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Research Article

Identification and Characterization of Metabolites of Irinotecan *in-vivo* and *in-vitro* matrixes by HPLC/LC-MS

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Abstract:

In the present study metabolite identification and characterization has done by using HPLC and LC-MS. During method development various mobile phases have tried for identification of metabolites. The matrixes selected for *in- vivo* study were urine because nearly all the metabolites of irinotecan were obtained in it. The extraction mixtures have selected to retain maximum amount of analyte with less effort. During experiment four extraction solvents were used in six different concentrations out of which TBME suit our method. *In-vitro* study done by Human Liver microsomes by using Phosphate buffer (pH 7.4) and NADPH as co-factors for initiation of enzymatic reaction

Irinotecan is a prodrug that is converted in the liver to an active metabolite, SN-38. It is eliminate in Bile and Faeces and thus its dose reduced in Hepatic Failure. Irinotecan act by inhibiting Topoisomerase-1.It is the enzyme which nicks, introduces negative supercoils and reseals the DNA strand. Conventionally, drug metabolite identification in the past has usually been based on the comparison of ultraviolet (UV) spectral data and high-performance liquid chromatography (HPLC) retention times of isolated 'unknown' metabolites with those of synthesised standards. Such a method of detecting and characterising drug metabolites is an uncertain, time-consuming and expensive process, as well as affording very limited structural information. Furthermore, Phase I metabolism of a drug candidate often results in only minor structural modification of the parent compound; these minor changes can make it particularly difficult to determine suitable chromatographic conditions to effect HPLC separation of metabolites. This study describes contemporary approach to identification and characterization of xenobiotic metabolites in complex biological fluids derived from drug metabolism studies.

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INTRODUCTION:

Bioanalytical method can be defined as an analysis of sample or analyte or drug in biological fluid like Plasma, serum, urine, blood and tissue etc. Quantitative determinations of drugs in biological samples, such as blood or plasma, play a significant role in evaluation and interpretation of bioequivalence data.

Scientist with reliable and accurate data has been a major driver and Bioanalysis has always played an important role in providing the pharmacokinetic challenge for the bioanalyst. Over the years the scope of the bioanalytical involvement has immensely broadened. Linking pharmacokinetic data with pharmacodynamic observations (PK/PD and biomarkers) or investigation of the fate of a drug in a living species (investigation of metabolite profiles) are only a few examples where additional bioanalytical expertise was built in. In all these experiments, the quality of data is paramount to allow interpretation and subsequent decision-making in a project. In the last years, closer involvement of bioanalysis in drug discovery was needed to lower the attrition rate in the later phases. Thorough investigation of pharmacokinetic and metabolic behavior of drugs in discovery and early development shifted the bioanalytical support from pre-phase I to lead optimization in drug discovery [1].

The bio-analytical testing procedures involve methods like gas chromatography (GC), high-pressure liquid chromatography (HPLC), combined GC and LC mass spectrometric (MS) procedures, such as LC–MS, LC–MS/MS, GC–MS, and GC–MS/MS, performed for the quantitative determination of drugs and/or metabolites in biological matrices. Selective and sensitive analytical methods for the quantitative evaluation of drugs and their metabolites (analytes) are critical for the successful conduct of preclinical and/or biopharmaceutics and clinical pharmacology studies [2-3].

LC-MS is frequently used in drug development at many different stages including Peptide Mapping, Glycoprotein Mapping, Natural Products Dereplication, Bioaffinity Screening, In Vivo Drug Screening, Metabolic Stability Screening, Metabolite Identification, Impurity Identification, Degradant Identification, Quantitative Bioanalysis, and Quality Control. The need for quantitative bio analysis

comes primarily from the requirement to demonstrate the exposure of preclinical species and human volunteers to candidate drug compounds during drug development studies. During the development of a drug molecule, it is essential to have a specific, sensitive bio-analytical method capable of resolved the dose compound from its metabolites and from any endogenous compounds in the matrix. Such resolution can be achieved at the isolation stage by chromatographic resolution or by specificity at the detector. Quantitative drug analysis is also important at the early drug discovery phase, so that candidate molecules' pharmacokinetic parameters can be determined. This allows the comparison of candidate development compounds as well as a compound's elimination and distribution in different sexes and species. The tabular form shows stages of drug development and their corresponding milestone and analysis emphasis [4].

To analyse metabolites of Irinotecan *in-vivo* and *in-vitro* matrixes following developed bioanalytical methods literature survey has been done before the final method was developed J. Gira´l dezhas developed "Simple and rapid determination of irinotecan and its metabolite SN-38 in plasma by high-performance liquid-chromatography: application to clinical pharmacokinetic studies." [4]. Alex Sparreboom carried out Liquid chromatographic determination of irinotecan and three major metabolites in human plasma, urine and feces [5]. Franck Desmoulin perform Isolation of an unknown metabolite of capecitabine, an oral 5-fluorouracil prodrug and its identification by nuclear magnetic resonance and liquid chromatography–tandem mass spectrometry as a glucuroconjugate of 59-deoxy-5-fluorocytidine,namely 29-(b-D-glucuronic acid) –59-deoxy-fluorocytidine[6]. Daniel F. Chollet developed Simultaneous determination of the lactone and carboxylate forms of the camptothecin derivative CPT-11 and its metabolite SN-38 in plasma by high-performance liquid chromatography.[7]

Materials and Methods

Drugs (Active Pharmaceutical Ingredients)

Name	Batch No.	Supplier
Irinotecan Hydrochloride	IRH-H4042	In-house
SN-38	SRH-H4023	In-house

Properties of Active Pharmaceutical Ingredients

Properties	Irinotecan	SN-38
Molecular weight (g/mol)	586.67	394.02
% Purity	99.0	99.0
Molecular formula	$C_{33}H_{38}N_4O_6$	$C_{22}H_{20}N_2O_5$

Chemicals and Reagents

Methanol (HPLC grade), Acetonitrile (HPLC grade), water (HPLC grade) and 0.02M Potassium Di Hydrogen Orthophosphate, 0.03M Di Potassium Hydrogen Orthophosphate were obtained from Merck chemicals and J.T. Baker respectively. Distilled water was prepared using a Milli-Q system (Millipore). Nylon syringe filters (0.45 µm) were from Millipore.

Mobile Phase

Mobile phase was prepared by Potassium Dihydrogen Orthophosphate buffer and acetonitrile in ratio of 43:57 v/v respectively. Buffer solution was prepared by dissolving 7.0 gm of potassium dihydrogen orthophosphate to 1000ml of HPLC grade water and pH is adjusted to 3.0 with Formic acid. The content of mobile phase and buffer solution were filtered before use through 0.45µ filter and degassed for 10min by sonication.

In-vitro study

Standard Stock Solution Preparation:

Stock Solution Irinotecan

Accurately weighed 10.00 mg of Irinotecan working standard was transferred to 10.0 ml volumetric flask. The content was dissolved in 3 ml of DMSO and made up to the volume mark with DMSO (100 % v/v), (1.0 mg/ml as Irinotecan).

Stock Solution of SN-38

Accurately weighed 10.00 mg of SN-38working standard was transferred to 10.0 ml volumetric flask. The content was dissolved in 3 ml of DMSO and made up to the volume mark with DMSO (100 % v/v), (1.0 mg/ml as ISN-38).

Method development

Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. During the method development, different methods were tried for the extraction of drug from the Urine.

Procedure

Test solution was prepared by diluting the stock 1:10 with phosphate buffer pH 7.4 to achieve specified concentration, containing 10%DMSO. Mix 10 µl NADPH Solution A, 2 µl NADPH Solution B, 20 µl working stock solution, 10 µl pooled Human Liver Microsomes and 158 µl 0.1 M phosphate buffer. Then keep the mix at 37^0C followed by addition of cold liver Microsomes. Withdraw 100 µl of sample called as zero min sample immediately after addition of liver Microsomes and terminate by the addition of equal amount of ACN and keep in ice for 30 min. then incubate the remaining mixture in a water bath at 37^0C with shaking for 60 min and terminate as done previously. Both the

samples are then centrifuged at 10,000 RPM for 10 minutes at a temperature of 40^0C. Then the supernatant is collected and store at -20^0C for further analysis by HPLC and LC-MS.

In-vivo study

Procedure

Drug formulation and administration

For metabolite studies, formulation was prepared by dissolving 12 mg of Irinotecan hydrochloride in 1ml water by vortexing for 5min and sonicate for 2min. and then dilute with 0.9% NaCl upto 3ml. then 1 ml of formulation was administered through a slow intravenous injection via the lateral tail vein obtain the dose of 4mg/kg.

Sample collection

Urine samples were withdrawn prior to dosing and at 0-6, 6-24, 24-30 and 30-48 hours after dosing and collected in a microtube. Samples were stored -20^0C until HPLC/LC-MS analysis.

Extraction Procedure

Urine samples were extracted by using TBME.100µl urine sample was taken in centrifuge tube, Add 1000µl of TBME. Keep the samples for 5 min. vortexing. Then the samples were kept in a centrifuge for 10 min. at 10,000 RPM at 4^0C. Remove the 900µl supernatant in another eppendrof tubes. Then samples were kept in an evaporator for 60 min. The samples were reconstituted by using 150µl Methanol: Water (1:1) and used for further analysis.

Results and Discussion

To identify and characterize the metabolites of Irinotecan by an suitable chromatographic and liquid chromatography-tandem mass spectrometric method, and also developed the method by this technique. The samples were prepared by suitable extraction technique in order to retain maximum quantity of analytes. The characterization of metabolites has done by their molecular mass by LC-MS.

METHOD DEVELOPMENT

In-Vitro Study

HPLC Interpretation

The identification of unknown peak at **R.T. 9.228 (Fig-1)** at zero minute and **R.T. 9.331 (Fig-2)** at sixty minutes it shows there is a formation of metabolite. As it is retained at zero minute implies that the formation of unknown metabolite1 is a very fast and spontaneous process in Human Liver Microsomes. But other metabolites are not formed which indicate that the other metabolites are formed after one hr. or they are metabolically stable by *in vitro* procedure or may be formed after sixty minutes.

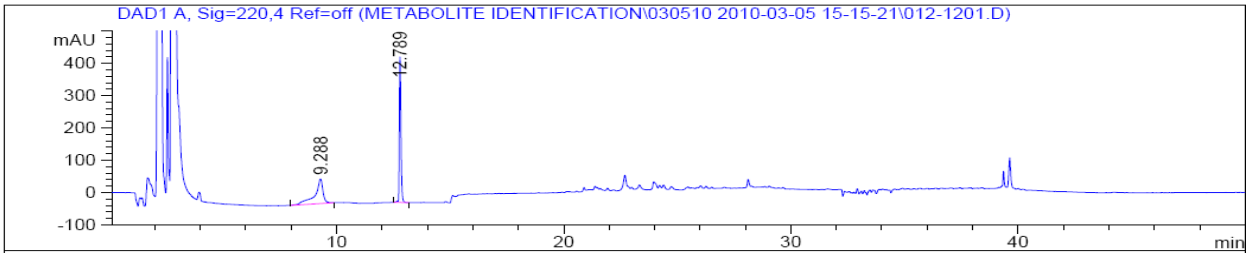


Fig 1- Irinotecan and metabolites at Zero Min

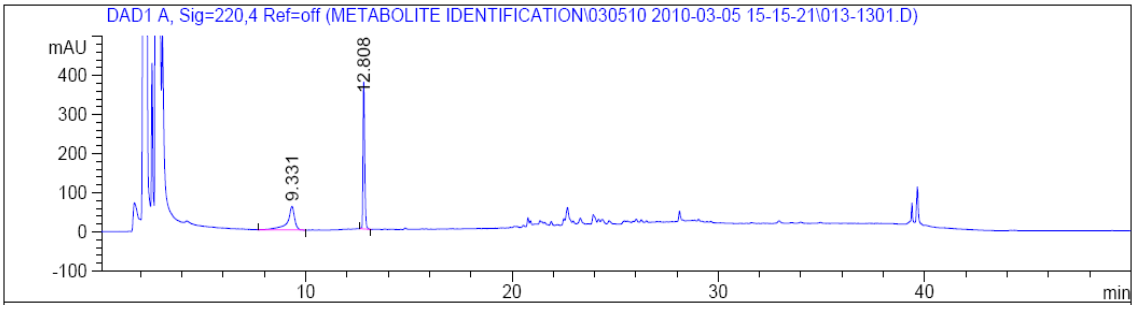
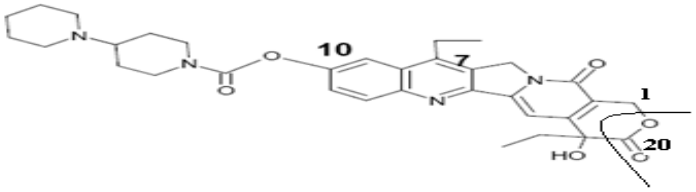


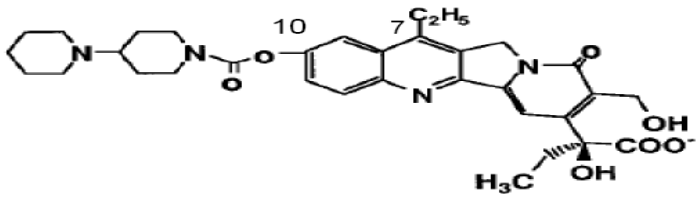
Fig 2- Irinotecan and metabolites at 60 MIN

LC-MS Interpretation

The molecular weight at retention time at 6.88 is 543.50 and at 6.92 is 543.49 which have characterized as Irinotecan and its metabolite PDP-5.



Structure of metabolite PDP-5



Structure of CPT-11 hydroxy acid (Irinotecan carboxylate)

***In-Vivo* Study:**

HPLC Interpretation

Name	Retention Time In Minutes			
	0-6 Hrs.	6-24 Hrs.	24-30 Hrs.	30-48Hrs.
Irinotecan	12.199	12.238	12.196	12.474
Metabolite1	13.002	-	-	-
Metabolite2	19.657	19.649	19.640	19.644
Metabolite3	13.871	13.793	13.040	13.792
Metabolite4	15.832	15.840	15.836	15.832
Metabolite5	-	-	-	11.474
Metabolite6	9.4	-	9.901	-

The identification of unknown peaks at 0-6, 6-24, 24-30, 30-48 Hours implies that their is a formation of 6 metabolites.

Metabolite 1- The metabolite was observed after 0-6 hrs indicates that its formation is spontaneous and it may characterized as minor metabolites.

Metabolite 2,3,4- These metabolites were observed during 0-6, 6-24, 24-30, 30-48 Hrs. implies that they may be active metabolites and may be characterized as major metabolites.)(Fig-3)

Metabolite 5- This metabolite was formed at 30-48 Hrs. it may be characterized as minor metabolites.

Metabolite6- It is also minor metabolite and as its retention time matches with PDP-5 which is obtained in vitro method.

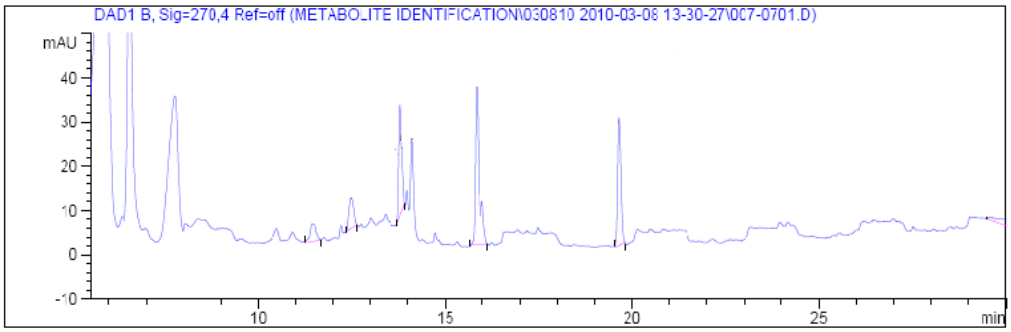


Fig 3- Irinotecan and its metabolites at 30-48 hrs.

LC-MS Interpretation:

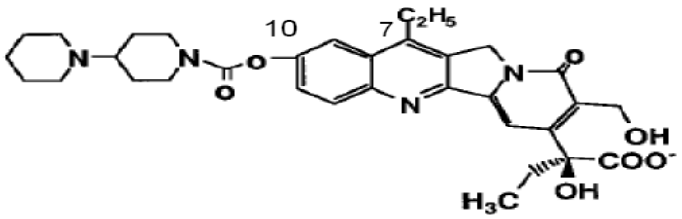
Name	Retention Time In Minutes			
	0-6 Hrs.	6-24 Hrs.	24-30 Hrs.	30-48Hrs.
Irinotecan	7.02	7.05	7.07	7.12
Metabolite 1	5.02	-	-	-
Metabolite2	6.05	6.07	6.07	6.08
Metabolite 3	8.52	8.52	8.55	8.50
Metabolite 4	7.37	7.38	7.40	7.12
Metabolite 5	14.62	14.63	-	-
Metabolite 6	-	5.98	5.98	5.85

MASS of Irinotecan and metabolites by *In-vivo* method.

METABOLITE	0-6 Hrs.	6-24 Hrs.	24-30 Hrs.	30-48 Hrs.	Characterization
Irinotecan	587.22	587.22	587.22	587.22	Irinotecan
Metabolite 1	605.24	-	-	-	CPT-11 Hydroxy Acid
Metabolite 2	619.39	619.32	619.39	620.18	APC
Metabolite 3	393.05	393.08	393.05	393.04	SN-38
Metabolite 4	569.11	569.44	569.40	569.40	SN-38G
Metabolite 5	520.09	520.09	-	-	NPC
Metabolite 6	-	541.25	541.25	541.29	PDP-5

The metabolites are characterized on the basis of their molecular weight.

Metabolite 1- CPT-11 Hydroxy acid is the hydroxylate form of parent drug irinotecan (CPT-11).



Structure of CPT-11 (Irinoteacn carboxylate)

Structure and fragmentation pattern of CPT-11 hydroxy acid (Irinotecan carboxylate)

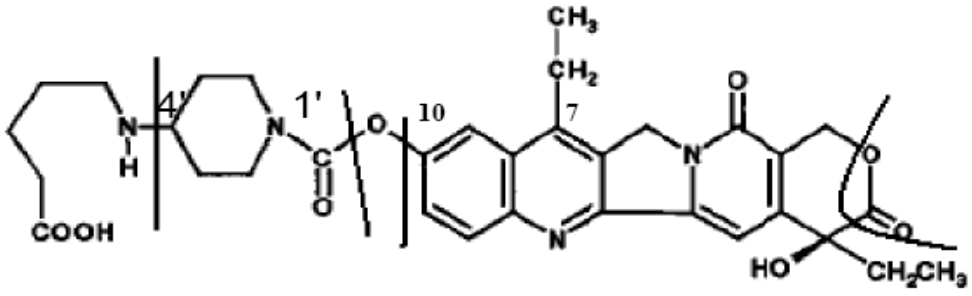
Metabolite 2- APC which is called as primary amine with proposed fragmentation pattern.

Fragment 1 having molecular weight 509 due to the breaking of aminopentanoic acid from **position 4'** of APC.

Fragment 2 having molecular weight 435.18 due to the breaking of carboxylic acid chain between piperidino ring and parent structure.

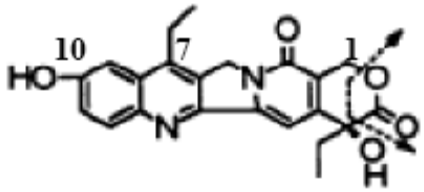
Fragment 3 of molecular weight 454.39 is due to separation of complete carboxylic acid chain from **position 10** of parent structure

Fragment 4 of molecular weight 575 is due the breaking of carboxylic acid from parent ring just like the 349 molecular weight fragment observed inSN-38.



Structure and fragmentation pattern of APC

Metabolite 3- SN-38 and it is the active metabolite of Irinotecan and it takes part in pharmacological action of parent drug. Fragment 349.09 is due to the removal of carboxylic acid from position 1 of SN-38.

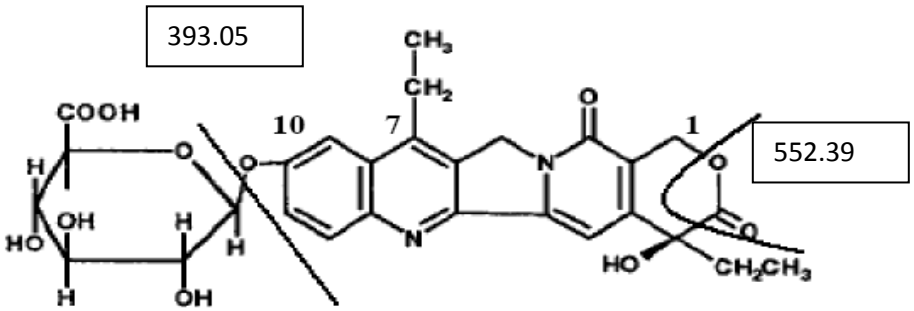


Structure and fragmentation pattern of SN-38.

Metabolite 4- SN-38G is the Glucuronide conjugation of SN-38.

In the mass spectra of SN-38G following fragments are observed Fragment having molecular weight 393.05 is due to the separation of Glucuronide moiety from **position 10** of SN-38.

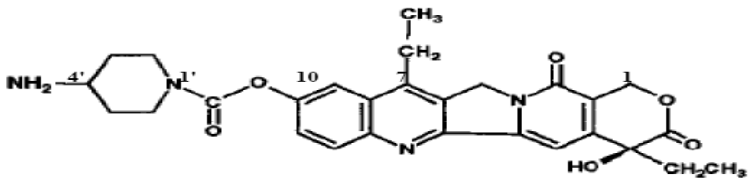
Fragment of molecular weight 552.09 is due the removal of carboxylic acid as the same case in SN-38.



Structure and fragmentation pattern of SN-38G.

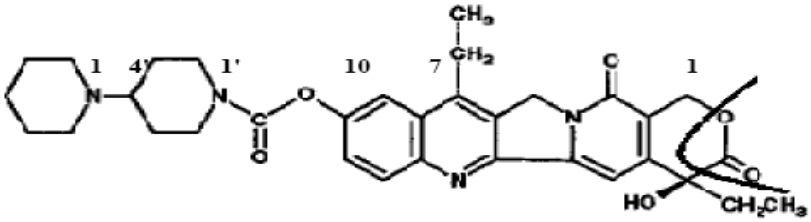
Metabolite 5- NPC

In NPC no fragmentation was observed but there may be possibility of breaking of Carboxylic acid from **position 1**.



Structure and fragmentation pattern of NPC.

Metabolite 6- PDP-5 is the metabolite obtained in both *in-vitro* and *in-vivo* in rat. It is the metabolite formed due the removal of Carboxylic acid from **position 1** of parent compound.



Structure and fragmentation pattern of PDP-5.

Conclusion

The present procedure is the most simple and comparative between Human liver microsomes and rat urine, it also compared the metabolite data by *in-vitro* and *in-vivo* method, also point out the differences between HPLC and LC-MS.

In present study metabolite identification and characterization has done by using HPLC and LC-MS. During method development various mobile phases have

tried for identification of metabolites. The matrixes selected for *in- vivo* study were urine because nearly all the metabolites of irinotecan were obtained in it. The extraction mixtures have selected to retain maximum amount of analyte with less effort. During experiment four extraction solvents were used in six different concentrations out of which TBME suit our method. *In-vitro* study done by Human Liver microsomes by using Phosphate buffer (pH 7.4) and NADPH as co-factors for initiation of enzymatic reaction.

The peaks obtained were characterized by their molecular mass on LC-MS which reduces the isolation steps. Only two metabolites PDP-5 and CPT-11 (Irinotecan in carboxylate form) are obtained and other metabolites not obtained which indicate that they may be stable by *in-vitro* metabolic stability or not formed during *in-vivo* procedure. Six metabolites were obtained during *in-vivo* study by urinary excretion. Out of which two metabolites namely NPC and CPT-11 hydroxy acid (irinotecan in carboxylate form) were obtained only during 0-6 hour sample collection. Hence they were called as minor metabolite. The metabolite PDP-5 is not obtained during 0-6 hour but continue in 6-24, 24-30, 30-48 hour, hence. The other three metabolites APC, SN-38, SN-38 Glucuronide were called as major metabolites. The metabolites Irinotecan carboxylate and PDP-5 were only oobserved during both *in-vitro* and *in-vivo* study. During in vitro study they are formed at zero minute which indicate that the reaction is spontaneous and metabolite formation is faster than *in-vivo*.

During characterization it is concluded that SN-38 is the active form of irinotecan and it has a fragment at 349 which is formed by removing carboxylic acid, and it has excreted as SN-38G during phase-II elimination reaction. The CPT-11 carboxylic acid is the carboxylate form of parent drug Irinotecan. The metabolite APC having molecular weight 619 is major metabolite obtained during phase II elimination. The PDP-5 is formed by eliminating carboxylic acid from irinotecan and has molecular weight 543. The primary amine NPC has eliminated after 6-hour through urine. In the present study there is a comparison between human liver microsomes and Rat. The rats were used for *in-vivo* study which helps to reduce metabolism study on human volnteers during clinical trials because this method helps in identification and characterization of all the metabolites in rat.

The future prospective is that the other matrix of rat like feces, plasma and bile used for the purpose of study. Although Plasma/ Serum are the preferred matrix for the characterization of parameters, Recent Years have witnessed the emergence of bile matrix as another tool for refining the pharmacokinetic. Lastly we conclude that there are total six metabolites are obtained. We also concluded the Different Molecular Weight of Drug which is used for characterize the different structure of Drug. The developed method was validated with respect to specificity, linearity, limit of detection and quantification, accuracy, precision and solution stability. The overall procedure can be used as part of a cleaning validation program in Pharmaceutical Manufacture of Irinotecan Hydrochloride. The increasing availability of high- resolution LC-MS instruments and enhanced functions of MDA software, we except that LC-MS based metaolomics technologies will be widely adopted and utilized in the Xenobiotic Metabolism Research Field. The validated method was first applied to Study the preclinical Pharmacokinetics of Irinotecan, SN38, and SN38-G in pigs. A validated and Novel Ultra high performance chromatographic method with tendem mass-spectrometric detection for the rapid quantitative determination of Irinotecan, SN38, SN38-G in human and protein plasma. The Resulting Preclinical and Clinical Application further support the usefulness of other method.

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