Online : ISSN 2349-669X
Print : ISSN 0973-9874

J.Pharm.Chem

CODEN: JPCOCM

Journal of Pharmacy and Chemistry

(An International Research Journal of Pharmaceutical and Chemical Sciences)
Indexed in Chemical Abstract and Index Copernicus (IC Value 5.28)

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Volume 12 • Issue 3 • July – September 2018

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Evaluation of *Punica granatum Roots* for *In Vitro* Anti Urolithiatic Activity

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ABSTRACT

Different extracts of *Punica granatum* roots have been evaluated for *in vitro anti-urolithiatic* activity. The obtained results reveal that, roots extract of *Punica granatum* possess a prominent antiurolithiatic property. Among various extracts used for *in vitro* model the methanol extract of *Punica granatum* roots exhibited favourable *antiurolithiatic* activity which is equipotent to the standard drug cystone. The percentage dissolution of calcium oxalate crystals by methanol extract was 43.54% at 25mg and 56.44% at 50mg, similarly aqueous extract also showed marginal *anti urolithiatic* activity with percentage dissolution ranging from 35.48% to 46.77% at 25mg and 50mg concentration respectively. The highest percentage dissolution of calcium oxalate crystals exhibited by methanol extract was 56.44 % at the dose 50mg almost near to standard i.e. 67.68%.

Keywords: Anti urolithiatic activity ; *Punica granatum* ; *In Vitro*

Introduction

The condition of having urinary calculi is termed as nephrolithiasis and urolithiasis. "Lith" referred as "stone". The calculi are mainly composed of calcium oxalate crystals which are formed due to presence of higher concentration of hydrogen ions, sodium ions and uric acid in filtrate. This condition is also known as super saturation. The supersaturated ions come out of solution and crystallize[1]. 5-7 million Indians suffer from renal stones every year[2] .The incidence rate in the western world is 0.5% and life time risk of developing urolithiasis is about 10–15%, but in the Middle East it can be as high as 20–25%.[3] The recurrence chances without preventive treatment is approximately 10% at 1 year, 33% at 5 years, and 50% at 10 years.[4,5]

Punicaceae is one of the plant family used in treatment of urolithiasis[6]. In India *Punica granatum* L. is used for treatment of urinary stones [7]. Around 50 ml of fruit juice is given before breakfast till stone expulsion or boiled rind of 1 fruit in one litre of water till 30 mins and filtered the same 250 ml BD / TID is given for 7 days.[8] In this view, the present study aimed to evaluate *Punica granatum* roots for *in vitro* anti-urolithiatic activity.

Punica granatum is a deciduous shrub of iran[9]. It is also commonly found in the Himalayas in northern India, China, USA and throughout the Mediterranean region. The *Punica granatum* has several anatomical compartments such as seed, juice, peel, leaf, flower, bark, and root which possess different pharmacological and toxicological chemical compositions such as antioxidants,anti-inflammatory agents anti carcinogens etc[10]., *Punica granatum* is used in different illness ranging from inflammation, rheumatism to the pain of a simple sore throat. It is also used as vermifugal agent for killing and expelling intestinal worms[12,13]. Other treatments include leprosy[13], snakebite[14], diabetes[15], burns[16] and fresh fruit can be used to lower fever[17]. *Punica granatum* has shown inhibitory effects on different types of cancers such as lung cancers[18], breast[19], prostate [20,21], and colon[22,23]. *Punica granatum* inhibited NF-k Band cell viability of prostate cancer cell lines in a dose-dependent manner in the LAPC4 xenograft model in *in-vitro* analysis[24]. *Punica granatum* inhibits CYP activity which is necessary for activation of procarcinogens, therefore inhibit carcinogenic effects[25]. *Punica granatum* chemical components such as 3,8-dihydroxy- 6H-dibenzo[b, d]pyran-6-one (urolithin A, UA) produced from Ellagitannins (ETs) also possess anti-cancer effects26. Fermented juice of *Punica granatum* fruit showed antioxidant activity whereas extracted juice from flowers showed reduction in blood sugar and lipid levels[27]. Flavonoids and tannins of fruit juice prevent growth of cancer cells[28] . Flavonoids present in the watery extract and fruit peel have estrogenic activity[29].

There is lot of research done on different parts of pomegranate plant and fruits. The medicinal use of *Punica granatum* roots is less commonly known. Hence, our study aimed to conduct *in vitro* evaluation of *Punica granatum* roots for the anti-urolithiatic activity.

Materials and Methods:

Extraction:

The shade dried roots of *Punica granatum* Linn (punicaceae) were pulverized to fine powder and around 2 kg subjected to continuous hot extraction with methanol (95%) into 15 batches of each 250 – 280g in a Soxhlet extractor. After complete extraction, the solvent was evaporated to concentration at room temperature. Finally it is dried under reduced pressure using flash evaporator.

Simultaneously around 2 kg of powdered drug was subjected for maceration with cold water for 7 days with occasional stirring followed by filtration to obtain aqueous extract as filtrate. The aqueous extract was subjected to concentration on water bath at low temperature followed by drying under reduced pressure in a flash evaporator.

Evaluation of *In Vitro* Anti-urolithiatic *activity*.

Materials:

1. Decalcified semipermeable egg membrane prepared freshly and subjected for *In vitro* anti-urolithiatic activity.
2. Methanol and aqueous extracts of roots of *Punica granatum* linn.were freshly prepared in DMSO solution.
3. Both methanol and aqueous extracts from roots of *Punicagranatum* were evaluated for *in vitro* anti urolithiatic activity at different doses 25 mg/ml and 50 mg/ml, separately.
4. Calcium oxalate crystals prepared artificially and the same used for *in vitro anti-urolithiatic* activity.
5. Cystone sample (50mg /ml) prepared in DMSO solution used as standard.

Procedure:

a. **Preparation of semipermeable membrane :**

The semi- permeable membrane was prepared by keeping whole fresh egg in freshly prepared 2M HCL for overnight, which caused complete decalcification of egg shell. Further, washed with distilled water, and carefully with a sharp pointer a hole is made on the top and the content squeezed out completely from decalcified egg. Washed thoroughly with water and stored in refrigerator in Tris buffer (pH of 7-7.4).

b. Preparation of calcium oxalate crystals :

Equimolar solution of calcium chloride di-hydrate dissolved in distilled water and sodium oxalate in 10 ml of 2 N sulphuric acid were prepared. Sufficient quantity of the above are allowed for reaction in a beaker. The resulting precipitate was calcium oxalate. The precipitate freed from traces of sulphuric acid by treating it with ammonia solution and washed with distilled water and dried at 60^0C for 4 hours.

c. Procedure for *in vitro* anti urolithiaticactivity :

In vitro anti-urolithiatic activity has been evaluated by calcium oxalate dissolution method[27]. Calcium oxalate crystals [28,29] and semi permeable membrane [30,31] required for the above study were prepared artificially by standard methods. Approximately 1 mg of prepared Caox crystals mixed with 1 ml of test samples viz Methanol extract, aqueous extract and standard (Cystone) samples separately. All the methanol extract, aqueous extract and cystone standard samples were prepared in DMSO solution at the concentrations of 25mg/ml and 50 mg/ml of each. The above samples individually packed in separate semi permeable membranes and sutured. The packed material in semi permeable membrane allowed to suspend in separate conical flask containing 100 ml 0.1 M Tris buffer. Simultaneously negative control (only CaOx 5 mg) has been prepared and subjected as above. All the above flasks were subjected for incubation at 37 ± 1^0 C for 07 hours.

Figure No.1: [A] Process of decalcification of egg [B] Prepared semi permeable membrane

Figure No.2: Photograph showing prepared.

Figure No.3: Photograph showing set up calcium oxalate crystals for anti-urolithiatic activity of test, standard, and negative control.

d. Estimatation of calcium oxalate by titrimetric method:

The obtained test samples and standard after incubatation were taken and removed the contents of semi-permeable membrane from each group into a test tube. To this added 2 ml of 1 N sulphuric acid and titrated with 0.9494 N $KMnO_4$ till a light pink color end point reached. 1 ml of 0.9494 N $KMnO_4$ equivalents to 0.1898 mg of calcium. Accordingly, the percentage dissolution of calcium was calculated.

Results:

The estimation of calcium oxalate was carried by performing titrimetric assay. Anti urolithiatic effect of extracts using *in vitro* model showed that, the methanol extract of *punica granatum* roots exhibited prominent i*n vitro* anti urolithiatic activity which is equipotent to the standard drug cystone. The percentage dissolution of calcium oxalate crystals by methanol extract was 43.54% at 25mg and 56.44% at 50mg, similarly aqueous extract also showed marginal *anti urolithiatic* activity with percentage dissolution ranging from 35.48% to 46.77% at 25mg and 50mg concentration respectively. The highest percentage dissolution of calcium oxalate crystals by methanol extract was seen at the dose 50mg which was 56.44 % which is nearly equipotent to standard i.e. 67.68%.

Discussion:

The dissolution profile of calcium oxalate crystals by titrimetric assay from various extract reveals that, methanol

Table - 1

Table showing the dissolution of calcium oxalate by test, standard and negative control at a concentration of 25mg

Sl. No.	Group	Volume of $KMnO_4$ (ml)	Weight of Calcium estimated (mg)	Weight of Calcium reduced (mg)	Percentage Dissolution
1	Negative Control	6.2	1.1767	-	-
2	Standard	2.5	0.4745	0.7022	59.67
3	Methanol extract	3.5	0.6643	0.5124	43.54
4	Aqueous Extract	4	0.7592	0.4175	35.48

Table - 2

Table showing dissolution of calcium oxalate by test, standard and control samples at concentration of 50mg

Sl. No.	Group	Volume of $KMnO_4$ (ml)	Weight of calcium estimated (mg)	Weight of Calcium reduced (mg)	Percentage Dissolution
1	Negative Control	6.2	1.1767	-	-
2	Standard	2.0	0.3796	0.7964	67.68
3	Methanol Extract	2.7	0.5124	0.6642	56.44
4	Aqueous Extract	3.3	0.6263	0.5503	46.77

extract of *Punica granatum* root showed dose dependent increased *in vitro* activity at 25mg and 50mg concentration. Whereas aqueous extract showed nominal *anti urolithiatic* activity compared to methanol extract. The highest percent *anti urolithiatic* activity was exhibited by methanol extract at 50mg concentration which was near to standard drug .This indicates that, the potent *in vitro anti urolithiatic* activity exhibited by methanol extract may be due to better calcium oxalate solubilising property of phytoconstituents present in the methanol extract.

However, the detailed phytopharmacological evaluation for the claimed *in vitro anti urolithiatic* activity of roots of *Punica granatum* is yet to be carried.

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Development and Validation of A Reverse Phase HPLC Method for Rapid Determination of Naratriptan Hydrochloride In Bulk and Fast Dissolving Oral Film Formulation

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ABSTRACT

Naratriptan hydrochloride is a selective 5-hydroxytryptamine drug used to provide instant relief from migraine. A simple, sensitive and rapid RP-HPLC method was developed and validated for determination of Naratriptan hydrochloride in bulk and fast dissolving oral film, using Cosmosil C18 column (250 mm 4.6 mm, 5 µ) as stationary phase, acetonitrile and water (80:20 v/v) as mobile phase with a flow rate of 0.8 mL/min. The analyte was determined using UV detector at 223 nm. The retention time was found to be 3.6 min. The developed method was validated for linearity, specificity, system suitability, precision, ruggedness, robustness and accuracy. The calibration curve was linear over a concentration range of 5-35 mcg/mL, with LOD and LOQ values of 0.3188 mcg/mL and 3.188 mcg/mL respectively. All the validation parameters were found to be within the acceptance criteria.

Keywords: Naratriptan hydrochloride, Cosmosil C18 column, fast dissolving oral film, RP-HPLC

Introduction

Migraine is a chronic neurological disorder characterized by recurrent, moderate to severe headache often in association with a number of autonomic nervous system related symptoms. Typically, it affects one half of the head, is pulsating in nature, and lasts for about 2 to 72 hours [1]. About 33% of patients suffering from migraine perceive an aura: a transient visual, sensory, language, or motor disturbance which signals that the headache will occur soon. Symptoms are disturbances such as altered mood, irritability, depression or euphoria, fatigue, craving for certain food, stiff muscles especially the neck, constipation or diarrhoea and sensitivity to smell or noise [2]. Also associated symptoms may include nausea, vomiting, and sensitivity to light, sound, or smell. The pain worsens with physical activity. Worldwide, migraine is the fifth leading cause of disability and is three times more common among females, often occurring between early and middle adulthood [3]. Naratriptan hydrochloride (Fig 1), a triptan drug used for the treatment of migraine, is a selective 5- hydroxytriptamine receptor 1 ($5HT_1$) subtype agonist [4].

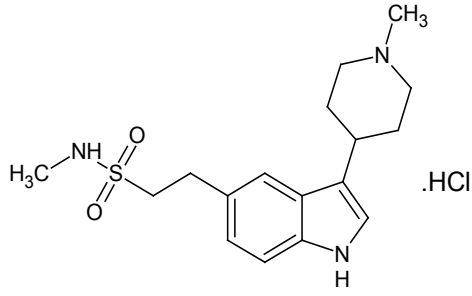

Fig 1: Structure of Naratriptan hydrochloride

N-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethanesulfonamide hydrochloride.

Molecular formula: $C_{17}H_{25}N_3O_2S$•HCl

Molecular weight: 371. 924 g/mol

Naratriptan hydrochloride (NH) belongs to group II triptans [5]. Orally administered naratriptan reaches its peak plasma concentration in 2 to 3 h and has a long half-life of 6h. The oral bioavailability of NH is 74%. The oral dose of NH should not exceed 5 mg in 24 h and may be administered in divided doses twice or thrice a day. 50% of an administered dose of naratriptan is excreted unchanged in urine and about

30% is excreted as metabolic products of cytochrome P450 oxidation [6]. It also has a lower headache recurrence rate and is less likely to cause drug interaction [7,8].

As reported in the literature, very few analytical methods have been developed for the estimation of NH in pharmaceutical dosage form and biological fluids like HPLC-UV, LC-MS, and UPLC. A stability indicating method was developed for NH using acetonitrile and phosphate buffer in ratio of 20:80 v/v, (pH adjusted to 4.0 using orthophosphoric acid) with UV detection at 225 nm, injection volume of 20 µL and flow rate of 1 mL/min. Linearity range was found to be 0.1-25 mcg/mL with r^2=0.9991. The retention time was found to be 6 minutes [9]. A RP-HPLC was developed for NH, using ammonium phosphate monobasic buffer and acetonitrile in the ratio of 65:35 v/v and the retention time was found to be 7.2 min [10].

In the present investigation, an attempt has been made to develop a rapid, simple and sensitive reverse phase HPLC method for determination of NH in bulk and fast dissolving oral film.

Materials and methods

Materials

Naratriptan hydrochloride was procured as a gift sample from APOTEX Pvt Ltd, Bengaluru. HPLC grade Water and Acetonitrile (Fischer scientific) were used. Orthophosphoric acid and triethylamine of LR grade were used.

Instrumentation

Method development and validation for estimation of NH was carried out using Shimadzu LC (Grace Smart RPc18 model) equipped with detector (Shimadzu spd 10A UV-vis, Japan), pump (Shimadzu LC-10ATVP, Japan), injection valve (7725i Rheodyne 20 µl, USA), syringe (50 µl Hamilton, Switzerland), column (Cosmosil C18 coloumn (250 mm □ 4.6 mm, 5 µ) with a baseline chromatography data system N2000 software.

Preparation of mobile phase

The mobile phase was prepared by mixing acetonitrile and water in 80:20 v/v ratio respectively. It was sonicated for 10 min and filtered through 0.45 µ Millipore filter paper disc.

Preparation of standard solution

25 mg of NH was weighed accurately and transferred into a 25 mL volumetric flask, dissolved in the mobile phase (80:20 v/v) and volume made up to the mark with the same to get concentration of 1 mg/mL.

Preparation of working standard solution

A working standard solution was prepared by diluting 0.2 mL of standard solution to 10 mL using mobile phase to obtain a concentration of 20 mcg/mL.

Preparation of sample for fast dissolving oral film

Table - 1
Composition of fast dissolving oral film

Ingredients	Quantity
Naratriptan hydrochloride	2.5 mg
HPMC E-15	2.9 mg
Stevia extract powder	86 mg
Cross carmellose sodium	10 mg
Citric acid	10 mg
Propylene glycol	114 mg
Flavour	16 mcg

A 4 cm^2 strip containing 2.5 mg of NH was dissolved in 2.5 mL of mobile phase. Further, 0.2 mL of above solution was diluted to 10 mL using mobile phase and the concentration of NH present in the sample solution was estimated.

Method development and validation

The method was validated in terms of linearity, accuracy, precision, ruggedness and robustness as per ICH guidelines

Linearity

The linearity of the method was established by taking aliquots of 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 and 0.35mL respectively of dilute standard NH solution in to separate 10 mL volumetric flasks and making up the volume with mobile phase to obtain a concentration range of 5-35 mcg/mL. (Table 2 and Fig 2)

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD is defined as the smallest concentration of analyte that gives a measurable response (signal to noise ratio of at least 3) whereas, LOQ is the smallest concentration of analyte that can be quantified accurately (signal to noise ratio of at least 10). LOD and LOQ is calculated using the formula, LOD = 3.3 σ/s and LOQ = 10 σ/s where σ denotes standard deviation of response and s, slope respectively.

Accuracy

Accuracy of the developed method was determined by the method of standard addition. The sample was spiked with 75%, 100% and 125% of the selected concentration, viz. 20 mcg/mL

Precision

The precision of the method was studied with respect to

repeatability and intermediate precision. Intra-day precision was determined by six sequential injections of 20 mcg/mL of dilute standard NH solution. Intermediate precision was assessed by injecting dilute standard NH solution in triplicate on three different days. System suitability parameters such as retention time, peak height, peak area, tailing factor and number of theoretical plates were determined.

Ruggedness

Ruggedness of an analytical procedure is a measure of the ability of the method to remain unaffected when analysis is performed by different analysts or by the same analyst. It was analysed in triplicate using 20 mcg/ml concentration.

Robustness

Robustness of the method was evaluated by deliberate variation of chromatographic conditions like flow rate, wavelength, and pH. The pH of the mobile phase was varied by using either orthophosphoric acid or triethylamine.

Results and Discussion

A reverse phase HPLC method has been developed for determination of NH in bulk and fast dissolving oral film formulation. Preliminary trials were carried out using different mobile phase solvents like 0.05M ammonium phosphate buffer and acetonitrile (97:3 v/v), 0.05M glacial acetic acid and acetonitrile (50:50 v/v and 70:30 v/v), but they did not give a sharp, well resolved peak; finally acetonitrile and water with 80:20 v/v ratio was selected as it gave a single chromatographic peak with good resolution (Fig 3). The developed method was validated as per ICH guidelines. The retention time and tailing factor was found to be 3.6 min and 1.43 respectively. As indicated in (Table 6), all the system suitability parameters met the acceptance criteria. (Table 7).

Table 2
Data for calibration curve of Naratriptan hydrochloride

Sl. No.	Concentration (mcg/mL)	Average peak area
1	5	827564
2	10	1582515
3	15	2484270
4	20	3292249
5	2	4214841
6	30	5087508
7	35	5976041

Table 3
Data of calibration curve obtained, LOD and LOQ

Linearity	5-35mcg/mL
Regression equation	Y = 171040X + 60083
Correlation coefficient(R^2)	0.9994
LOD (mcg/mL)	0.3188
LOQ (mcg/mL)	3.1882

Calibration curve was found to be linear within a concentration range of 5-35 mcg/mL. Seven different concentrations of dilute standard NH solutions were prepared and 20 µL of each concentration was injected. The correlation coefficient (R^2) was found to be 0.9994 which indicated an excellent correlation between response and concentration. The slope and intercept were found to be 60083 and 171040 respectively. LOD and LOQ were found to be 0.3188 mcg/mL and 3.188 mcg/mL respectively. (Table 3)

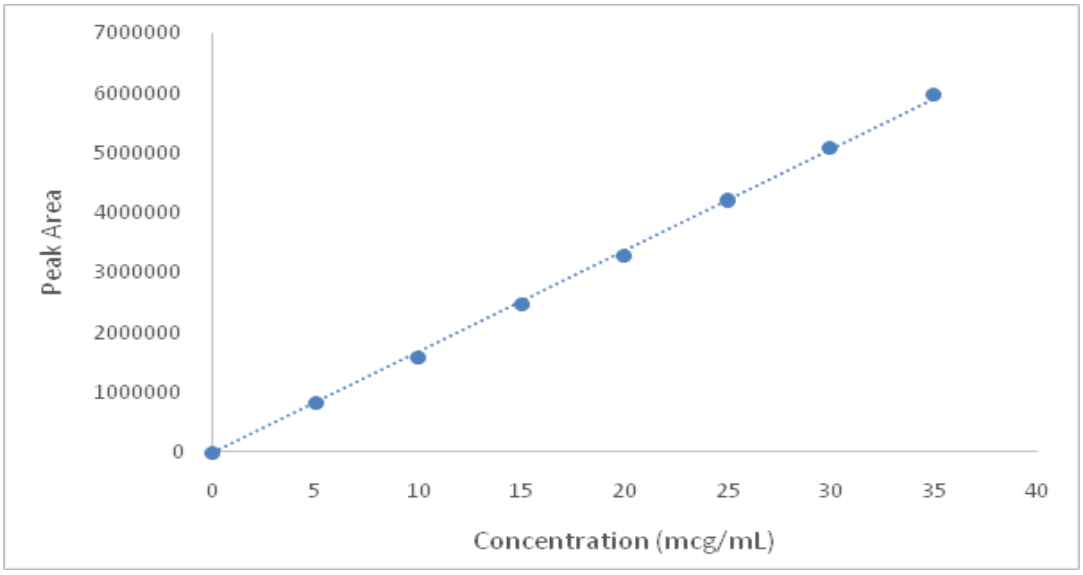

Fig 2: Standard curve of Naratriptan hydrochloride

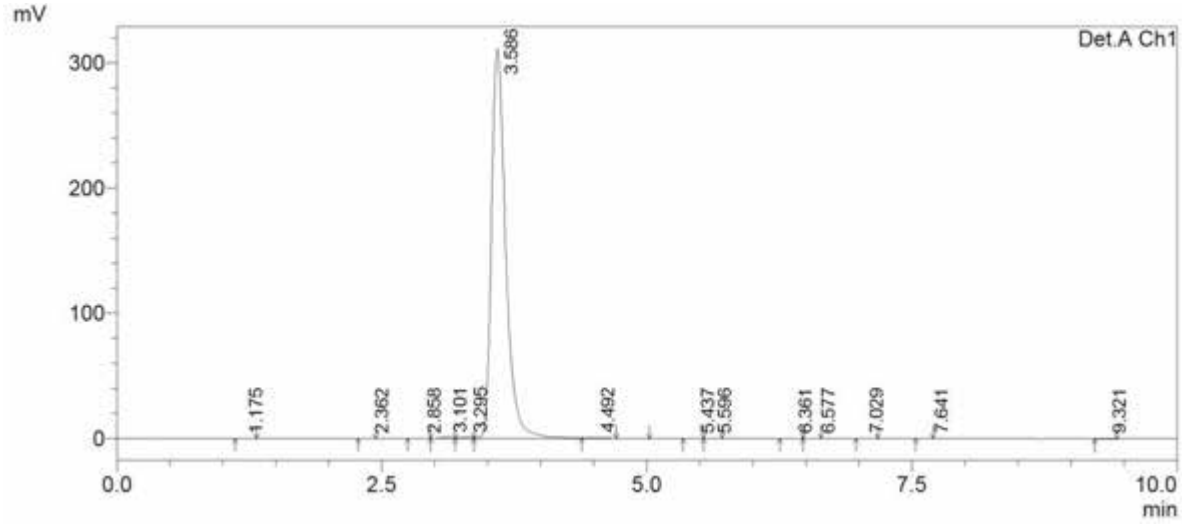

Fig 3: Chromatogram of Naratriptan hydrochloride

Accuracy of the method was determined from recovery data of spiked samples and percent recovery was found to be 95.12%, 99.74% and 100.02% for 75%, 100% and 125% respectively. (Table 4) .

Table - 4
Results of Accuracy

Sl. No	Percentage level of standard	Peak area	SD	% RSD	Sample concentration (mcg/mL)	Standard concentration (mcg/mL)	Amount of standard (mcg/mL)		Recovery (%)
							Spike	Found	
1.	75%	2429077	657.84	0.0270	10	5	15	14.75	95.12%
2.	100%	3288094	1592.45	0.0484	10	10	20	19.97	99.74%
3.	125%	4115925	1560.09	0.0379	10	15	25	25.00	100.02%

Table 5
Results of Intra-day precision

Time	Peak area	Retention time
1	3254936	3.58
2	3271923	3.58
3	3264849	3.58
4	3245800	3.58
5	3261987	3.58
6	3273871	3.58
12	3274030	3.58
Mean	3263914	3.58
SD	10637	1
%RSD	0.326	0.289

Table 6
Results of Inter-day precision

Sl. No	Peak area		
	Day -1	**Day – 2**	**Day – 3**
1	3245639	3235639	3245639
2	3237020	3258020	3234020
3	3252974	3253974	3252874
Mean	3245211	3249211	3244178
SD	7985.60	11926.52	9511.56
%RSD	0.246	0.367	0.293

Precision of the method was established from intraday determination and ruggedness from inter-day determinations respectively. The relative standard deviation (RSD) of <1% indicated a good precision of the method. (Table 5,6).

Table 7
Data of system suitability parameters

System suitability parameter	Acceptance Criteria	%RSD	Criteria met/not met
Injection precision for retention time (min)	RSD ≤ 1%	0.000	Met
Injection precision for peak area (n=6)	RSD ≤ 1%	0.123	Met
USP tailing factor (T)	T = ≤ 2.0	1.454	Met
Theoretical Plates (N)	N = ≥ 2000	2275.202	Met

Table - 8
Data showing results of robustness with respect to flow rate

Change in flow rate		
Flowrate 0.72mL/min	**Peak area**	**Retention time (min)**
1	3572503	3.81
2	3578933	3.81
3	3579707	3.81
Mean	3577047.667	3.81
SD	3954.7775	0.0058
%RSD	0.1105	0.1522
Flowrate 0.8mL/min	**Peak area**	**Retention time (min)**
1	3245800	3.58
2	3261987	3.58
3	3273871	3.58
Mean	3260552.667	3.58
SD	14090.3599	0.0058
%RSD	0.4321	0.1681
Flowrate 0.88mL/min	**Peak area**	**Retention time (min)**
1	2958736	3.13
2	2955263	3.13
3	2958315	3.13
Mean	2957438	3.13
SD	1895.3308	0.0057
%RSD	0.0640	0.1821

Table - 9
Data showing results of robustness with respect to wavelength

Change in Wavelength		
223 nm	**Peak area**	**Retention time (min)**
1	3356671	3.43
2	3359929	3.43
3	3357581	3.43
Mean	3358060.333	3.43
SD	1681.060	0.0057
%RSD	0.0500	0.1661
225 nm	**Peak area**	**Retention time (min)**
1	3245800	3.58
2	3261987	3.58
3	3273871	3.58
Mean	3260552.667	3.58
SD	14090.3599	0.0057
%RSD	0.4321	0.1652
227 nm	**Peak area**	**Retention time (min)**
1	3186204	3.41
2	3188942	3.41
3	3185992	3.41
Mean	3187046	3.41
SD	1645.4020	0.0057
%RSD	0.05162	0.1671

Table 10
Data showing results of robustness with respect to pH

Change in pH		
pH 3.0	**Peak area**	**Retention time (min)**
1	3028996	3.28
2	3059897	3.27
3	3022423	3.28
Mean	3033772	3.2766
SD	14346.1772	0.0057
%RSD	0.4728	0.1739
pH 3.2	**Peak area**	**Retention time (min)**
1	3245800	3.58
2	3261987	3.58
3	3273871	3.58
Mean	3260552.667	3.58
SD	14090.3599	0.0057
%RSD	0.4321	0.1652
pH 3.4	**Peak area**	**Retention time (min)**
1	3055906	3.61
2	3050832	3.62
3	3082876	3.61
Mean	3063204.667	3.613333
SD	17223.7448	0.00577
%RSD	0.5622	0.1577

Robustness of the method was evaluated by small deliberate changes in the optimised parameters of chromatographic conditions like flow rate, pH and wavelength of detection on retention time and tailing factor. The % RSD was calculated for each parameter and it was found to be <1%. (Table 8,9,10)

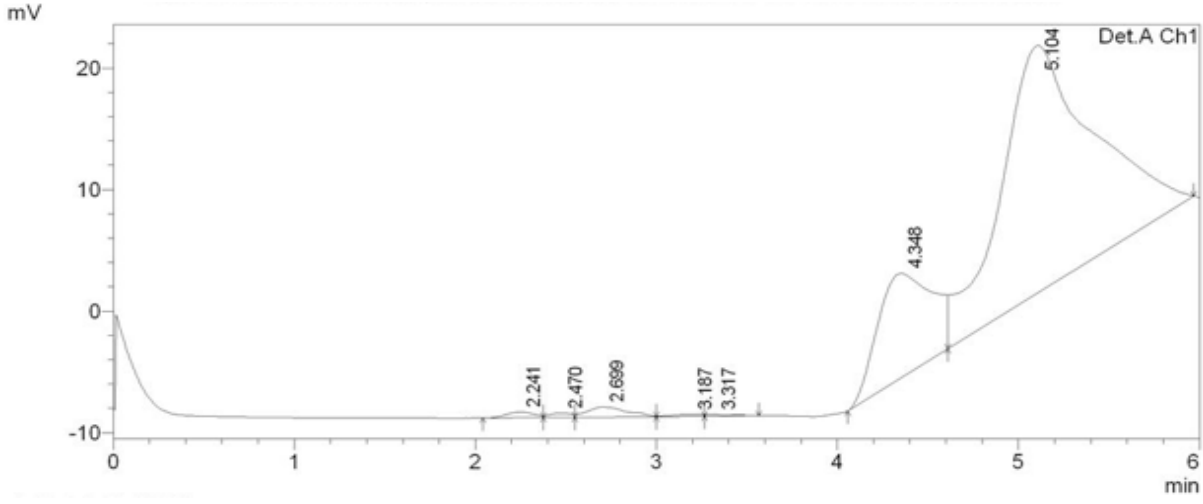

Fig 4: Chromatogram of placebo

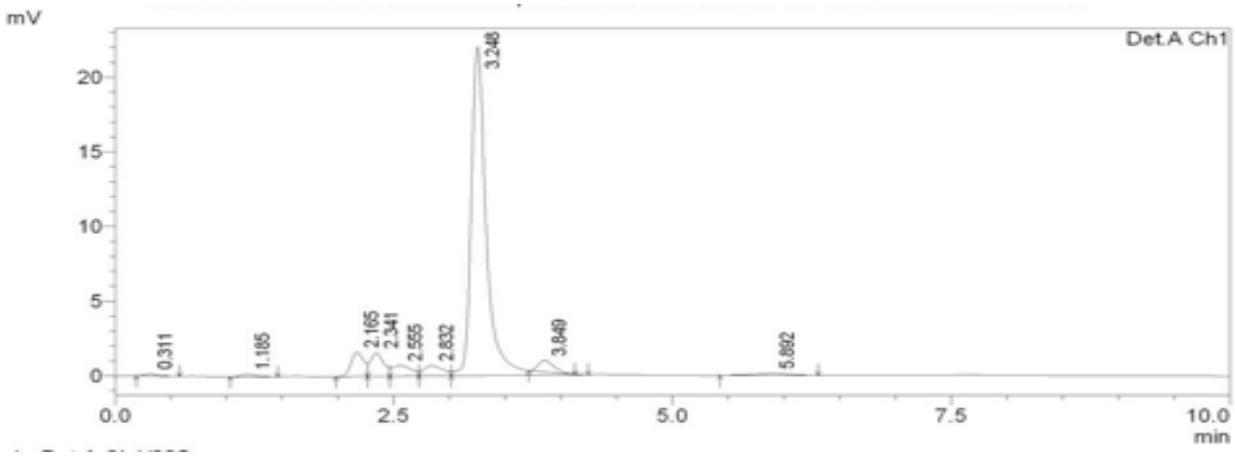

Fig 5: Chromatogram of fast dissolving oral film

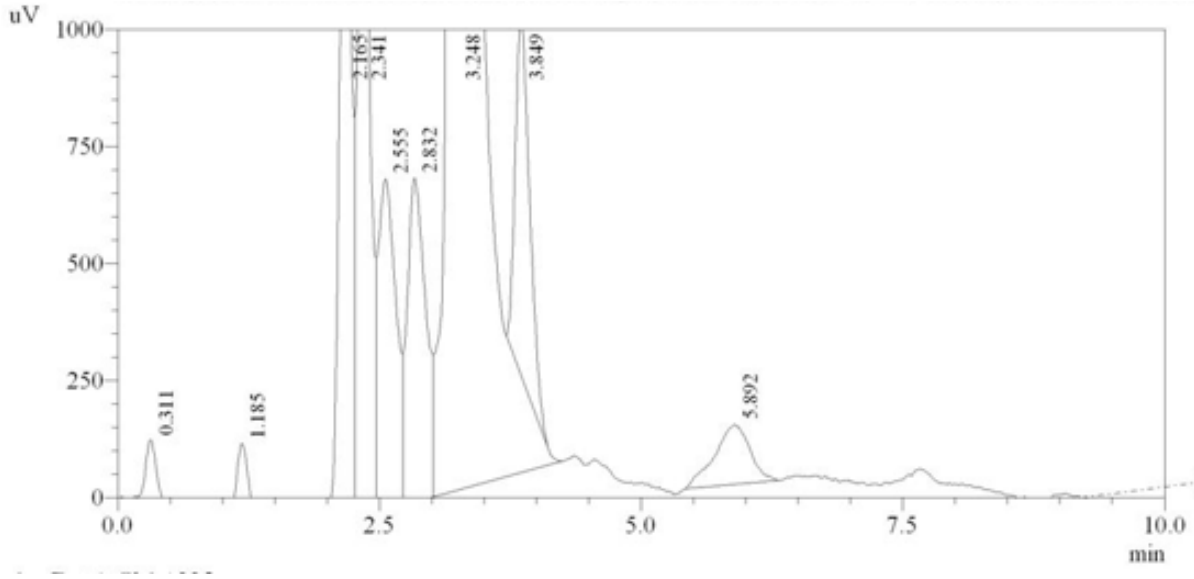

Fig 6: Chromatogram of fast dissolving oral film (zoom image)

Comparison of chromatograms of standard NH and fast dissolving oral film formulation indicated the presence of NH peak with retention time of 3.8 min in the chromatogram of sample, which confirms the presence of drug in the formulation. NH content in the formulation was found to be 98% (Fig 4,5,6). The results clearly indicated that the developed method is simple, rapid, sensitive, precise, accurate and robust.

Conclusion

The proposed, rapid reverse phase HPLC method was found to be simple, sensitive, precise, specific and robust. The method is best suited for routine determination of NH in bulk and fast dissolving oral film formulations. The developed method can be adapted for analysis of NH tablets and NH immediate release tablets.

Acknowledgement

The authors are grateful to APOTEX Pvt Ltd, for providing a sample of Naratriptan hydrochloride and also to Government College of Pharmacy, Bengaluru, for providing laboratory facilities to carry out this research work.

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Development of Technology for the Manufacture of Sweet Cheese

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ABSTRACT

Unripened fresh cheese is one of the popular varieties among the cheeses having a wider marketing. The popularity of this type of cheese is attributed to great taste, convenience and versatility of use, and nutritional value. A formulation of raw sweet cheese using milk coagulum, sugar, cream, flavor and additives was standardized by studying the effects of varying levels of rennet-2, 3 4 and 6 ml per per litre of milk, setting time- 40, 60 and 90 min, sugar -10, 15, 20 and 25%; cream- 2.5, 5, 7.5%; skim milk powder - 12, 15 and 18%; cocoa powder -1, 2 and 3%; vanilla essence – 0.50, 1.0 and 1.5%; grinding time- 20, 30, 40 min and manual grinding. Out of these, based on the sensory evaluation, the parameters optimized were: rennet level, setting time, sugar, cream, SMP, cocoa, vanilla essence and grinding time of 4 ml, 40 min, 25%, 2.5%, 12%, 1%, 1.5% and 20sec, respectively. The sensory scores of attributes of the raw cheese viz. colour and appearance, body and texture, flavour and overall acceptability were in 'like very much' range.

Key words: Sweet cheese, rennet , cream , sugar, flavour.

Introduction

Milk products have played a significant role in the economic, social, religious and nutritional well-being of our people since time immemorial. It is estimated that about 50 to 55 per cent of milk produced is converted by the traditional sector into variety of Indian milk products, using processes such as heat and acid coagulation, heat desiccation, non-fermentation and fermentation.

Cheese consumption and production in the world continued to increase over the past; however in India cheese is slowly gaining market especially because of popularity of products like pizza. The popularity of cheese is attributed to its typical taste, the availability of new and different varieties, convenience and versatility of use, and its nutritional value. Varieties of fresh unripened cheeses like Ricotta, Feta, Cottage, Cream, baby Gouda, Bricotta and Quark cheeses are making their presence in Indian market.

Consumption of cheese has been demonstrated to reduce the risk of dental caries through various mechanisms (Kashket and DePaola, 2002). Cheese can be an important source of calcium and many other nutrients found in milk especially for persons who have difficulty digesting lactose or milk sugar (Suarez et al., 1995). Most of the varieties of cheese are unsweetened, very few being added with sugar. Some products like junkets made with milk coagulum can be called as sweet cheese products. Kesavan et al. (2014) reported a junket type product prepared from rennet coagulation and microwaving of milk. Praveen Kumar (2010) and Kelly Donlea (2007) reported the use of Ricotta cheese in the preparation of sweet cheese product. There is a scope for developing more varieties of sweet cheese with different flavours which can have high market potential and nutritional value.

In this paper development of a sweet cheese from rennet coagulum of milk is being reported by adding sugar, cream and flavor to milk in the cheese making process.

Materials and Methods

Materials

Raw milk was collected from cattle yard of National Dairy Research Institute (NDRI), Bangalore, India. It was standardized to fat- 3.5% and SNF- 8.5%. Good quality fresh cream was available from Experimental Dairy of NDRI, Bangalore. Sugar and skim milk powder (SMP) required for product standardization were procured from the local market. Meito rennet (fungal rennet) produced by Aspergillus species and procured from Arun & Co., Mumbai

was used for coagulating milk. Rennet solution was prepared @ 2gm/100ml of distilled water which was used in the study later. Vanilla essence (Bush Boake Allen) was procured from the local market. Cocoa powder - commercially available Cadbury's plain cocoa powder - was used.

Methodology

Preparation of raw sweet cheese

Milk was pasteurized (65˚C for 15 min) and cooled to about 35°C. The pasteurized milk was placed in a coagulating vessel and added with desired quantity of rennet solution. The coagulum obtained after keeping the renneted milk for a certain time was cut with a knife for facilitating separation of whey and the coagulum transferred to muslin cloth which was the kept in a refrigerator for 24 h to facilitate complete whey drainage. The coagulum obtained after 24 hrs was taken for the preparation of the product. 100 gm of coagulum was weighed to which the following ingredients were added. Finely powdered sugar (finely powdered), cream and different flavors (vanilla and cocoa) were added to the product, and grinding completed. This was named raw sweet cheese.

Parameters Optimization

The following levels of various parameters were tried in order to arrive at best possible formulation:

Rennet level: 2, 3, 4 and 6 ml for one litre milk

Setting time: 40, 60 and 90 min

Sugar level: 10, 15, 20 and 25% of coagulum

Cream (fat) level: 2.5, 5.0 and 7.5% of coagulum

Skim milk powder level: 12, 15 and 18% of coagulum

Cocoa flavor: 1, 2 and 3% of coagulum

Vanilla flavor: 0.5, 1 and 1.5 ml per 100 gm coagulum

Grinding time: 20, 40, and 60 seconds using mechanical agitator, and manual grinding with a ladle

Analyses

Chemical

Moisture content, ash ,titratable acidity and fat contents of the sweet cheese were determined by gravimetric method described in BIS (1981), protein by standard micro Kjeldahl method (AOAC, 2005) , pH by a digital pH-meter (Digisun Electronics, Hyderabad, and Model: DI 707). Water activity of the sample was measured using the water activity meter (Rotronic, Switzerland).

Microbiological

The total bacterial count, coliform count and yeast and mold counts of the product were estimated by pour plate technique as per the method of BIS (1981).

Sensory

The organoleptic quality of the sweet cheese was evaluated by a panel of judges on a 9- point hedonic scale wherein a score of 1 represented 'dislike extremely' and score of 9 represented 'like extremely' (Amerine et al., 1965).

Rheological

TA.XT plus Stable Micro System, England was used for measuring firmness and consistency of sweet cheese. A p/25 probe was used during experiment. The probe was calibrated to a distance of 50 mm above the platform on which sample was to be kept for analysis. The raw sweet cheese taken in a 50 ml beaker was positioned centrally over the platform and the computer was allowed to execute the program by activating "run a test" option, then the sample was penetrated by the plunger up to 5 mm depth after attaining a trigger force of 5 gm to yield a force – time curve. The height of the force peak obtained was taken as hardness and area of the peak as consistency.

Statistical

Data of sensory and rheological analyses obtained in the study were subjected to statistical analysis by one way ANOVA using MS-Excel package version 2007. The differences among treatments were measured at 5% level of significance.

Results and Discussion

Production of raw sweet cheese included various parameters such as rennet, fat, setting time, sugar, grinding time, skim milk powder and flavors. Their effects on the cheese quality and the results on their optimization are presented.

Rennet level

The color & appearance scores of sweet cheese prepared with varying levels of rennet were 7.45, 7.41, 7.49 and 7.41 for 2, 3, 4 and 6ml rennet levels, respectively (Table 1). Similarly, the body and texture scores were 7.26, 7.37, 7.45 and 7.29 which were statistically significantly different from each other ($p<0.05$). Use of optimum rennet is essential to produce desirable textural characteristic in cheeses. In the case of raw sweet cheese also, it has enhanced the textural attribute (Wium and Qvist, 1998). However, there was no statistically significant effect of rennet level on flavor, colour and appearance and overall acceptance scores as seen from the data in Table-1. The 4 ml rennet level was chosen as it had a desirable chewy body and texture and scored highest for that attribute (7.45). However, this was not reflected in instrumentally measured attributes (hardness, consistency and adhesiveness) which were found to be statistically not significant. This is because texture analyser

could not measure variations in granularity caused by rennet coagulation and subsequent grinding action. Okigbo et al. (1985) reported that high-chymosin concentration at reduced pH decreased coagulation time without substantially increasing curd firmness.

Setting time

The results pertaining to sensory evaluation scores of raw sweet cheese prepared with various setting times are presented in Table 2. Statistical analysis showed that all the sensory scores were not significantly affected by setting time levels ($P \leq 0.05$). Hence, the least value of 40 min was used for setting time of the milk for raw sweet cheese and variation of time for setting of the coagulum did not have any effect on the instrumentally measured textural parameters and sensory attributes of the product because the cheese coagulum was ground thoroughly after setting process was complete. The values ranged as follows: hardness 0.051-0.057 N, consistency 0.164-0.172 N.sec.and adhesiveness 0.008 – 0.014 N.sec.

Johnson et al. (2001) found increase in firmness of curd as the renneting time increased. Muliawan (2008) reported that Mozzarella cheese which is a rennet coagulum exhibited pseudoplastic behavior and shearing action thinned down the coagulum leading to no influence on the acceptance levels.

Sugar level

Four sugar levels viz. 10, 15, 20 and 25% were used for the preparation of sweet cheese. The results are presented in the Table 3. The statistical analysis showed that the sugar levels ($P \leq 0.05$) affected the flavor and overall acceptability. There was no much influence of sugar level on the color and appearance and body and texture scores. The flavor scores 6.13, 6.33, 6.6 and 7.3 respectively. These scores were statistically significant indicating that sugar level did influence the flavor and overall acceptance scores ($P<0.05$) .The overall acceptance scores were 6.26, 6.6, 6.93 and 7.33 respectively for the four treatments. Raw sweet cheese was prepared by using 25% sugar level.

It was seen that the variation in sugar level did not have any effect on objective textural characteristics. The values ranged from 0.043- 0.082 N for hardness, 0.127-0.247 N.sec for consistency and 0.004 – 0.006 N.sec. for adhesiveness. Sugar level is expected to affect the instrumental measurements, however, because of shearing action the effect of sugar might have diminished.

Cream (Fat) level

The color and appearance scores of sweet cheese prepared with different levels of cream ranged from 7.5-7.67; body and texture scores ranged from 7.30- 7.56, flavor scores from7.33-7.56 and overall acceptance scores from 7.40-7.76 (Table 4). These scores were not statistically significant indicating that fat level did not affect the sensory scores (Drewnowski et al., 1989). It was also seen that there was no significant different in textural characteristics measured by texture analyser. The values ranged from 0.021-0.029 N for hardness, 0.059-0.091 N.sec for consistency and 0.003 – 0.005 N.sec for adhesiveness. Hence, the lowest 2.5% fat level was optimized based on the sensory score.

SMP level

Different skim milk powder (SMP) levels were tried in order to enhance the consistency of sweet cheese. The results of sensory acceptance are presented in the Table 5. It was seen that there was no significant difference in sensory acceptance of the sweet cheese samples made with different levels of SMP. Apparently the judges did not mind incorporation of SMP at different levels. From these results, it is clear that SMP level did not effect any changes in the product. Hence, raw sweet cheese was prepared by using the lowest level 12% SMP level. However, texture analyser measurements (hardness: 0.038 – 0.041 N, consistency: 0.124 -0.189 N.sec. and adhesiveness: 0.012-0.014 N.sec) indicated that they significantly ($p<0.05$) varied with the SMP levels. Probably, the product became slight soggy resulting in lower textural values (Table5).

Cocoa level

Cocoa powder @ 1, 2 and 3% was added to the rennet coagulum to prepare cocoa flavored raw sweet cheese. In this experiment only cocoa level was varied as above and all other parameters were kept constant with a background vanilla flavor @1.5%. The color and appearance scores were 7.27, 7.60, 7.80; body and texture scores were 7.22, 7.57 and 7.8. The flavor scores were 7.1, 7.45 and 7.62, respectively for 1, 2 and 3% cocoa levels. The overall acceptance scores were 7.1, 7.45 and 7.72 (Table 6). From these results it is clear that cocoa level did not influence any changes in the textural attributes of the product. Hence, raw sweet cheese was prepared by using the lowest level 1% cocoa level. Though increased cocoa level might enhance the brown colour, the judges did not show any positive influence by increase in cocoa level. Colour intensity of the product was not measured instrumentally.

Vanilla essence

Three vanilla essence levels were tried viz. 0.5, 1.0 and 1.5% (v/w) and the results are presented in Table 7. From these results it was seen that the flavor acceptance score was affected and showed significant difference ($p<0.05$). The flavor scores were 7.25, 7.57 and 7.85, respectively for 0.5, 1.0 and 1.5% vanilla levels. The essence level of

1.5% scored highest with a significant difference. Hence, this level was chosen for further trials. There was no any significant effect of vanilla level on instrumental textural characteristics. Hardness values ranged from 0.017 – 0.02 N, consistency 0.047 -0.096 N.sec. and adhesiveness 0.003-0.004 N.sec.

Grinding time

Grinding of the coagulum was carried out so that optimum body and texture is developed in the final product. The grinding was done manually and by mechanical stirrer for different times. The results are presented in the Table 8. The color and appearance scores were between 7.31-7.92, body and texture scores were between 7.20 – 7.45, the flavor scores 7.24 -7.51 and the overall acceptance scores 7.25 - 7.75. These scores were not statistically significant indicating that grinding time did not affect the color and appearance; body and texture; and flavor.

It was seen that the cheese prepared with hand tended to have different color and there was more whey drainage compared to others, it gave a grainy texture. Hence, the lowest 20 sec was optimized based on the sensory score. It was seen that the grinding time did not affect objective textural characteristics of the product.

Physico-chemical and microbiological analysis of the product

The manufacture process of sweet cheese with optimized parameters is depicted in Fig 1 and the physico-chemical characteristics are presented in Table 9. The raw sweet cheese had the values of protein, acidity, pH, aW, moisture content, fat, and ash of about 13.4%, 2.7%, 6.2, 0.9, 60 %, 14% and 1.1%, respectively. The microbiological analysis was done for the fresh sample for total bacterial count, coliform count and yeast and mold count. The TBC obtained was on lower side as the experiments were carried out under controlled conditions. The water activity of the product was 0.90 because of sugar content which may impart a good shelf life to the product.

Conclusion

There is a growing demand for nutritious and safe and accessible products. Trials were carried out for developing methodology for raw sweet cheese. The method was standardized mainly based on the sensory and texture analysis. The proposed manufacturing method may be adopted by the small-scale industries. Future studies are necessary to determine the shelf-life of the product under standard and commercial conditions. Scope also exists for preparation of baked product from the raw sweet cheese samples.

Acknowledgement

The first author gratefully acknowledges staff of Dairy Technology Section of NDRI, Bangalore for all help in conducting the project work.

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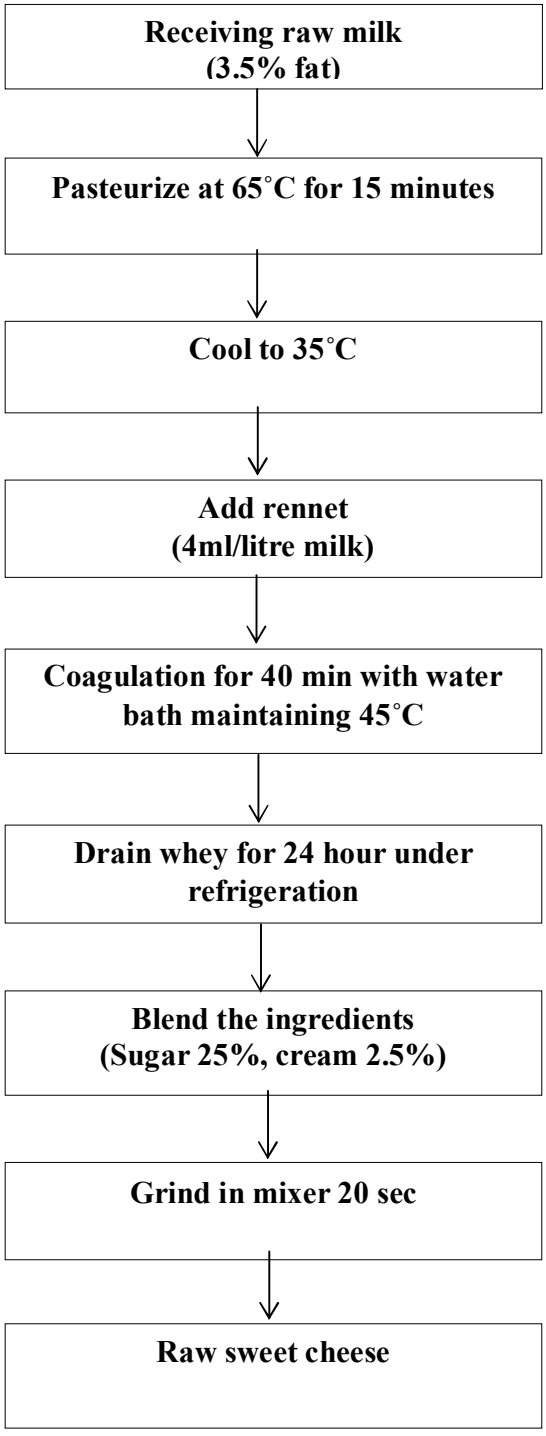

Fig. 1: Flow chart for the method of preparation of raw sweet cheese

Table - 1

Effect of rennet level on the sensory* and textural characteristics of raw sweet cheese

Particular	Rennet level (ml)			
	2	3	4	6
Sensory characteristic				
Color & appearance	7.45 ^{NS}	7.41 ^{NS}	7.49 ^{NS}	7.41 ^{NS}
Body & texture	7.26^{c}	7.37^{b}	7.45^{a}	7.29^{c}
Flavor	7.31 ^{NS}	7.30 ^{NS}	7.40 ^{NS}	7.18 ^{NS}
Overall acceptability	7.38 ^{NS}	7.39 ^{NS}	7.48 ^{NS}	7.31 ^{NS}
Textural characteristic				
Consistency, N.sec	0.329 ^{NS}	0.320 ^{NS}	0.299 ^{NS}	0.315 ^{NS}
Adhesiveness, N.sec	0.027 ^{NS}	0.034 ^{NS}	0.025 ^{NS}	0.028 ^{NS}

Note- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p\geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table - 2

Effect of setting time on the sensory* and textural characteristics of raw sweet cheese

Particular	Setting time (min)		
	40	60	90
Sensory characteristic			
Color & appearance	7.50 ^{NS}	7.83 ^{NS}	7.85 ^{NS}
Body & texture	7.10 ^{NS}	7.83 ^{NS}	7.73 ^{NS}
Flavor	7.43 ^{NS}	7.73 ^{NS}	7.63 ^{NS}
Overall acceptability	7.43 ^{NS}	7.86 ^{NS}	7.63 ^{NS}
Textural characteristic			
Hardness, N	0.051 ^{NS}	0.057 ^{NS}	0.057 ^{NS}
Consistency, N.sec	0.164 ^{NS}	0.171 ^{NS}	0.172 ^{NS}
Adhesiveness, N.sec	0.008 ^{NS}	0.004 ^{NS}	0.014 ^{NS}

Note- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p\geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table 3

Effect of sugar level on the sensory* and textural characteristics of raw sweet cheese

Particular	Sugar level (%)			
	10	15	20	25
Sensory characteristic				
Color & appearance	7.16 NS	7.26 NS	7.23 NS	7.30 NS
Body & texture	6.96 NS	7.00 NS	6.96 NS	7.13 NS
Flavor	6.13[a]	6.33[ab]	6.60[b]	7.30[c]
Overall acceptability	6.26[a]	6.60[ab]	6.93[b]	7.33[b]
Textural characteristic				
Hardness, N	0.082 NS	0.069 NS	0.075 NS	0.043 NS
Consistency, N.sec	0.247 NS	0.190 NS	0.184 NS	0.127 NS
Adhesiveness, N.sec	0.005 NS	0.004 NS	0.006 NS	0.006 NS

Note- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p \geq 0.05$. * Acceptance score on 9-point Hedonic scale

Table - 4

Effect of fat level on the sensory* and textural characteristics of raw sweet cheese

Particular	Fat level (g)		
	2.5%	5%	7.5%
Sensory characteristic			
Color & appearance	7.50 NS	7.50 NS	7.67 NS
Body & texture	7.30 NS	7.56 NS	7.46 NS
Flavor	7.33 NS	7.56 NS	7.33 NS
Overall acceptability	7.40 NS	7.76 NS	7.43 NS
Textural characteristic			
Hardness, N	0.029 NS	0.028 NS	0.021 NS
Consistency, N.sec	0.091 NS	0.083 NS	0.059 NS
Adhesiveness, N.sec	0.005 NS	0.005 NS	0.003 NS

Note- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p \geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table - 5

Effect of SMP level on the sensory* and textural characteristics of raw sweet cheese

Particular	SMP level (g)		
	12%	15%	18%
Sensory characteristic			
Color & appearance	7.90 ^{NS}	7.80 ^{NS}	7.70 ^{NS}
Body & texture	7.72 ^{NS}	7.74 ^{NS}	7.66 ^{NS}
Flavor	7.50 ^{NS}	7.80 ^{NS}	7.64 ^{NS}
Overall acceptability	7.68 ^{NS}	7.76 ^{NS}	7.64 ^{NS}
Textural characteristic			
Hardness, N	0.041^{ab}	0.048^{b}	0.038^{a}
Consistency, N.sec	0.189^{a}	0.149^{c}	0.124^{b}
Adhesiveness, N.sec	0.014^{a}	0.016^{a}	0.012^{c}

Note:- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p\geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table - 6

Effect of cocoa level on the sensory* and textural characteristics of raw sweet cheese

Particular	Cocoa level (g)		
	1%	2%	3%
Sensory characteristic			
Color & appearance	7.27 ^{NS}	7.60 ^{NS}	7.80 ^{NS}
Body & texture	7.22 ^{NS}	7.57 ^{NS}	7.80 ^{NS}
Flavor	7.10 ^{NS}	7.45 ^{NS}	7.62 ^{NS}
Overall acceptability	7.10 ^{NS}	7.45 ^{NS}	7.72 ^{NS}
Textural characteristic			
Hardness, N	0.049 ^{NS}	0.045 ^{NS}	0.043 ^{NS}
Consistency, N.sec	0.142 ^{NS}	0.121 ^{NS}	0.116 ^{NS}
Adhesiveness, N.sec	0.016 ^{NS}	0.013 ^{NS}	0.013 ^{NS}

Note- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p\geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table - 7

Effect of vanilla level on the sensory* and textural characteristics of raw sweet cheese

Particular	Vanilla level (ml)		
	0.5%	**1%**	**1.5%**
Sensory characteristic			
Color & appearance	7.75 ^{NS}	7.75 ^{NS}	7.80 ^{NS}
Body & texture	7.05 ^{NS}	6.90 ^{NS}	7.12 ^{NS}
Flavor	7.25^{a}	7.57^{b}	7.85^{c}
Overall acceptability	7.27 ^{NS}	7.52 ^{NS}	7.67 ^{NS}
Textural characteristic			
Hardness, N	0.020 ^{NS}	0.018 ^{NS}	0.017 ^{NS}
Consistency, N.sec	0.096 ^{NS}	0.076 ^{NS}	0.047 ^{NS}
Adhesiveness, N.sec	0.004 ^{NS}	0.004 ^{NS}	0.003 ^{NS}

Note- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p\geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table 8

Effect of grinding time on the sensory* and textural characteristics of raw sweet cheese

Particular	Grinding time (sec)			
	20	30	40	Hand
Sensory characteristic				
Color & appearance	7.40 ^{NS}	7.71 ^{NS}	7.92 ^{NS}	7.31 ^{NS}
Body & texture	7.31 ^{NS}	7.45 ^{NS}	7.68 ^{NS}	7.01 ^{NS}
Flavor	7.25 ^{NS}	7.40 ^{NS}	7.51 ^{NS}	7.35 ^{NS}
Overall acceptability	7.75 ^{NS}	7.47 ^{NS}	7.25 ^{NS}	7.31 ^{NS}
Textural characteristic				
Hardness, N	0.033 ^{NS}	0.038 ^{NS}	0.037 ^{NS}	0.036 ^{NS}
Consistency, N.sec	0.102 ^{NS}	0.104 ^{NS}	0.102 ^{NS}	0.097 ^{NS}
Adhesiveness, N.sec	0.007 ^{NS}	0.01 ^{NS}	0.011 ^{NS}	0.007 ^{NS}

Note:- Values with different superscripts in a row are significantly different ($p<0.05$); NS: not statistically significant at $p\geq 0.05$. * Acceptance score on 9-point Hedonic scale.

Table 9

Physico-chemical and microbiological characteristics of raw sweet cheese

Characteristics	Values
Protein content	13.4%
Acidity	3.6%
pH	6.2
Water activity	0.90
Moisture content	60%
Fat	14%
Ash	1.1%
TBC/gm	10330
Coliform count/gm	Not detected in 10^{-1}dilution
Yeast & mold count/gm	20

Core Benefits and challenges of 505 (b) (2) for Pharmaceutical Products

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ABSTRACT

A 505(b) (2) application is a new drug application (NDA) describes in section 505(b) (2) of the federal food, drug and cosmetic Act.505 (b) (2). It is submitted under section 505 (b) (1) of the Act and approved under section 505 (c) of the Act. This guidance also provides further information and amplification regarding FDA's regulations at 21 CFR 314.54. Section 505 (b) (2) was added to FFDC Act by the drug price competition and patent term restoration Act of 1984 (Hatch-Waxman Amendments). The 505 (b) (2) is relatively low risk because the drug has already been proven to be safe. It is low cost because there are fewer studies. It is also faster due to fewer studies and if done right, a drug can make it to market in as little as 3 years. It is very important in the economic climate today that our clients quickly get these drugs on the market.

Keywords: 505 (b) (1), 505 (b) (2), NDA, 21CFR 314.54, FFDC, FDA.

Introduction:

The Section 505 of the Act describes three types of new drug applications: (1) an application that contains full reports of investigations of safety and effectiveness (section 505(b)(1)); (2) an application that contains full reports of investigations of safety and effectiveness but where at least some of the information required for approval comes from studies not conducted by or for the applicant and for which the applicant has not obtained a right of reference (section 505(b)(2)); and (3) an application that contains information to show that the proposed product is identical in active ingredient, dosage form, strength, route of administration, labelling, quality, performance characteristics, and intended use, among other things, to a previously approved product (section 505(j)). Note that a supplement to an application is a new drug application.

Drug development researchers may be aware about new drug applications (NDA) and Abbreviated new drug applications (ANDA) are two of the FDA's regulatory pathway for how prescription drugs can be approved and ultimately reach the market. NDA's are new drugs that have not yet been approved and not available in market considered the guideline as 505 (b) (1) application and ANDA's are the generic products consider the guideline 505 (b) (2) which is already exited in market. However, there is an additional pathway that's hybrid between the NDA and ANDA known as 505 (b) (2)1.

505 (b) (1) or NDA is the format that manufacturers send a formal proposal to the FDA that a new drug should be approved and made available for use by patients in the United States. NDA submission includes information about the drug being evaluated including the ingredients, how it's made, pre-clinical (animal model) study results, clinical trial results in humans, what the drug does in the body, and how it will be packaged2. It takes a great deal of time and resources for a manufacturer to complete all the necessary requirements to submit a successful NDA to the FDA for review.

ANDA stands to get approval for a generic version of a drug that is already existed in the market. For this Manufacturer providing evidence to the FDA that the generic product is comparable to the currently approved product which is in market through analytical chemistry and bioequivalence evaluations. The approved indication, dose route, and strength for the generic will be the same as the original or reference product. The pathway is abbreviated because preclinical and clinical trials are not required. These studies were performed by the manufacturer of the original product and reviewed by the FDA as part of the approval process, so it would be redundant for the generic manufacturer to complete similar trials again3. This data deals saves time and resources for the manufacturer compared to the NDA.

505 (b) (2) pathway provides by manufacturers certain types of drugs with an opportunity to acquire FDA approval without performing all the work that's required with an NDA. These drugs are not strictly generics, but are often not entirely novel new molecular entities either. 505 (b) (2) can be an option for drugs with a new aspect related to indication, dosage form or regimen, strength, combination with other products,orotheruniquetraits1.

The key future of 505 (b)(2) pathway is that it allows a manufacturer to submit their product for FDA review by including data and/or study results originally collected by another manufacturer or researcher. The manufacturer of the 505 (b) (2) eligible product needs to build a connection between their version of the product, or the active ingredients in it, and the reference product. For example, this could

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include data and results of bioanalytical testing, pre-clinical studies, or even clinical trial results. If successful in their effort to include supporting evidence from other researchers in their submission, the manufacturer of the 505 (b) (2) candidate won't have to re-run these studies themselves. While the 505 (b) (2) path allows for using the research of others as a component of their FDA submission, the manufacturer of the 505 (b) (2) product may still need to complete some of their own research in other areas to help fulfil all the various requirements of the FDA to earn approval4.

The main aspect of gaining approval through the 505 (b) (2) is that the approved product is eligible for 3-5 years of market exclusivity. A drug approved via a full NDA is normally granted a 5 year market exclusivity period, while a generic product approved through the ANDA pathway may earn 6 months of market exclusivity if it's the first generic approved. ᵒ During the period of market exclusivity the product will be protected from competitors.

APPROVAL PATHWAYS 505 (b):

505 (b) (1) Regulatory approval pathway:

The 505(b) (1) regulatory pathway is the traditional New Drug Application (NDA) to obtain the approval of a new drug whose active ingredients have not previously been approved. This type of submission requires extensive research including both clinical and nonclinical studies that contains complete reports of investigations of safety, effectiveness, quality of drug product. Because of the substantial amount of research and data that is required, 505(b) (1) NDA submissions can take many years to complete, and require a significant amount of resources to get approved. In order to prove safety and efficacy, manufacturers are required to complete extensive clinical trials, consisting of three phases (Phase I, Phase II, and Phase III). Phase I trial is conducted in tiny range of healthy volunteers starting from 25-100 to determine dose ranging. Phase II trial includes testing of drug on patients to assess efficaciousness and safety, conducted in patients starting from 100-300. Phase III trial determines a drug's therapeutic effect and conducted in patients starting from 1000-3000. When all three phases are over, the manufacturer submits an NDA containing results from these studies to the FDA. The FDA scientists reviewed the new NDA to assess whether the trials demonstrate the product's benefit, compared with its risks. Information submitted may include not only the results of the various clinical trials, but also the raw data that was used to generate the conclusions. The drug company is able to do this because it either conducted the studies itself or paid for the studies. This is called the "right of reference".

By the time an NDA is approved, the manufacturer has invested numerous years and many millions of dollars for development, clinical trials and regulatory approval. As compensation, the FDA grants the company the exclusive right to manufacture the product for a period of time under patent protection. Manufacturers are granted patent protection for 20 years from the date of the first filing of the patent application.

505 (b) (2) Regulatory Pathway for New Drug Approvals:

This guidance is intended to serve as a foundational guidance to assist applicants in determining which one of the abbreviated approval pathways under the Federal Food, Drug, and Cosmetic Act (FD&C Act) is appropriate for the submission of a marketing application to FDA. Many potential drug product developers are not familiar with the different abbreviated approval pathways for drug products under the FD&C Act — the abbreviated approval pathways described in section 505(j) and 505(b) (2) of the FD&C Act (21 U.S.C. 355(j) and 21 U.S.C. 355(b) (2), respectively) — or the types of data and information that are permitted to support approval under those pathways.

Categories of 505(b) (2) Application:

505 (b) (2) address the following the situation in which the application should be filed. Approval of the application will require review of studies beyond those that can be considered under section 505(j) (ANDA).

1. **Change in Dosage form:** An application for a change of dosage form from one formulation route in to another formulation route, such as a shift from a solid oral dosage form to a liquid oral or parenteral that relies to some extent upon the Agency's finding of safety and/ or effectiveness for an approved drug. (e.g., Tramadol orally-disintegrating Tabs Ondansetron thin film sublingual dosage form).

2. **Change in Strength:** An application where a change in strength of already approved compound .After changing strength it should show significant improvement in its therapeutic indication to be eligible for 505 (b) (2) application. (e.g. Antara; micronized fenofibrate Caps).

3. **Change in Route of administration:** An application for a change in the route of administration, Ex: Shifting from oral route to parenteral route or Parental route to oral route. (e.g., Duraclin; epidural clonidine, Protonix I.V.)

4. **Change in Substitution of an active ingredient in a combination product:** An application for a change in one of the active constituents of an approved combination product for another active ingredient that bears or has not been previously approved. (e.g. Pexeva; paroxetine (e.g., Pexeva; paroxetine mesylate, Nexium; esomeprazole)

5. **Change in Formulation:** An application for a proposed drug product that contains a different quality or quantity of an excipient(s) than the listed drug where the studies required for approval are beyond those considered limited confirmatory studies appropriate to a 505(j) application. (e.g., Doxil liposomal injection; doxorubicin)

6. **Change in Dosing Regimen.** An application for a new dosing regimen Ex: A dose change from thrice daily to once in a day. (e.g., BID to QD)

7. **Changes in Active ingredient.** An application for a change in an active ingredient such as a different salt, chelate, complex, racemate, clathrate, ester or an enantiomer of an active ingredient in a listed drug containing the same active moiety. (eg. S-Omeprazole & E-Omeprazole).

8. **New molecular entity (NME):** In some cases a new molecular entity may have been studied by parties other than the applicant and published information may be pertinent to the new application. This is significantly possible if the NME is the prodrug of an approved drug or the active metabolite of an approved drug. In some cases, information on a drug with similar pharmacological effects could be considered critical to approval. (e.g., Thalidomide, quinine sulfate).

9. **Combination product.** An application for a new combination of active constituents, where two of them are already approved individually. (e.g., Lotrel, Tekturna HCT Janumet).

10. **Change in Indication:** An application where a new therapeutic indication was identified for an already approved drug (RLD). (e.g., Cenestin; conjugated estrogen, sildenafil)

11. **Rx/OTC switch:** An application where a drug is switched from a prescription category to OTC (over the counter) indication (e g loratadine omeprazole).

12. **Naturally derived or recombinet active ingredient:** An application for a drug product containing an active pharmaceutical ingredient obtained from animal or plant sources where clinical trials are necessary to indicate that the active ingredient is the same as an active constituent in a listed drug. (e.g., Omnitrope; rhGH)

13. **Non monograph Indication:** An application for a drug product that is not described in OTC monograph, Assigned as new dosage form.

Which Can't Be Submitted As 505(b) (2) Applications:

An application that's a reproduction of a listed drug and eligible for approval beneath under section 505(j) for approval (i.e. ANDA) isn't eligible for applying beneath 505 (b) (2) application. The rate & extent to which the active ingredient (s) the absorption is unintentionally less than the listed drug. The extent of absorption is less than the listed drug.

Patented and Non patented Market Exclusivities for 505 (b) (2) applications6:

Patents are issued by the US Patent and Trade Office based on intellectual property. A patent lasts for 20 years yet, can be challenged and found invalid. Exclusivity based on patents provides the patent owner the right to exclude others from specific uses of their products for a specified period. By getting product approval, the sponsor must provide patent certification, i.e., authenticating statements claiming the drug or a method of using the drug that is submitted in the NDA. Marketing Exclusivity is granted by FDA as an incentive to conduct studies. Marketing Exclusivity means that a generic cannot be marketed during the applicable term. Moreover, for 3-year exclusivity, an generic cannot be approved until the 3 years expires, while for 5-year exclusivity a generic application cannot be filed until the 5 years expired. The term ranges from 3-7 years and can be extended by 6 months in some cases by paediatric studies. There are 505(b) (2) applications that receive no exclusivity – those applications that did not need to conduct a clinical study. These drugs are approved on the basis of only bioavailability studies. An example that receives no exclusivity would be developing an extended release oral product when the reference listed drug (RLD) is an immediate release drug product.

Strategies for Developing 505 (b) (2) Products:

For small drug companies, the 505(b)(2) pathway for a new product could prove an attractive business model for the simple reason that it takes much less time, cost and risk to get the product onto the market compared to innovator drugs, and could yield significantly higher returns on investment compared to generic drugs. A good strategy could mean the difference between a successful, i.e., profitable, and unsuccessful product.

The following are key strategic considerations for a 505(b) (2) product:

- Extent of innovation/modification made to the innovator product: these modifications decide whether the product is applicable for a 505(b) (2) review or not, and help determine the number of years of market exclusivity granted.
- Thorough analysis of available data: before embarking on manufacturing a 505(b)(2) product, a company should thoroughly analyse the data available, including the scientific basis of approval of the reference drug, published literature, particularly since the innovator drug was approved, market competition, etc. (The amount of available data previously submitted to FDA determines whether this is a viable project.)
- Development strategy: careful analysis of data should lead to a list of the additional studies that may be required for a given 505(b)(2) product; bridging studies are required to show that changes to the innovator product lead to the desired impact on safety, efficacy and tolerance of the proposed drug product.
- FDA discussions: there is no substitute for robust discussions with the relevant FDA review division regarding the proposed and executed development strategy; FDA offers significant advice regarding

final requirements for an approval, and it has been statistically demonstrated that companies that involve FDA in discussions early in their product development plans and implement the agency's suggestions increase their chances for first cycle approval almost three-fold, leading to enormous time and cost savings and, hence, higher returns on investment.

- Implementation of strategy: exhaustive implementation planning is the path to success; timelines should be diligently observed and any deviations aggressively addressed.
- Cost control: cost incurred depends upon the preclinical and clinical studies required, amount of information available regarding the reference drug, advancements in analytical technology and various other such factors; bridging studies should be scientifically justified and strategically executed to control cost.
- Marketing and branding strategy: as 505(b) (2) products are generally more expensive than generic versions of the innovator drug, the manufacturer should have a robust marketing plan.

Table 1: Comparison of 505 (b) (2), NDA and 505 (b) (j)

	505 (b) (2)	505 (j)	NDA
Use Fee	Yes/No	Yes	Yes
Studies	Partial	BA/BE	Full
NCE	No	No	Yes
New Ingredients	Yes	No	Yes
New Formulation	Yes	No	Yes
Patented	Yes	No	Yes
Market Exclusivity	3-5 Years	6 months	5 Years

Advantages of 505(b) (2) type NDA[7]:

- Marketed as branded products rather than generic.
- Relatively low risk because of existing safety and efficacy information.
- Lower cost due to smaller scope and number of potential studies.
- Increased speed due to fewer studies
- Wide range of drug substance with better market possibilities are made available for fast approval of drug under 505(b) (2) pathway.
- Unlike ANDA not affected by discontinuation of RLD.
- Earns patent and exclusivity.
- Insulated from high market competition.
- An opportunity in Drug efficacy Study Implementation (DESI) drugs.

Challenges of 505(b) (2):

A faster and less expensive pathway to approval is ideal; however, as with any NDA application, there are challenges. For example, the FDA's 1999 Draft Guidance on 505(b) (2) applications is largely directed at drugs that may rely on an RLD for safety and efficacy data if equivalence is demonstrated. The guidance does not speak directly to unapproved drug-specific issues,

For example:

- using the literature to prove safety and efficacy when there is no RLD on record with FDA
- using the literature to find well controlled Phase 3 studies containing adequate details about the protocol, statistical analysis plan and data.

It's critical for the success of the application that the 505(b) (2) application present the FDA with all of the safety and efficacy in a traditional NDA. Therefore, it is vital that sponsors align themselves with knowledgeable strategic partners that can help assess the best NDA pathway and successfully navigate the process in the most efficient manner.

Table 2: Few examples of drugs approval as per 505 (b) (2) guideline

TRADE NAME	FORMULATION & DRUG	COMPANY	TYPE
Altocor®	Extended-release tablets Lovastatin	Andrx	New dosage form
Avinza®	Extended release Morphine	Elan	New dosage form
Doxil®	liposomal injection Doxorubicin	Janssen	New dosage form
Zyrtec D®	Certirizine and pseudoephedrine	Pfizer	New Combination
Stalevo®	carbidopa/levodopa/entacapone,	Orion	New formulation
Thalomid®	Thalidomide	Celgene	New indication
Rid®	Piperonyl butoxide and pyrethins	Pfizer	New dosage form
Canasa®	Suppositories Mesalamine	Axcan	New delivery mechanism
Luxiq®	Foam Betamethasone Valerate	Connectics	New delivery mechanism

Conclusion:

Pharmaceutical products drug development approvals are tightened by regulatory authorities. Because of this medicinal services are highly expensive. For this reason Pharmaceutical organizations are under weight to build R& D with shorter and less excessive way i.e. fast track approaches for Pharmaceutical products development. 505 (b) (2) application is meant to promote innovation by eliminating spare repetition of clinical trials. Exploitation of this pathway drug will simply enter into market with less value on investment. Consequently this 505 (b) (2) is tackle by creating qualified medication in new therapeutic area.

Acknowledgement:

The authors are thankful to Dr. P.Sathyanaraya Rao sir, Managing Director and Mr. R. Vijayaranga Rao sir, General Manager of Simson Life Sciences, Hyderabad, India for giving his valuable suggestions, motivation and encouragement to carry out this article.

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