ADME DIVISION

REPORT ID: ADME/CACO/CRO/101/VERSION 17

COMPARATIVE STUDY OF ABSORPTION OF CURCUMINOIDS IN TWO PRODUCTS USING CACO-2 CELL LINES

Aim

To study the comparative absorption of the following two products containing curcumin by using Caco-2 cell lines:

- **Cumerone ®** (CB/09001)
- **Curcumin (95% curcuminoids)** (CL/09)

Introduction

Oral absorption of a compound is critical for its bioavailability. This absorption is mainly controlled by the passage across the intestinal epithelium. Thus prediction of intestinal absorption by means of an *in vitro* model mimicking intestinal epithelium may offer a powerful tool for the early selection of molecules during the process of drug development[1] . One such model is based on Caco-2 cells derived from a human colon carcinoma which undergo spontaneous enterocytic differentiation in culture and become polarized cells with well-established tight junctions, resembling intestinal epithelium in humans. The Caco-2 cells when grown on a suitable filter form a monolayer which retains many features of absorptive intestinal cells such as microvillus structure, hydrolysis enzymes and carrier mediated transport system for sugars, amino acids and several drugs [2 3] . The drug permeability through the Caco-2 monolayer therefore is expected to correlate well with that of intestinal membrane *in vivo* and thus can be used for the study / prediction of drug absorption. The present study was performed as per the Millipore protocol with slight modifications[4].

Curcuminoids (mixture of three compounds viz., curcumin, demethoxycurcumin and bisdemethoxycurcumin) are the major active principles of the Indian medicinal plant *Curcuma longa*. Extracts standardized to curcuminoids are being widely used in food, nutraceutical, and complementary medicine industry. However, oral bioavailability of curcumin has been estimated to be less than 1% [5] which hinders the efficacy of products containing curcuminoids. Therefore methods to improve oral absorption of curcuminoids attain special significance. The present study provides a comparative evaluation of absorption of a classical curcuminoids extract, **Curcumin (95% curcuminoids)** (CL/09) against a newly developed proprietary extract, **Cumerone ®** (CB/09001) using Caco-2 cell line.

Materials:

- Acetaminophen [Cat # 190091, store at 2-8^{o}C]
- Hank's Balanced Salt Solution [Himedia, Cat # TS1020, store at 2-8^{o}C]
- Millicell inserts [Millipore, Cat # PIHP012050]
- Lucifer yellow [Sigma, Cat # L0259, store at RT]
- 24 well plate [Falcon, Cat # 353047]
- Caco-2 cell lines [ATCC]
- Pipettes, Reagent reservoir
- CO_2 incubator [Binder]
- TEER Equipment [Millipore, Cat no # MERS00001]
- FLUOstar OPTIMA [BMG Labtech]
- HPLC system [Shimadzu system, LC,2010A*HT*]
- **Cumerone ®** (CB/09001)
- **Curcumin (95% curcuminoids)** (CL/09)

Preparation of solutions:

Acetaminophen:

- Stock A [5mM]: 3.77mg in 5mL of HBSS
- Stock B [1mM]: 200µL of stock A diluted to 1mL with HBSS
- Working solution [0.5mM] was prepared by diluting Stock B 1:1 with HBSS

Test Sample Preparation:

The following two samples were evaluated:

1. Cumerone ® (CB/09001)
2. Curcumin (95% curcuminoids) (CL/09)

The above samples (100mg each) were dissolved in 25mL of HBSS. The samples were sonicated for 20 minutes and then filtered using 0.4 micron filter. The concentration of the filtrate was measured by HPLC and was considered as the initial concentrations and 200µL from these stocks was added to the apical side of the inserts which were placed on 600µL of the media.

Method for Caco cell analysis

1. The Caco-2 cells were grown on 12mm inserts as per SOP #**NR/ADME/SOP/CACO/01.** After 21 days of culture the cells were ready for the permeability study.
2. The Trans Epithelial Electrical Resistance of each insert was measured as per the SOP **#NR/ADME/SOP/TEER/01.**
3. The Lucifer Yellow transport on the cell monolayer was conducted on insert as per SOP **#NR/ADME/SOP/LY/01.**
4. The monolayer was washed with sterile HBSS, pH 7.4 for 3 times.
5. After washing, the HBSS was removed from the inserts and receiver plate.
6. 200µL of Acetaminophen and the Curcuminoids samples were added into separate inserts to determine the rate of drug transport.
7. The wells of the receiver plate were filled with 600µL HBSS and incubated at 37°C in CO_2 incubator.
8. The samples from the receiver plate were collected at one hour interval for 3hrs.
9. The volume of sample (300µL) taken from the receiver plate was replaced by same volume of fresh HBSS (300µL) and kept back in the incubator.
10. After 1 and 3 hours of incubation time samples were collected from the basolateral side and at zero time sample was taken from apical side of the insert.
11. The amount of sample transported across the monolayer was quantified by HPLC.

The samples were analysed by HPLC and apparent permeability was calculated according to the below formula:

$Papp = dQ/dt \times 1/AC_0$

dQ/dt: mass transfer per unit time

A: exposed filter surface area

C_0: the initial concentration in the donor compartment

HPLC Method of Analysis

Reverse phase analytical HPLC was conducted on a Shimadzu LC-2010 CHT with UV-visible/ PDA detector set at 425 nm and fitted with a C18 column (Phenomenex, USA, 250 x 4.6mm, 5m). The mobile phase consisted of KH_2PO_4 (0.001N) and H_3PO_4 (0.05%) in HPLC grade water (A) and acetonitrile (B) utilizing the following gradient solvent system over a run time of 14 min - 60% A in B for 3 min, 20-15% A in B for next 3 min, 15-20% A in B for next 2 min, 20-60% A in B for next 2 min and 60% A in B for next 4 min until completion of the run. The flow rate of the mobile phase was 2.0 ml/min. The required concentrations of standards were prepared in methanol whereas the samples in HBSS were diluted 1:1 with methanol and 50µl of each solution was injected into HPLC. The detection limit for the curcuminoids was 1 nanogram / mL.

Results

Table 1: Trans Epithelial Electrical Resistance (TEER) readings (Ωcm^2)

Permeability study by using Caco-2 cell lines - TEER readings				
Inserts	**Raw data**		**Raw data - Blank**	**TEER value(Ωcm^2)**
1	Blank	165	0	
2	Blank	165	0	
3	1	1259	1094	656
4	2	1238	1073	644
5	3	1267	1102	661
6	4	1290	1125	675
7	5	1201	1036	622
8	6	1283	1118	671
9	7	1287	1122	673
10	8	1255	1090	654
11	9	1226	1061	637
12	10	1236	1071	643
13	11	1254	1089	653
14	12	1263	1098	659
15	13	1280	1115	669
16	14	1258	1093	656
17	15	1232	1067	640
18	16	1271	1106	664

Table 2: **Lucifer yellow transport data**

	RFU*		
	Raw data	**Raw data - Blank**	**% Transport**
Blank	62		
Equilibrium	6723	6661	**0.39%**
Basolateral	88	26	

* Relative Fluorescence Units

Percentage transport of Lucifer yellow was calculated using the following equation:

$$\text{Percentage transport} = \frac{(RFU_{test} - RFU_{blank})}{(RFU_{equi} - RFU_{blank})} \times 100$$

Permeability in Caco-2 cell line:

The TEER values for all the inserts were found to be more than 500Ωcm^2 (Table 1). The percentage transport of Lucifer yellow (Table 2) was performed for the insert showing least TEER value and was found to be less than 1%. Both these tests together confirmed the monolayer integrity and suitability of the insert for permeability studies.

Solubility:

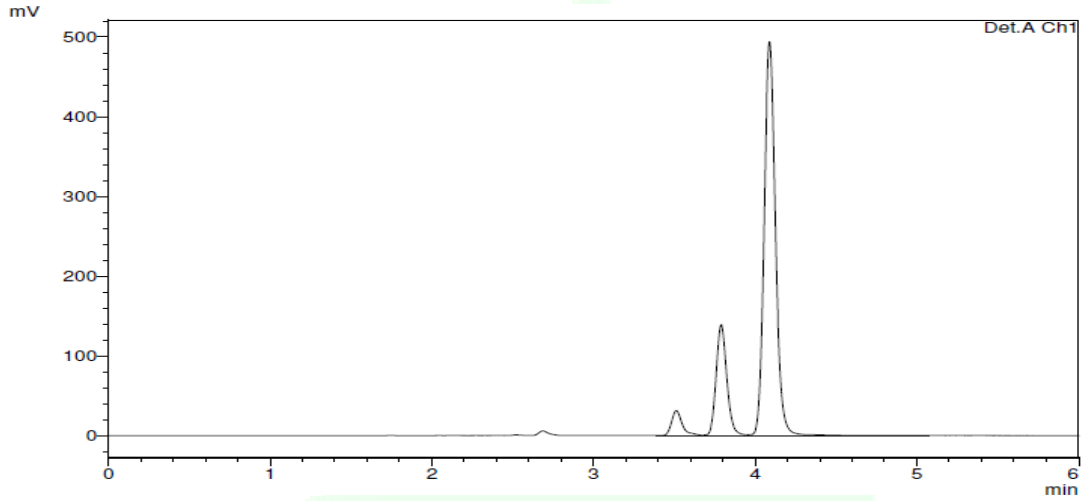
Concentration of curcuminoids in the filtered solution (after sonicating 4mg/ml concentrations of samples in HBSS) of

1. **Cumerone®** (CB/09001) was 74.88ug/ml
2. **Curcumin (95% curcuminoids)** (CL/09) was below detectable limits by HPLC .

These results indicate that the solubility of curcuminoids in **Cumerone®** (CB/09001) are dramatically increased in comparison to **Curcumin (95% curcuminoids)** (CL/09). It is evident that while **Curcumin (95% curcuminoids)** (CL/09) has little or no solubility below the limits of detection of 1ng/ml,, even when the curcuminoids are soluble (as shown in the apical side in Fig.3) there is little or no permeation across the membrane (as shown on the basolateral side in Fig.s 4 & 5), when tested at the same initial concentrations of 4mg/ml. The solubility of both demethoxy-curcumin and bisdemethoxy-curcumin in HBSS buffer is greater in comparison to curcumin in pure methanol.

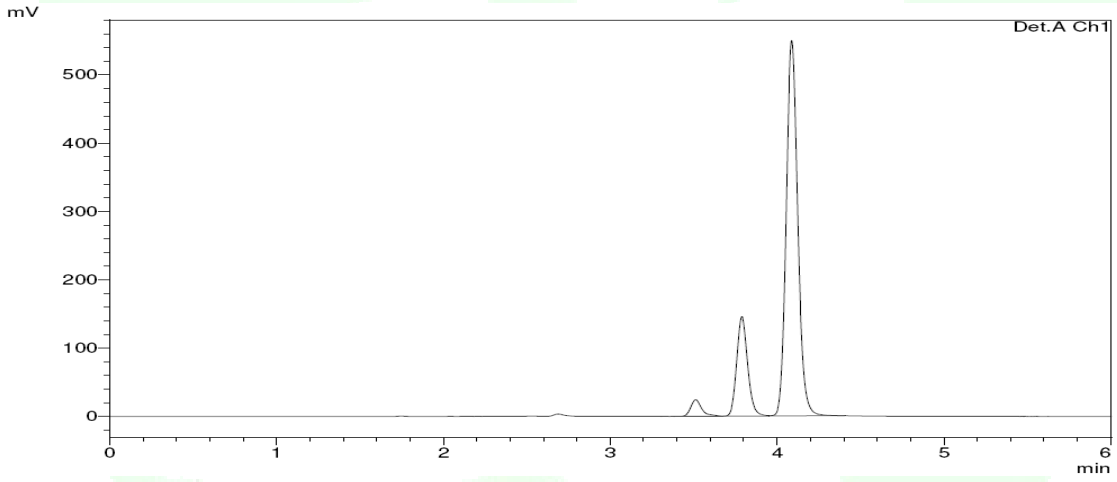
The percentage of different curcuminoids in methanol is shown in the following chromatograms:

Fig 1 Cumerone® (CB/09001) in methanol



Sample	Bisdemethoxy curcumin	Demethoxy curcumin	Curcumin
CB/09001 in methanol	4.55	20.10	75.40

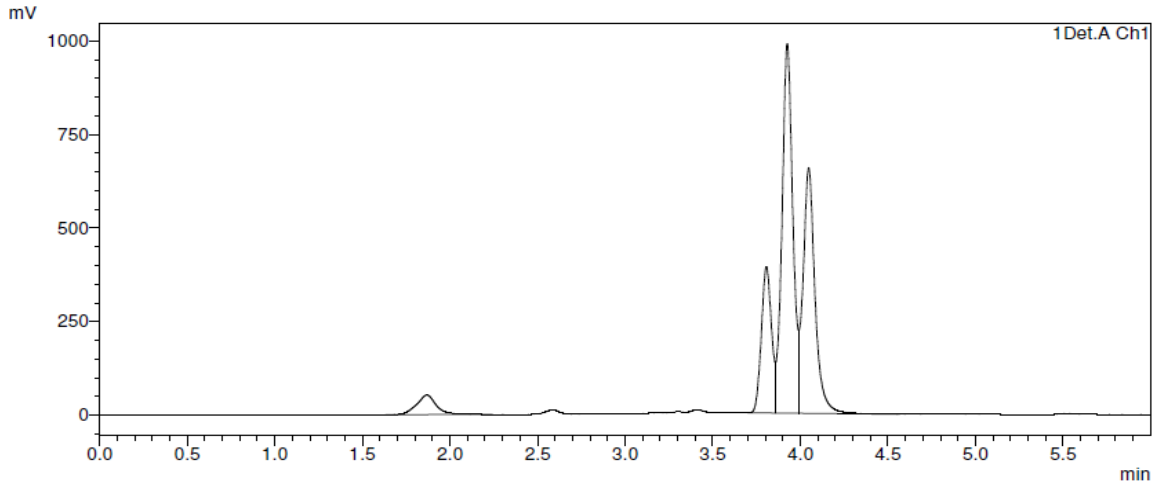
Fig 2 Curcumin (95% curcuminoids) (CL/09) in methanol



Sample	Bisdemethoxy curcumin	Demethoxy curcumin	Curcumin
CL/09 in methanol	3.20	19.40	77.40

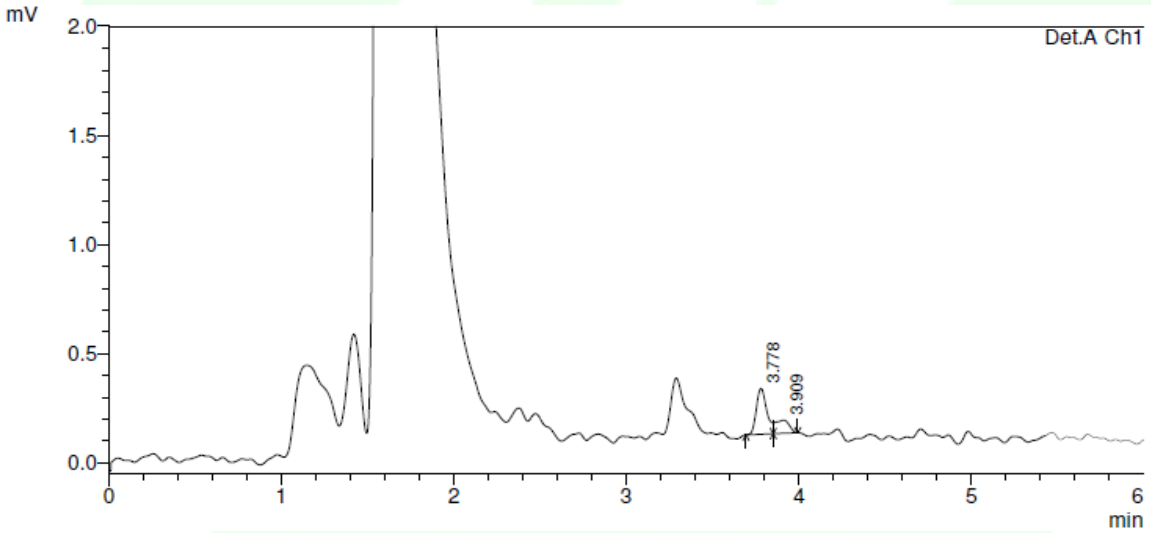
The percentage of different curcuminoids in apical and basolateral samples is shown in the following chromatograms:

Fig 3 Cumerone® (CB/09001) (Apical side) in HBSS buffer



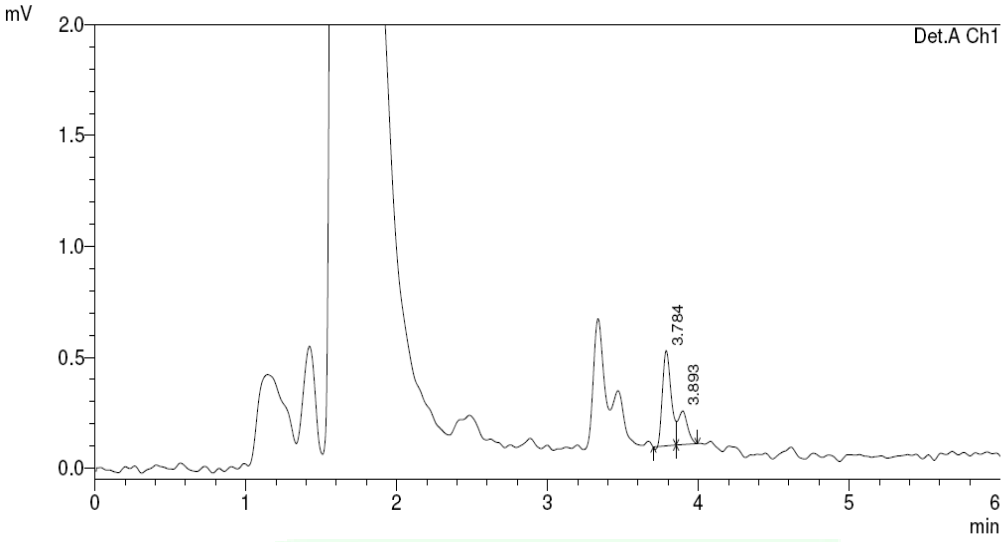
Sample	Bisdemethoxy curcumin	Demethoxy curcumin	Curcumin
Apical	17.68	47.41	34.91

Fig 4 Cumerone® (CB/09001) (Basolateral side 1h)



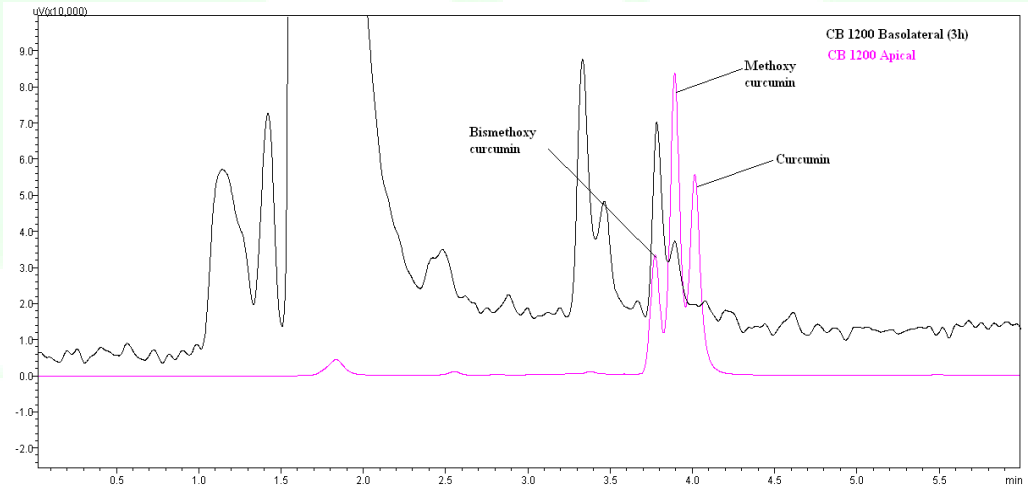
Sample	Bisdemethoxy curcumin	Demethoxy curcumin	Curcumin
Basolateral (1h)	72.54	27.46	ND

Fig 5 Cumerone® (CB/09001) (Basolateral side 3h)



Sample	Bisdemethoxy curcumin	Demethoxy curcumin	Curcumin
Basolateral (3h)	71.74	28.26	ND

Fig 6 Overlap of Apical and Basolateral (3h) Chromatograms comparing Cumerone® (CB/09001)



*The Y-axis scale is applicable only to the basolateral chromatogram

Table 3: **Percentage of curcuminoids in apical and basolateral samples**

Sample CB/09001	**Bisdemethoxy curcumin**	**Demethoxy curcumin**	**Curcumin**
Apical	17.68	47.41	34.91
Basolateral (1h)	72.54	27.46	ND
Basolateral (3h)	71.74	28.26	ND

Table 4: **Apparent permeability coefficient value of Acetaminophen**

Concentration (µmoles)			**Flux (µmoles/s)**	**P_{app} ($x10^{-6}$cm/s)**	
1h	**2h**	**3h**			
0.03672	0.04356	0.08280	0.00000709	23.6333	23.37 ± 0.38
0.03870	0.04386	0.08148	0.00000693	23.1000	

P_{app} = Flux x $1/AC_0$

= 0.00000709**µmoles/s** x { 1/ (0.6**cm^2** x 0.5**mM**}

= 0.00000709 x {1 / (0.3)}

= 23.6333 x 10^{-6}cm/s

The apparent permeability coefficient of the standard (Acetaminophen) as given in Table 4 was found to be in good agreement with the reported values[6].

Table 5: **Apparent permeability coefficient values of Cumerone® (CB/09001) sample**

Samples	Conc:*	CAR** (µg)			Flux (µg/s)	Papp ($x10^{-7}$cm/s)	
		1h	2h	3h		Values	Mean
Cumerone® (CB/09001)	64.42mg/L (64.42µg/mL)	0.00387	0.00779	0.01335	0.000001221	0.3158	**0.3162**
		0.00403	0.00645	0.01388	0.000001224	0.3166	

* Initial concentration on the apical side ** Cumulative Amount Released

P_{app} = Flux x $1/AC_0$

= 0.000001221**µg/s** x { 1/ (0.6**cm^2** x 64.42**mg/L**)}

= 0.000001221 x { 1/ (38.652)}

= 0.3158 x 10^{-7}cm/s

P_{app} = Flux x $1/AC_0$

= 0.000001224**µg/s** x { 1/ (0.6**cm^2** x 64.42**mg/L**)}

= 0.000001224 x { 1/ (38.652)}

= 0.3166 x 10^{-7} cm/s

The analysis of curcuminoids in apical basolateral solutions was carried out as per the same method described above. . The P_{app} value for **Cumerone®** (CB/09001) was 0.3166 x 10 $^{-7}$ cm /sec while apparent permeability of **Curcumin (95% curcuminoids)** (CL/09) could not be calculated since the amount was less than the HPLC detection limit.

Conclusion:

Poor aqueous solubility of curcumin is an important factor in hindering its absorption in the gastrointestinal tract. Curcumin is known to undergo extensive intestinal and hepatic biotransformation by sulphation, glucuronidation and reduction resulting in its overall low bioavailability in rats and humans[7]. Various formulations have been researched with the aim of improving both the solubility as well as the bioavailability of curcumin[8].

The results of this study clearly demonstrate that the proprietary formulation **Cumerone®** (CB/09001) containing curcuminoids, yields a dramatic increase in the solubility of curcuminoids when compared to the solubility of curcuminoids in **Curcumin (95% curcuminoids)** (CL/09). Also observed with **Curcumin (95% curcuminoids)** (CL/09), is evidence that even when these curcuminoids are solubilised, there is little or limited permeability across the Caco-2 membrane.

In examining the solubility of three types of curcuminoids in HBSS buffer, the solubility of both demethoxy-curcumin and bisdemethoxy-curcumin were greater than the solubility of curcumin and it is these two compounds that have been found to permeate the Caco-2 monolayer.

Cumerone® has exhibited an apparent permeability (P_{app}) coefficient of 0.3166 x 10^{-7}cm /sec when compared to an undetectable permeability of **Curcumin (95% curcuminoids).** This clearly indicates that **Cumerone®** (CB/09001) shows an increased absorption across Caco-2 cell line. The peaks appearing at 3.3 min are likely to be metabolites of demethoxy-curcumin and bisdemethoxy-curcumin as these have been identified as the curcuminoids with the greater solubility.

The overall results of this study indicate that **Cumeron®** (CB/09001) demonstrates a probable oral absorption in humans of 5 - 15 % of the oral dose (as calculated in Ref.3) and also a dramatic increase in solubility in comparison with **Curcumin (95% curcuminoids)** (CL/09) when measured in physiological HBSS buffer.

References:

[1] Hidalgo I.J *et al*., *Gastroenterology*. 96:736-749, 1989

[2] Shinji Yamashita *et al*., *Pharm Res*. 14:486-491, 1997

[3] Shinji Yamashita *et al*., *European Journal of Pharmaceutical Sciences*. 10:195-204, 2000

[4] Jeanne E Philips *et al*., Millipore Protocol Note

[5] Yang et al., *Journal of Chromatography*, 853: 183-189, 2007

[6] Anthony M. Marino et al., *International Journal of Pharmaceutics*. 297: 235-241, 2005

[7] Christopher R. Ireson et al *Cancer Epidemology, Biomarkers & Prevention*.11:105-111, 2002

[8] Preetha Anand et.al. *Molecular Pharmaceutics* 4: 807-818, 2007

Sherly Varghese	Dr Shekhar Dethe	Dr. Amit Agarwal
Analyst	**Report Verification**	**Authorized signatory**