 nm

Conc. (µg/mL)	Absorbance	Conc. (µg/mL)	Mean absorbance ± S.D.*	Mean Conc. (µg/mL) ± S.D.*	% R.S.D.
20	0.074	20	0.072± 0.001	18.6 ± 0.5	0.66
	0.073	19			
	0.071	17			
30	0.075	21	0.077 ± 0.002	23.6 ± 0.1	0.4
	0.078	24			
	0.080	26			
40	0.091	37	0.0089± 0.001	36± 1	2.7
	0.090	36			
	0.088	35			

n=3*

5.5.1. C. LOD & LOQ:

Table: 5.16. LOD & LOQ of Asiatic Acid by UV-Visible Spectroscopy Method

Drug	LOD (µg/ml)	LOQ (µg/ml)
Asiatic Acid	3.3	10

5.6. UV Spectroscopy Result for Estimation of Asiatic Acid from Plasma & Sample Based on Amide Bond Formation

The developed UV spectroscopy method for estimation of asiatic acid in plasma or sample based on formation of amide bond. Firstly, carboxylic group of asiatic acid were activated using 1-Ethyl-3-(3- dimethylaminopropyl) carbodiimide, which leads to formation of o-isoacylurea, followed by nucleophilic attack of amine group of p-toludine on above intermidate, resulted in to formation of amide bond. The resulting solution was estimated by UV spectroscopy at 267 nm. The calibration curve was prepared from different concentration range of 0.05 µg/ml to 2.0 µg/ml.

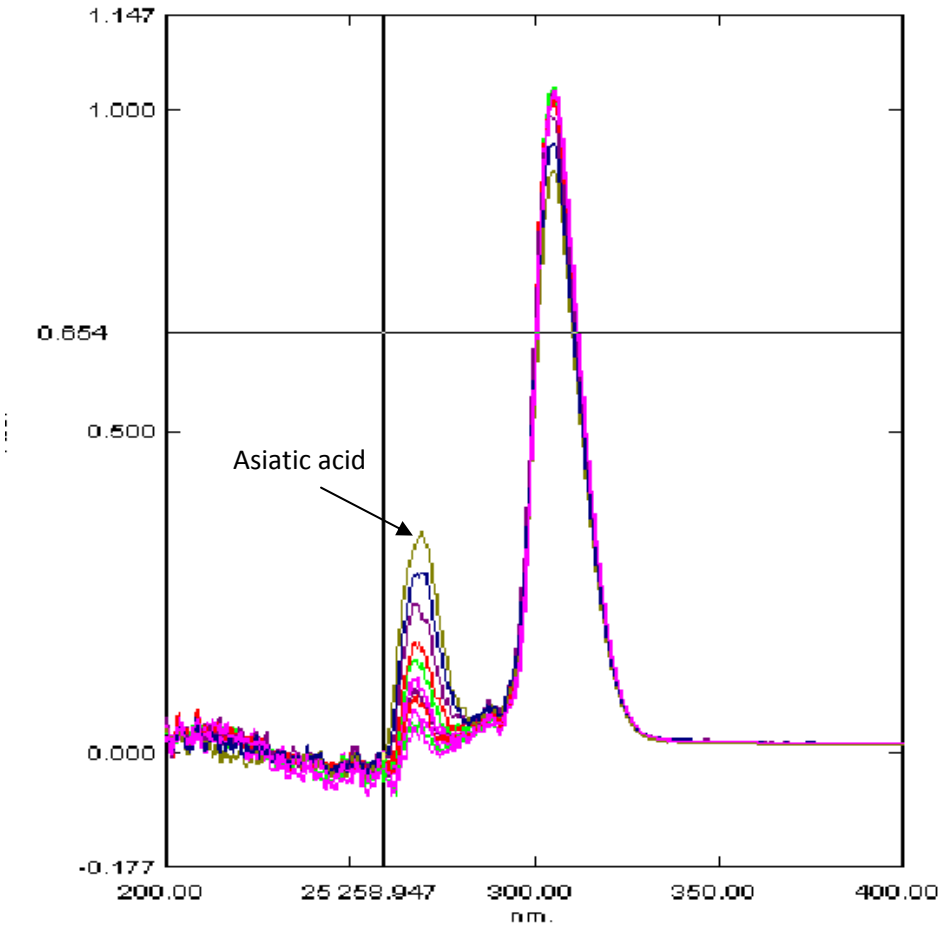


Fig.5.14. UV Spectra of Asiatic Acid at 267 nm

Table: 5.17. Calibration Curve of Asiatic Acid by Formation of Amide Bond at 267 nm

Conc. (µg/mL)	Mean Absorbance ± S.D.*	% R.S.D.
0.05	0.057±0.001	1.75
0.1	0.067±0.001	1.49
0.2	0.079±0.0005	0.63
0.4	0.090±0.0015	1.66
0.8	0.110±00020	1.8
1.0	0..129±0.001	0.77
1.5	0.147±0.0025	1.7
2.0	0.171±0.0026	1.52
Linearity Equation		y = 0.056x +0.063
Correlation Coefficient		0.979
Slope		0.056
Intercept		0.063

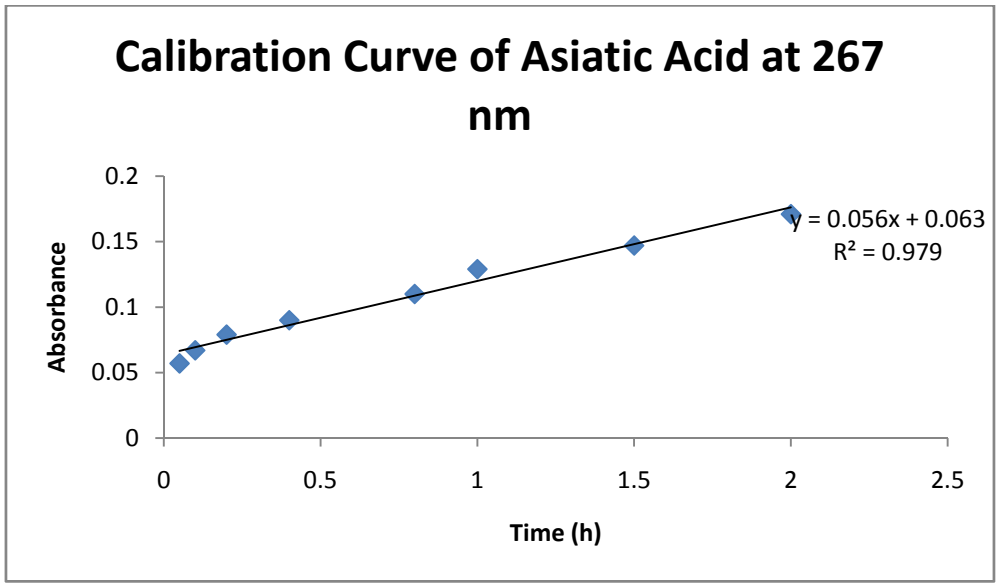


Fig.5.15. Calibration Curve of Asiatic Acid at 267 nm

5.6.1. Validation Parameter for UV Spectroscopy Method:

5.6.1. A. Linearity:

Asiatic acid sowed linear response in range 0.05-2.0 µg/ml in methanol with correlation co-efficient of 0.979 at 267 nm.

5.6.1. B. Precision:

A) Intraday Precision:

Intraday precision was determined by measuring the area at three different concentrations levels for three times in a day. The concentrations selected were 0.1, 0.2 & 0.8 µg/ml for asaitic acid. The % RSD was found to be 1.03%, 0.6% & 1.8%, respectively.

Table: 5.18. Intraday Precision of Asiatic Acid at 267 nm

Conc. (µg/mL)	Absorbance	Conc. (µg/mL)	Mean absorbance ± S.D.*	Mean Conc. (µg/mL) ± S.D.*	% R.S.D.
0.1	0.067	0.17	0.066± 0.001	0.145 ± 0.001	1.03
	0.065	0.14			
	0.066	0.15			
0.2	0.072	0.25	0.072 ± 0.001	0.25 ± 0.0015	0.6
	0.074	0.28			
	0.071	0.23			
0.8	0.111	0.87	0.11± 0.001	0.85±0.015	1.8
	0.110	0.85			
	0.109	0.84			

B) Interday Precision:

The concentrations selected were 0.1, 0.2 & 0.8 µg/ml for asaitic acid. The % RSD was found to be 1.19%, 0.71% & 2.5%, respectively.

Table: 5.19. Interday Precision of Asiatic Acid at 267 nm

Conc. (µg/mL)	Absorbance	Conc. (µg/mL)	Mean absorbance ± S.D.*	Mean Conc. (µg/mL) ± S.D.*	% R.S.D.
0.1	0.061	0.079	0.062± 0.001	0.091 ± 0.1	1.19
	0.062	0.095			
	0.063	0.1			
0.2	0.070	0.22	0.069 ± 0.0015	0.215 ± 0.0015	0.71
	0.071	0.23			
	0.068	0.19			
0.8	0.105	0.77	0.11± 0.001	0.79±0.02	2.5
	0.108	0.82			
	0.106	0.79			

5.6.1. C. LOD & LOQ:

Table: 5.20. LOD & LOQ of Asiatic Acid by UV Spectroscopy Method

Drug	LOD (µg/ml)	LOQ (µg/ml)
Asiatic Acid	0.05	0.17

In conclusion, a simple, sensitive and selective derivatization UV method for the determination of total concentration of asiatic acid in plasma & sample was developed & Validated. The assay had been successfully applied to pharmacokinetic study of asiatic acid in rat plasma after oral / i.v. administration asiatic acid.

5.7. HPLC Result for Estimation of Asiatic acid in Plasma & Organs:

Asiatic acid has absorption at low UV wavelength range. But if UV wavelength is set at 200 nm, interferences from endogenous substances may be significant and may affect the specificity of HPLC. In this paper, a novel precolumn derivatization HPLC method is present, with UV detection at 248 nm for the investigation of total concentration of asiatic acid in plasma after i.v. administration of asiatic acid loaded BSA Nanoparticles. p-Toluidine (PTD) as the derivatizing reagent can be coupled with the free carboxylic acid group of AA.

After derivatization, the maximum absorption wavelength of the derivative was 248nm at which interferences became insignificant and the response was greatly enhanced compared with unmodified asiatic acid detected at 200 nm. Moreover, a homologue of ursolic acid (UA) was used as an internal standard (IS). Analysis was conducted on a C18 column using gradient elution in a water–methanol system.

In summary, a sensitive and selective HPLC method with UV–vis detection was established and successfully applied to a biodistribution study in wistar rats after i.v. administration of asiatic acid loaded BSA Nanoparticles.

For calculation of Asiatic acid from BSA Nanoparticles in organs was carried out by following equations using peak area of particular standard concentration of asiatic acid (sigma Aldrich).

$$\frac{Au}{As} = \frac{Cu}{Cs}$$

Where,

Au = area of unknown concentration of asiatic acid

As = area of standard concentration of asiatic acid

Cu = concentration of unknown sample

Cs = concentration of standard asiatic acid

For standard concentration of asiatic acid,

0.3 ml of 1000µg/ml of standard asiatic acid was taken in microtube, than added 0.3 ml of blank plasma in to above solution.

5.7.1. Determination of Retention Time of Asiatic Acid:

The different concentration of standard solution of asiatic acid was injected individually into HPLC and analyzed as per optimized HPLC condition to determine Rt of asiatic acid. Peak of asiatic acid in methanol chromatogram was confirmed by comparison of Rt of asiatic acid in methanol and Rt of isolated asiatic acid in powder. The retention time of asiatic acid & ursolic acid as internal standard were found to be 10.45 min & 36.55 min respectively.

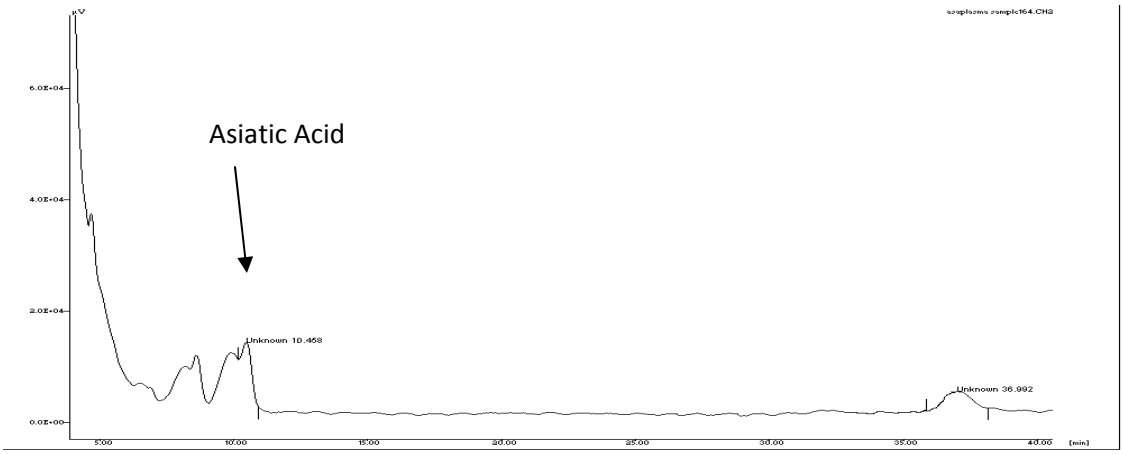


Fig. 5.16. HPLC Chromatogram of Standard Asiatic Acid at 255 nm

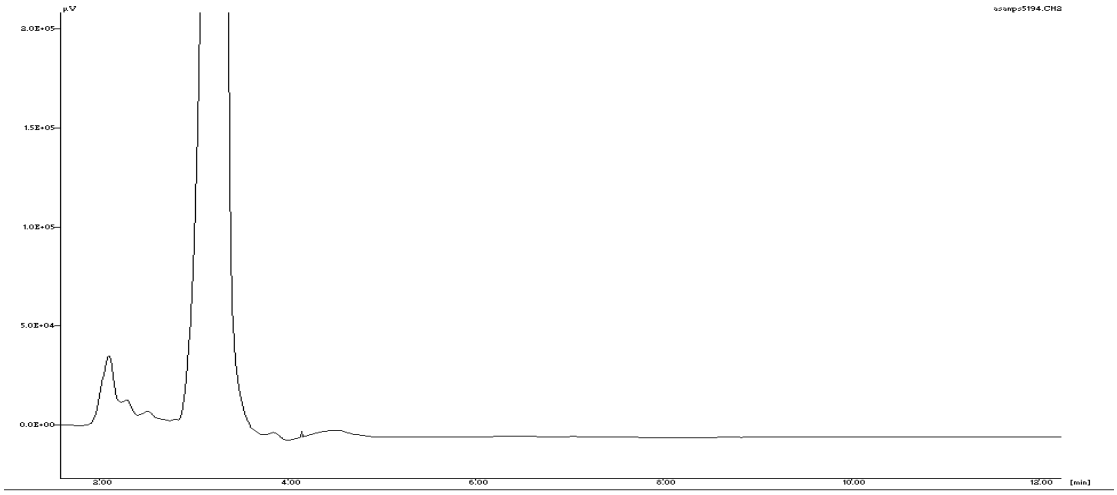


Fig. 5.17. HPLC Chromatogram of Blank Treated Plasma at 255 nm

Table: 5.21. HPLC Chromatogram Data for Asiatic Acid in Blood & Blank Plasma & Ursolic Acid as Internal Standard.

Name	Retention time (min.)	Area (µv.sec)	asymmetry	Plates	% area	Resolution
asiatic acid in blood plasma after i.v. injection	10.45	123179	1.32	6234	45.91	0.000
ursolic Acid as IS	36.993	145108	0.93	4789	54.09	19.9331
Standard asiatic acid in blank plasma	10.35	336943	1.46	5398	80.07	0.0000

In conclusion, a simple, sensitive, precise and selective precolumn derivatization HPLC method with UV detection at 255 nm for the determination of total concentration of asiatic acid in plasma was developed. The assay had been successfully applied to study the biodistribution study in wistar rats after i.v. administration of asiatic acid formulations.

5.8. Summary of Validation Parameters:

Validation parameters of asiatic acid are summarized in table 5.22.

Table: 5.22. Summary of Validation Parameters for Developed Spectrophotometric, HPLC & HPTLC Method

Parameter	UV-Visible Spectroscopy by Vaniline Assay	UV Spectroscopy by Amide Bond Formation	HPTLC	HPLC
Wavelength	535	267	615	220
Linearity	10-300µg/ml	0.05-2.0µg/ml	500-3000µg/spot	200-1800µg/ml
Equation	Y=0.001x + 0.054	y = 0.056x +0.063	Y=1.637x + 786	Y=7239x + 93424
R^2	0.990	0.979	0.990	0.993
Slope of Straight Line	0.001	0.056	1.637	7239
Intercept	0.054	0.063	786	93424
LOD (µg/ml)	3.3	0.05	200	0.00062
LOQ (µg/ml)	10	0.17	600	0.002
Interday Precision (%RSD, n = 3)	1.25	1.46	0.148	0.126
Intraday Precision (%RSD, n = 3)	3.25	1.14	0.173	0.12

5.9. Characterization of Asiatic Acid Loaded BSA Nanoparticles for Brain Targeting Delivery:

5.9.1. % Process Yield:

Asiatic acid to polymer (BSA) proportions were evaluated at different ratio and yield of the production was found 86.66% ± 1.66% to 70% ±1.0%. Further, the process was carried out at the ratio of 1:5 as it showed desirable process yield with minimum particle size.

5.9.2. Particle Size, %Entrapment Efficiency and %Drug Loading Efficiency:

The effect of the concentration of asiatic acid on the entrapment efficiency and drug loading efficiency was studied by taking polymer concentration constant (100 mg) with variable drug concentration in range of 20% - 100%. The entrapment efficiency was found to be 11.59% to 60% after drug content analysis. The entrapment & loading efficiency was found lesser when asiatic acid to polymer ratio was 1:1 and 1:2; while it found to be satisfactory with ratio of 1:5. Hence, drug to polymer ratio was optimized with 1:5 i.e. 20% w/w asiatic acid to achieve desirable entrapment efficiency within the BSA nanoparticles. The Particle size, % entrapment efficiency & % drug loading efficiency of optimize asiatic acid Loaded BSA nanoparticles was 228.66±2.51 nm,60%±1.0% & 16.33%±1.52% respectively.

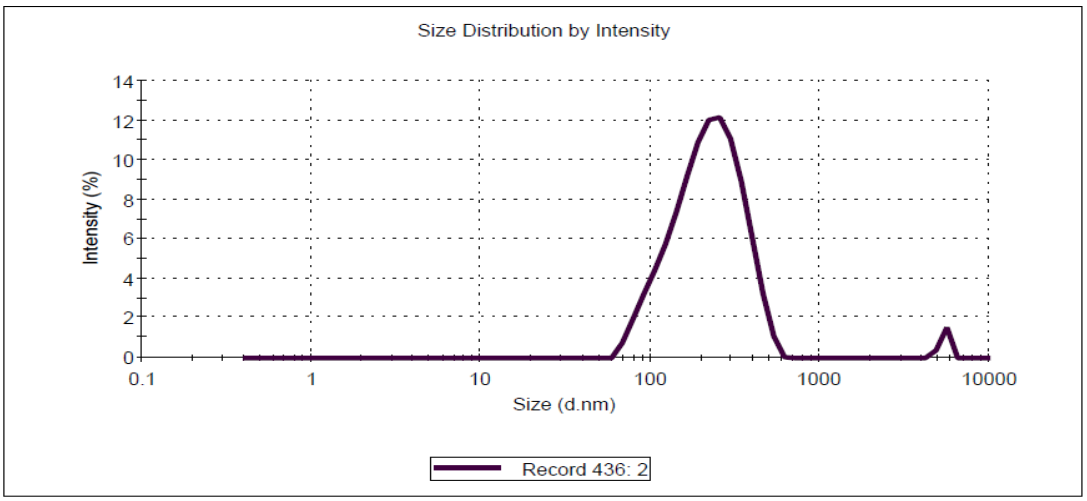


Fig. 5.18. Particle Size Analysis Report of Asiatic Acid Loaded BSA Nanoparticles

Table: 5.23. Results of Asiatic Acid Loaded BSA Nanoparticles in Organic Phase & in Aqueous Phase

Type of Process	Modified Variable	Variable Value	% Process Yield	% Entrapment Efficiency	% Loading Efficiency	Particle Size (nm)	Zeta Potential (mV)
Asiatic Acid in organic Phase	Amount of Asiatic acid (Polymer amount 100 mg)	20 mg	86.66% ± 1.66%	60% ± 1.0%	16.33% ± 1.52%	228.66 ± 2.51	-23.6±1.1
		50 mg	70% ± 1.0%	15.48% ± 0.50%	9.22% ± 1.07%	226±1.0	-29.7±1.0
		100 mg	76.66% ± 1.52%	11.66% ± 1.52%	6.02% ± 0.16%	410±1.0	-21.5±1.1
Asiatic Acid in Aqueous phase	Amount of Asiatic acid (Polymer amount 100 mg)	20 mg	71.33% ± 1.52%	49.33% ± 1.41%	17.12% ± 0.82%	226.66±2.08	-22.5±1.4
		50 mg	66.33% ± 1.523%	16.16% ± 0.76%	6.46% ± 1.71%	340±1.0	-23.3±1.4
		100 mg	61% ± 1.0%	11.59% ± 0.52%	6.62% ± 1.13%	408.66±1.527	-19.7±1.6

5.9.3. Zeta Potential Analysis:

The zeta potential is measured as the charge of particles that exhibited critical impact on the stability of particle in dispersion form. Larger absolute value of zeta potential implicated the higher stability of particles. In case of charged particles, as the zeta potential increase, the repulsive interactions will be larger leading to the formation of more stable particles with a more uniform size distribution (Muller *et al.*, 2001). The mean of zeta potential of optimized asiatic acid Loaded BSA Nanoparticles conjugated and non conjugated was -23.7 to -29.5 mV, respectively. The observed zeta potential values were highly sufficient to form stable nanoparticles suspension. Conjugation of glutathione to the nanoparticles further reduced the zeta potentials due to masking of surface charge of nanoparticles and exposed carbonyl functionality.

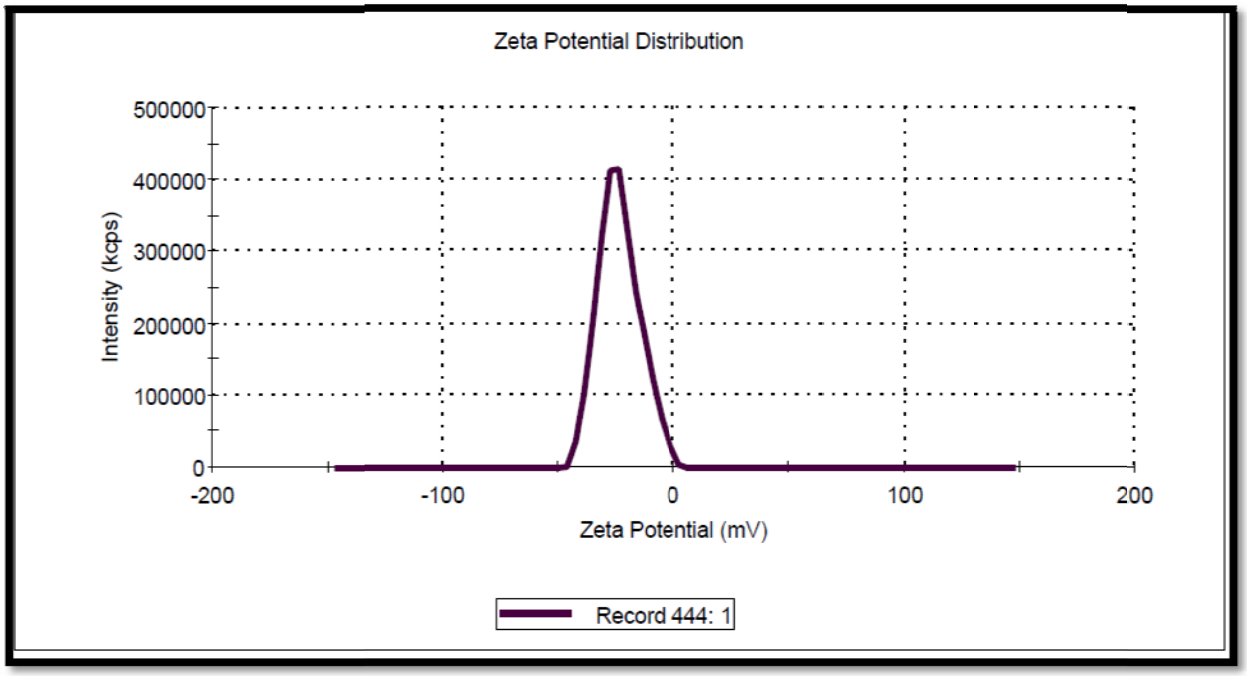


Fig.5.19. Zeta Potential Analysis Report of Asiatic acid Loaded BSA Nanoparticles

5.9.4. Quantitative Estimation of Glutathione with Ellman's Reagent

Glutathione was explored as a vector for enhancing drug delivery to brain. Further, glutathione conjugation on nanoparticles were validated using Ellman's reagent as each glutathione molecule have a single thiol group which has easily estimated spectrophotometrically at 412 nm. The approximately number of glutathione unit per nanoparticle is shown in table.

As per the formula described by Leila *et al.* 2003, the amount of thiol functions [n], present in the sample represents the approximate number of glutathione units coupled to the surface of BSA nanoparticles.

$$n = a \times N \times (d\ (4/3)\ \pi r^3)$$

Where,
n= amount of thiol functionality i.e. glutathione unit per nanoparticle
a= mol of –SH per gm of PLA
d= density of nanoparticle
r= mean radius of nanoparticles
N= Avogadro number (6.011×10^{23})

Table: 5.24. Number of Thiol Group Present per BSA Nanoparticles

Concentration of EDAC (per gm of BSA nanoparticles)	Thiol groups (Glutathione) µmol per gm of BSA	Thiol groups per BSA Nanoparticle	Particle size (nm)	Zeta Potential (mv)
60 mg	33.90±10.58	735.06±198	341±1.08	-33.21±1.21

From the previous study (Patel *et. al.*, 2012), it showed that conjugation reaction was optimized at variable concentration of EDAC and at different incubation time. Experimental results showed that high concentration of EDAC did not affect the conjugation reaction; rather it might promote reaction between glutathione units. Longer incubation time of nanoparticles with EDAC reduced the number of the number of conjugated glutathione.

5.9.5. *In Vitro* Release Studies

Dialysis bag technique was used to determine drug release and release kinetic was established for the optimized nanoparticulate formulations. The drug release showed a biphasic release pattern. The initial brust release observed within first 60 minutes because drug molecules absorbed on the surface layer of the particles dissolved instantaneously when it came in contact with release medium. Additionally high surface to volume ratio of the nanoparticle geometry caused more surface area exposure to release media which also promoted initial drug release. Subsequently, lateral release was observed over period of 24 hours in a sustained manner due to diffusion of the dissolved asiatic acid within the BSA core of nanoparticle. The control profile of asiatic acid showed that 95-99% of the drug released within 5-6 hours in phosphate buffer media with pH 4.0 & 7.4.

Result of the dissolution data revealed that conjugation of glutathione to the NPs did not alter release of asiatic acid significantly.

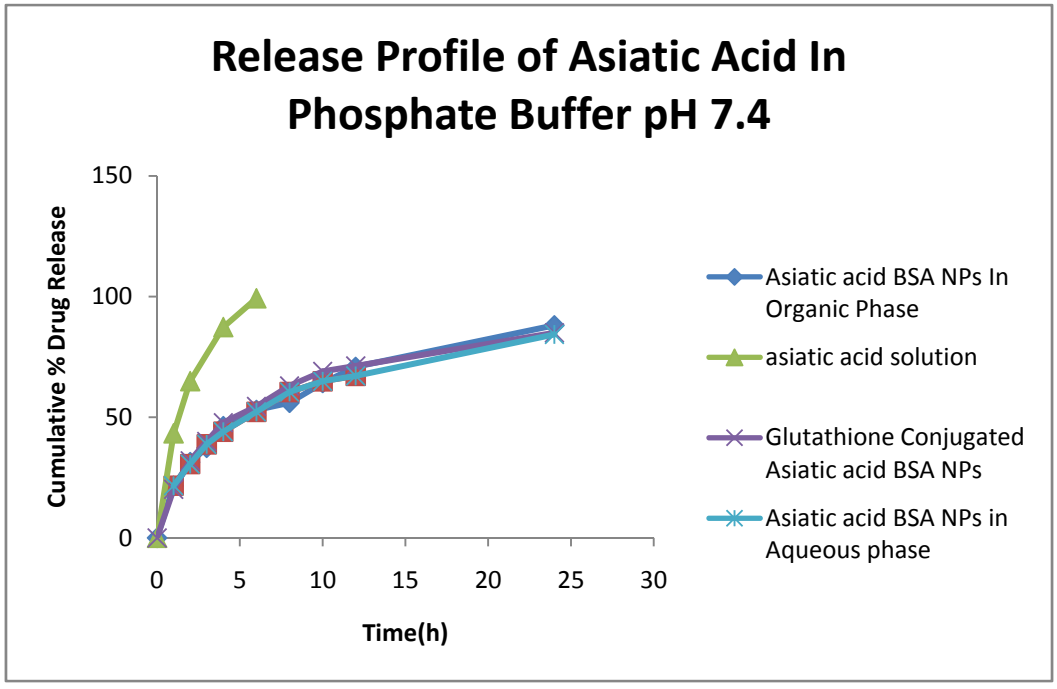


Fig.5.20. *In vitro* Release Profile of Asiatic Acid in Phosphate Buffer pH 7.4 from Different Formulations

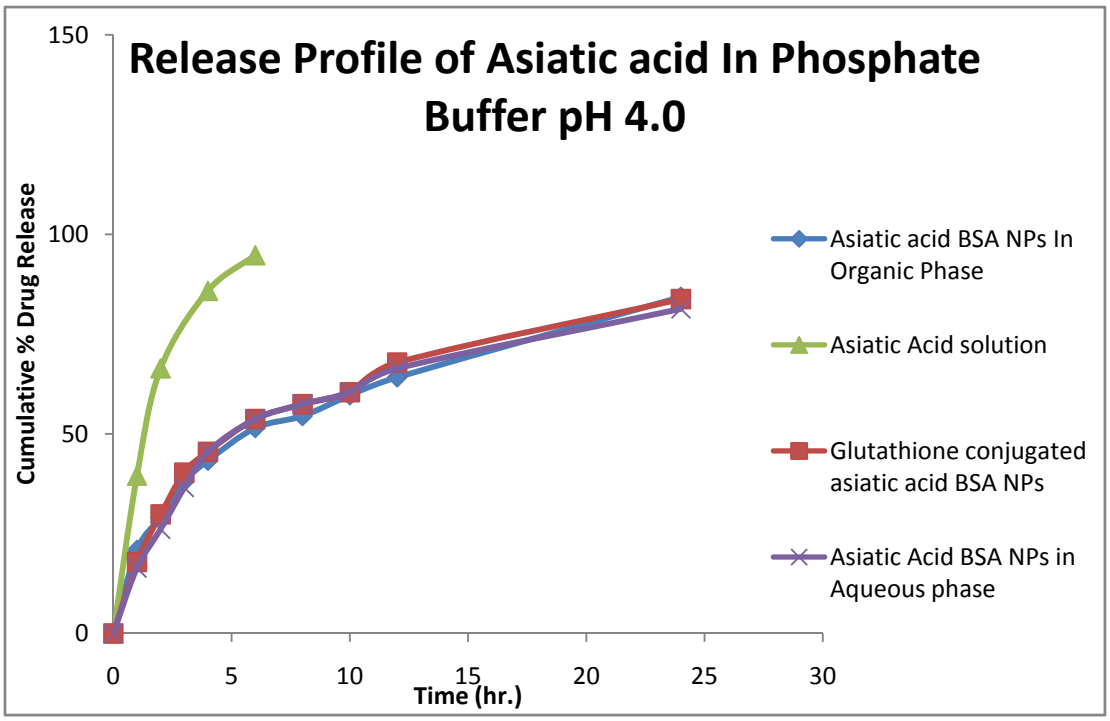


Fig.5.21. *In vitro* Release Profile of Asiatic Acid in Phosphate Buffer pH 4.0 from Different Formulations

5.9.6. Bio Distribution Study:

Biodistribution pattern of different formulation were carried out using asiatic acid as a model compound. The asiatic acid concentrations were measured in different organs after administration in rats. Asiatic acid in three foams i.e. as solution as BSA nanoparticle and as a glutathione conjugated BSA nanoparticles in dose equivalent to 75mg/kg of asiatic acid were given intravenously to wistar rats. The major part of the asiatic acid was found to be distributed in the reticuloendothelial (RES) organs, as particulate substance or drug carrier with an average size below 7 μm are normally taken up by these organs particularly by the kupffer cells of the spleen, liver and kidney. This can be ascribed to the activity of the RES, which tends to clear the particles from blood rapidly.

The concentration of asiatic acid was measured in brain and other visceral organs homogenates by novel pre column derivatization HPLC method. Collodial particles are normally taken up by RES organs hence higher concentration was observed in the liver with asiatic acid entrapped nanoparticles. In spleen, the concentration of asiatic acid achieved after the administration of the solution, non conjugated & glutathione conjugated nanoparticles was 1.13%, 5.01% & 4.51% at 2 h., respectively. Where as in lungs it was 20%, 18% & 14% respectively. The maximum concentration of asiatic acid in brain was achieved by ligand conjugated nanoparticles at 5 h. than the asiatic acid alone solution.

Table: 5.25. Asiatic acid Concentration in Different Organs After Intravenous Injection of Different Formulation of Asiatic Acid at 2, 5 & 24 h

Sr no.	Organ	Formulations		
		Asiatic Acid solution (%w/w)	Asiatic Acid loaded BSA NPs (%w/w)	Glutathione conjugated Asiatic acid loaded BSA NPs (%w/w)
At 2 hours				
1	Brain	4.15±0.05%	1.36±0.015%	1.59±0.01%
2	Kideny	6.46±0.01%	9.69±0.051%	6.4±0.01%
3	Spleen	1.13±0.015%	5.01±0.01%	3.1±0.0254%
4	Lungs	5.33±0.20%	5.72±0.24%	4.51±0.015%
5	Liver	10±0.21%	11.21±0.01%	09±0.025%
At 5 hours				
1	Brain	1.26±0.02%	10.02±0.01%	13.41±0.007%
2	Kidney	5.51±0.07%	8.3±0.03%	4.21±0.01%
3	Spleen	4.53±0.21%	6.66±0.15%	5±0.1%
4	Lungs	3.5±0.1%	5.5±0.07%	4.3±0.18%
5	Liver	4.5±0.1%	16.32±0.325%	14.32±0.15%
At 24 hours				
1	Brain	-	3.71±0.03%	6.45±0.02%
2	Kidney	-	1.42±0.005%	1.09±0.1%
3	Spleen	-	3.21±0.01%	0.13±0.053%
4	Lungs	-	2.06±0.02%	1.06±0.01%
5	Liver	-	5.21±0.001%	3.21±0.02%

The result of the studies showed that, the composite nanoparticles were able to deliver significantly higher concentration of Asiatic acid in the brain from the control groups, indicating potential penetration capacity of glutathione conjugated nanoparticles were compared with Asiatic acid solution and Asiatic acid loaded BSA nanoparticles. Colloidal particles are normally taken up by RES organs hence; higher concentration was observed in the other visceral organs.

The plausible meachanisms of glutathione conjugated nanopaerticles for vtransport of drug molecules across the blood brain barrier is receptor mediated endocytosis. Glutathione act as an endogenous ligand for NMDA and AMPA receptors which are abundantly expressed in brain and also inhibits expression of p-gp on BBB. Additionally, glutathione also modulates the permeability of BBB by acting on tight junction proteins occluding and claudin, which are present on the surface of BBB.Further, glutathione inhibits the enzyme PTP and hence promote phosphorylation of tyrosine which ultimately results in to opening of tight junction for permeability of hydrophilic molecules. (Patel *et al.*, 2012; Varga *et al.*,1997)

5.9.8. Differential Scanning Colorimetry (DSC):

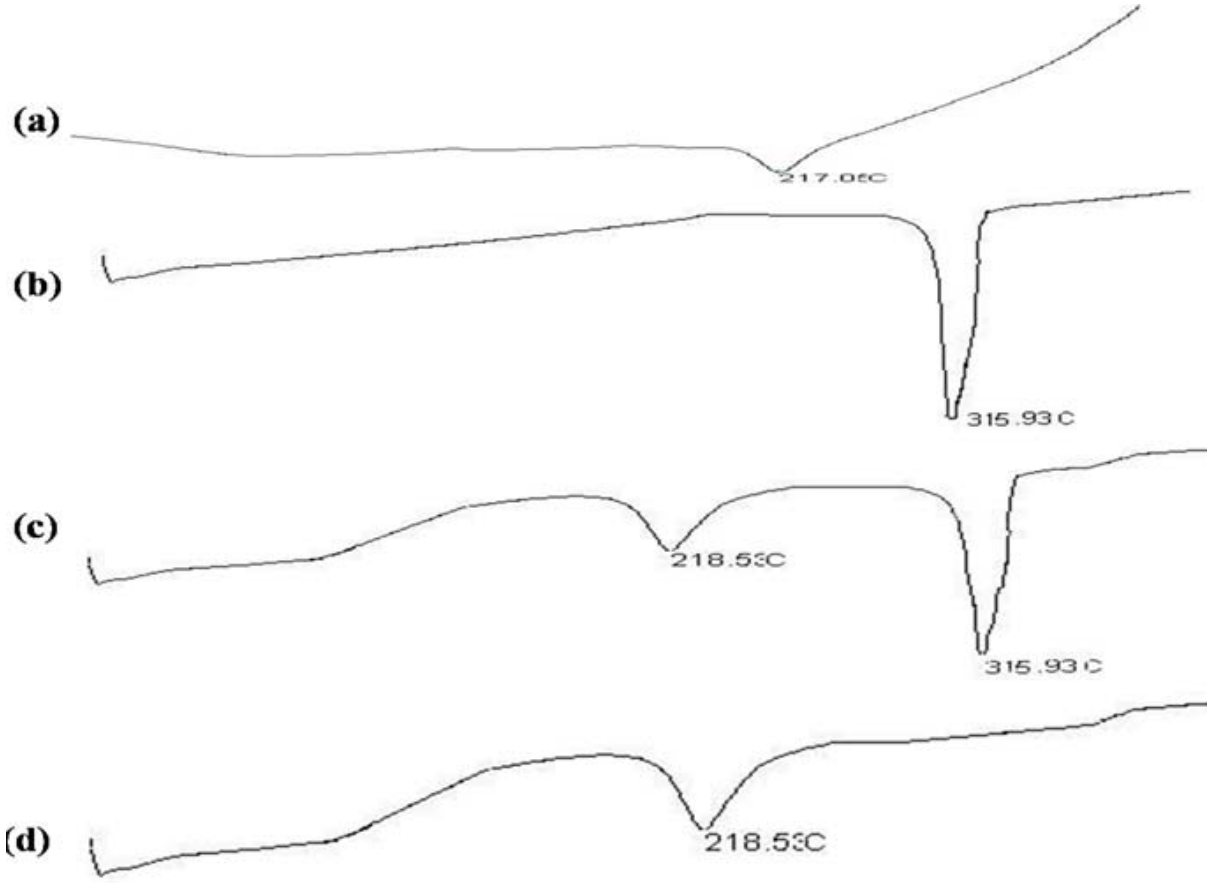


Fig. 5.22. DSC of Asiatic Acid (a). BSA (b). Asiatic Acid (c). Physical Mixture & (d). Formulation of NPs

From the above thermogram, it has been observed that the endothermic peak of asiatic acid was found to be absent in nanoparticles formulation, indicating drug is molecularly disperse in BSA matrix. Similarly, in case of physical mixture of asiatic acid & BSA no addition peak was observed so compatibility between asiatic acid & BSA in solid state.

Summary:

Asiatic acid is a pentacyclic triterpene isolated from a variety of plants, including *Centella asiatica*, *Shorea robusta*, and reported as one of the major active constituent responsible for the their various pharmacological activities. One of the major pharmacological activity reported for asiatic acid is neuroprotective, cognitive behaviour. asiatic acid was isolated by three different methods like conventional, ultrasonicator & microwave extraction. The samples were estimated by developed HPTLC method at 615 nm for scanning of plates using anisaldehyde sulphuric acid spraying agent. The highest % yield of asiatic acid was achieved by ultrasonicator probe extraction at 40 minutes. For estimation of asiatic acid in plasma or organs, a simple & novel precolumn derivatization method was developed using p-toludine as derivatization agent and EDAC as coupling agent to carboxylic group of asiatic acid. From the HPLC chromatogram, the retention time of asiatic acid was found to be 12.00 minutes & for ursolic acid (0.4µg/ml) was found to be 36 minutes. The UV spectroscopy method was developed for estimation of asiatic acid in plasma/formulation on basis of two step carbodiimide reaction. asiatic acid to polymer proportions were evaluated at different ratio and yield of the production process was found to 86.66% to 70%. Further, the process was carried out at ratio of 1:5 as it showed dersirable process yield with minimum particle size. The mean particle size of optimized asiatic acid Loaded BSA Nanoparticles (% entrapment efficiency & % drug loading 60%±1.0% to 16.33%±1.52%) was found to be 228±2.52 nm as shown in results suggesting uniformity in particle size distribution. Conjugation reaction with glutathione showed the mean particle size of NPs to 341±1.04 nm. The effect of the concentration of asiatic acid was studied by taking polymer concentration constant(100 mg) with variable drug concentration in range of 20 – 100 %w/w. the entrapment efficiency found to be lesser in ratio of 1:1 and 1:2. Hence, drug to polymer ratio was optimized with 1:5 i.e. 50%w/w asiatic acid to achieve desirable entrapment efficiency. The zeta potential is measured as the charge of particles and impact on the stability of particle in dispersion foam. The zeta potential of optimized BSA nanoparticles of Asiatic acid with glutathione and without glutathione was -23.5mV & -33.1 mV Conjugation to glutathione to the nanoparticles further reduced the zeta potential due to masking of surface charge of nanoparticles and exposed carbonyl functionality. For ligand based drug delivery of asiatic acid to brain, glutathione was explored as a vector for enhancing drug delivery to brain & asiatic acid Loaded BSA Nanoparticles was conjugated with glutathione by two

step carbodiimide conjugation technique. Further, glutathione conjugation on nanoparticle were validated using Ellman's reagent as each glutathione molecule have a single thiol group which was easily estimated spectrophotometrically. For *in vitro* release studies of asiatic acid solution , asiatic acid loaded BSA naoparticles & glutathione conjugated BSA nanoparticles were carried out using dialysis bag technique for 24 hours in phosphate buffer at physiological pH & endo-lysosomal compartment pH respectively. from the studied, the release profile of asiatic acid was found to be extended release(87.06±1.36% to 85.29%±0.56%). Further, glutathione conjugated nanoparticles have not altered the release pattern substantially in comparison to non conjugated nanoparticles. The biodistribution pattern of different formulations was carried out using asiatic acid as a model compound. asiatic acid in three different forms i.e. solution, nanoparticles & glutathione conjugated in dose equivalents to 75mg/kg of Asiatic acid were given intravenously to wistar rats at different time interval 2hr., 5 hr., & 24 hr. The concentration of asiatic acid in brain and other visceral organs at different time interval were measured by novel precolumn HPLC method. The concentration of asiatic acid solution, asiatic acid loaded BSA nanoparticles & glutathione conjugated NPs were found to be 4.14%w/w, 1.38%w/w & 1.58%w/w at 2 hours, 1.25%w/w,10%w/w & 13.42%w/w at 5 hours respectively. At 24 hours, there was no concentration found in brain for Asiatic acid alone solution. The concentration of asiatic acid Loaded BSA NPs and Glutathione conjugated BSA Loaded asiatic acid in brain at 24 hours were found to be 3.68% and 6.45% respectively. The possible mechanisms of glutathione conjugated nanoaprticles for transport of drug molecules across the blood brain barrier is receptor mediated endocytosis. Glutathione act as an endogenous ligand for NMDA and AMPA receptors which are abundantly expressed in the brain and also inhibits expression of p-gp modulation on BBB.

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