Pharmacokinetic and Tissue Distributions of Naringenin and Naringenin Nanosuspension

Sumathi Rajamani[1], Tamilarazi Sengodan[1], Arun Radhakrishnan[2], Nikhitha Shanmukhan[2]

[1]Department of Pharmaceutics, Nandha College of Pharmacy, Erode, Tamil Nadu, India, [2]Department of Pharmaceutics, JSS College of Pharmacy, Udagamund, JSS Academy of Higher Education and Research, Mysuru, Karnataka, India

Abstract

Aim: The purpose of this study was to investigate the pharmacokinetic properties and tissue biodistribution of naringenin nanosuspension (NAR-NS) with that of NAR solution (NAR-S) after oral administration. **Materials and Methods:** NAR is a major antioxidant flavonoid present in citrus fruits and herbs. It is an auspicious drug based on its good bioactivity, but the use of NAR is clinically hindered because of its poor solubility and bioavailability. Hence, it was formulated as a NS. A simple and rapid high-performance liquid chromatography method for the determination of NAR-S and NAR-NS present in plasma and tissue has been developed and validated. Biological samples were processed by simple protein precipitation. **Results:** NS formulation showed significantly improved solubility and oral bioavailability of NAR. **Conclusion:** Thus leads to a wider clinical application.

Key words: High-performance liquid chromatography, nanosuspension, naringenin, pharmacokinetic studies, tissue distribution

INTRODUCTION

Flavonoids are an extensive major class of natural substances found in fruits and vegetables.[1] Naringenin (NAR) is the main flavanone naturally occurring in citrus fruits.[2,3] NAR has aroused great interest worldwide because of its various biological activities. However, its relative insolubility in water and instability cause extremely low oral absorption, which greatly restricts its clinical application [Figure 1].[4]

In our previous studies, we prepared NAR-NS coated with soya lecithin and tocopherol polyethylene glycol 1000 succinate (TPGS) by high-pressure homogenization method and investigated its physicochemical properties.[5-10] The combination of soya lecithin and TPGS as stabilizers yields NS with the smallest average particle size.[11,12] As a continuation of our research, the present studies further evaluate the effects of particle size on the pharmacokinetics and tissue distribution of NAR-NS with NAR-S after oral administration. A number of high-performance liquid chromatography (HPLC) methods have been developed for the determination of NAR in biological fluids and tissues.[13,14] However, some of these methods are time-consuming, expensive, or there is no internal standard. A simple, sensitive, precise, and linear method by HPLC was established for simultaneous determination and quantification of NAR-S and NAR-NS.[15,16] In addition, the validation parameters including linearity, selectivity, repeatability, reproducibility, recovery, decision limit, and detection capability of the method were determined.

MATERIALS AND METHODS

Chemicals

NAR was purchased from Zim Laboratories Limited, Nagpur. Soya lecithin was purchased from Glenmark Generics Limited, Mumbai, India. TPGS was obtained from Ludwigshafen, Germany. Ammonium formate and acetonitrile of HPLC grade were purchased from Tianjin SiYou Chemical Agent Co., Ltd. (Tianjin, China). Caffeine was purchased from RdH

Address for correspondence:
Sumathi Rajamani, Department of Pharmaceutics, Nandha College of Pharmacy, Erode, Tamil Nadu, India. Phone: +91-7402222019. E-mail: sumoraji@gmail.com
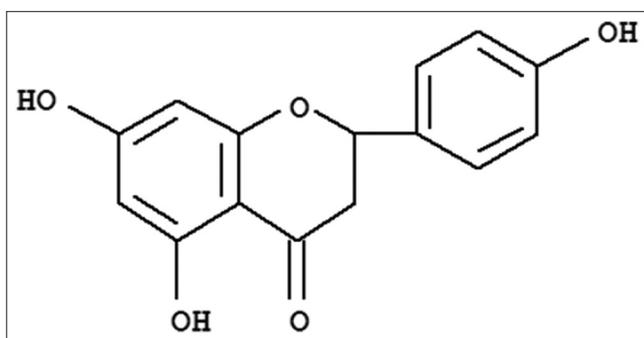
Figure 1: Chemical structures of naringenin

Laborchemikalien GmbH & Co. KG (Seelze, Germany). All other chemicals and solvents were of analytical reagent grade, and Milli-Q plus water (Millipore, Bedford, MA) was used throughout the study. The NAR-NS coated with soya lecithin and TPGS was prepared as previously described.

Instrumentation and HPLC conditions

The HPLC apparatus was equipped with a pump (LC-10AT, Shimadzu, Japan), UV detector (SNAR-10A, Shimadzu, Japan), vacuum degasser, automatic injector (SIL-10A, Shimadzu, Japan), and Cosmosil C18 column (5 μm particle size, 150 mm × 4.6 mm I.D., Waters, MA). Mobile phase consists of ammonium format pH 4.3 and acetonitrile (70:30 v/v), flow rate 1.0 ml/min for NAR assay in serum and tissue homogenate. Stationary phase consists of C18 RP column of dimensions (5 μm particle size, 250 mm × 4.6 mm I.D.). All samples were analyzed under isocratic elution at a flow rate of 1 mL/min. Chromatograms were monitored at 290 nm, and the column temperature was maintained at 30°C.

Bioanalytical method development

An aliquot (100 μl) of plasma was placed in an Eppendorf tube and mixed with 100 μl of drug solution (containing different concentrations of 10–100 μg/mL). To this, a fixed volume of 100 μl of internal standard (caffeine with concentration of 10 μg/ml) was added and vortexed for 5 min, followed by addition of acetonitrile as protein precipitating agent. The samples were vortexed for 5 min followed by centrifugation for 10 min at 5000 rpm at 4°C. A fixed volume of 10 μl of supernatant from each sample was injected into C18 reverse phase HPLC column. Calibration of NAR was established by plotting peak ratios of NAR to caffeine versus response factor. In all the cases, three replicate samples were determined. Quality control (QC) samples were prepared at low, medium, and high concentrations of 10, 50, and 100 μg/ml, respectively, for plasma and different tissue homogenates in the same manner as the calibration standards. These solutions were prepared fresh before use.[17,18]

Extraction recovery

The absolute recovery was measured as the response of a processed spiked matrix standard expressed as a percentage of the response of a pure standard, which has not been treated and indicates the response for the entire amount of analyte that is present in the sample.[19-21]

Evaluation of pharmacokinetic profile

Animals and dosing

New Zealand white rabbits (weighing 2.5 ± 0.2 kg) and Wister strain rats (weighing 200–250 g), supplied by the Experimental Animal Center of Nandha College of Pharmacy, Erode-52, Tamil Nadu, India, were used for pharmacokinetic and biodistribution studies. At first, the animals were acclimatized in metabolic cages at a temperature of 25 ± 2°C and a relative humidity of 40–70% under natural light/dark conditions for at least 7 days with water and a solid diet freely available before dosing. Before the experiment, the animals were kept under fasting overnight. All the described procedures were reviewed and approved by the Institutional Animal Ethics Committee (NCP/IAEC/2015-2016-04).

Pharmacokinetics study

Twelve rabbits divided into two groups were used for pharmacokinetic study. The dose of each sample: NAR-S and NAR-NS (20 mg/kg, expressed as NAR equivalent doses) was given through gavage administration. At pre-determined time points (30 min, 2, 4, 8, and 12 h), 1 mL of blood sample was collected from rabbits through the marginal ear vein into heparinized tubes. The samples were centrifuged at 3000 rpm for 15 min at 4°C immediately for isolation of the plasma. Samples were stored at −20°C until further analysis.

Determination of NAR in rabbit plasma by HPLC

Protein precipitation method was used for the determination of NAR in rabbit plasma. For the measurement of plasma NAR, 50 μl of internal standard solution (caffeine, 10 μg/ml, in distilled water) was added to 0.2 ml plasma. After vortex mixing for 1 min, 500 μl of acetonitrile was added and again vortex mixed for 1 min. After centrifugation at 10,000 rpm for 10 min, the supernatant was collected and then injected for HPLC analysis. Quantification was based on the peak area ratio of R. The pharmacokinetic parameters of NAR-S as well as NAR-NS groups were calculated using non-compartmental method. The area under the plasma concentration-time curve from zero to the last measurable plasma concentration (area under curve [AUC_{0-t}]) was calculated by the linear trapezoidal rule.[22]

Tissue distribution assay of NAR-NS

NAR-S and NAR-NS were dispersed in water for oral administration (210 mg/kg twice daily) for 17 doses through

gastric gavage which were administrated to 16 rats. Animals were euthanized at the time points of 10 min, 1 h, 3 h, and 6 h after dose (*n* = 4 at each time point). Dissected organs and tissues included the liver, spleen, kidneys, lungs, heart, brain, testicle, uterus, and ovary from each rat and washed with saline. Tissues were stored at −20°C until analysis. The tissue samples were homogenized in 10mL deionized water, and 200 µl of homogenate was taken in 2 ml Eppendorf tube. Tissue proteins were precipitated by adding acetonitrile up to 2 ml. Tissue samples could stabilize for 30min and centrifuged at 15,000 rpm for 15min. The supernatant was collected, and the pellet was discarded. The supernatant was evaporated at 45°C in nitrogen gas stream and was reconstituted in 100 µl of mobile phase and injected into the column for the detection of NAR (290nm).[23,24]

Pharmacokinetic analysis

Pharmacokinetic analysis was carried out using the WinNonlin 6.1 Professional software (Pharsight Corporation, NC, USA). Non-compartmental extravascular analysis method was employed for the pharmacokinetic parameter analysis.

Statistical analysis

All pharmacokinetic parameters of NAR-S and NAR-NS were expressed as mean ± standard deviation (SD). The data were analyzed with GraphPad Prism statistical analysis using student's unpaired *t*-test. Values <0.05, <0.01, and <0.001 were considered significant. The areas under the tissue distribution NAR were calculated by the log-linear trapezoidal method.

RESULTS AND DISCUSSION

Method development and optimization

Several stationary and mobile phases were tried. Columns such as C18, C8, and CN were tried with mobile phase consisting of acetonitrile:0.1 M ammonium acetate solution:acetic acid (30:69:1, v/v; pH 4.9) for NAR at a flow rate of 1 and 1.5 ml/min and detection at 290 and 292 nm. Method parameters such as mobile phase composition, its pH, and flow rate were optimized based on peak characteristics and run time. Finally, we used a Cosmosil C18 Column (5 µm, 150 mm × 4.6 mm, Waters, MA) using a mixture of ammonium formate pH 4.3 and acetonitrile (70:30 v/v), flow rate 1.0 ml/min. The column effluents were monitored at 290 nm.

Method validation

All methods used on biological samples were validated following the guidelines of the US Food and Drug Administration. The validation parameters included selectivity, accuracy, precision, specificity, linearity, and recovery.

Selectivity

Selectivity was evaluated by comparing six individual blank plasma and spiked plasma samples. The purpose of this assay was to detect the level of interference in blank plasma at the retention time of the NAR-S, NAR-NS, and IS.

Precision and accuracy

Accuracy and intra- and inter-day precision were calculated using the QC samples. Five replicates of each sample were injected on 3 consecutive days, and the concentration was then calculated from the values on the calibration curve. The accuracy was expressed with a relative error (RE), and the precision was expressed as the relative standard deviation (RSD). The acceptable range of both the RE and RSD was 15%.

Specificity

The specificity of the method was determined as there was no interfering peak at the retention time of NAR in blank plasma chromatogram. The representative chromatograms for the determination of NAR in plasma and tissues are shown in Figures 2-5. The retention time of IS was about 3.93 min and NAR-S and NAR-NS was 15.6 min. It was indicated that analytes were well separated; there were no obvious interference peaks located at the retention times of analyses in the blank chromatograms.

Figure 2 shows representative chromatograms of plasma; (1) Blank plasma, (2) blank plasma spiked with IS, (3) chromatogram of NAR-S, and (4) chromatogram of NAR-NS.

Linearity of calibration curve and lower limit of quantification (LOQ)

The calibration curves showed linearity over the concentration range of 10–100 µg/mL in rabbit plasma and rat tissue homogenates with a correlation coefficient ($y = 0.024x + 0.031$, $R^2 = 0.9996$). Typical linear regression equations, correlation coefficients, and linear ranges of NAR in plasma and each tissue are listed in Table 1 and Figure 6. The precision and accuracy of the method indicated that all coefficients of variation and the RE were below 7.1% and 12.7%, respectively. The limit of detection (LOD) and the LOQ were defined as a signal-to-noise ratio of 3 and the lowest concentration of the linear regression, respectively. The LOQ and LOD for NAR in serum were 24.09 and 8.28 ng/mL, respectively.

Recovery

The extraction recovery of NAR was in the range of 87.08–91.01% which suggested that the recovery of this method was consistent and reproducible. The average extraction recovery
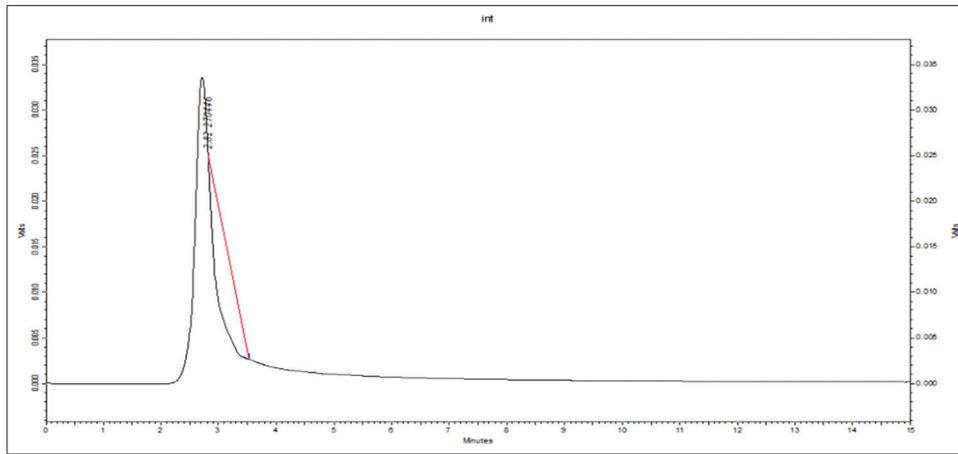
Figure 2: High-performance liquid chromatography of blank plasma
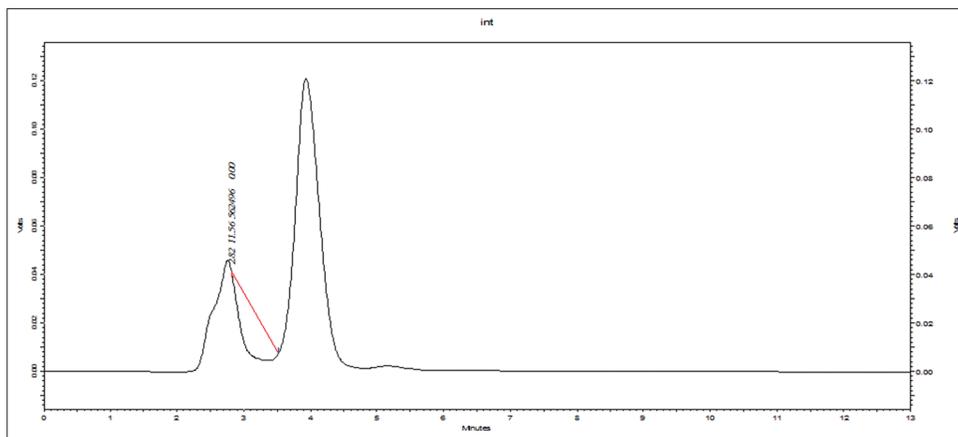
Figure 3: High-performance liquid chromatography of IS
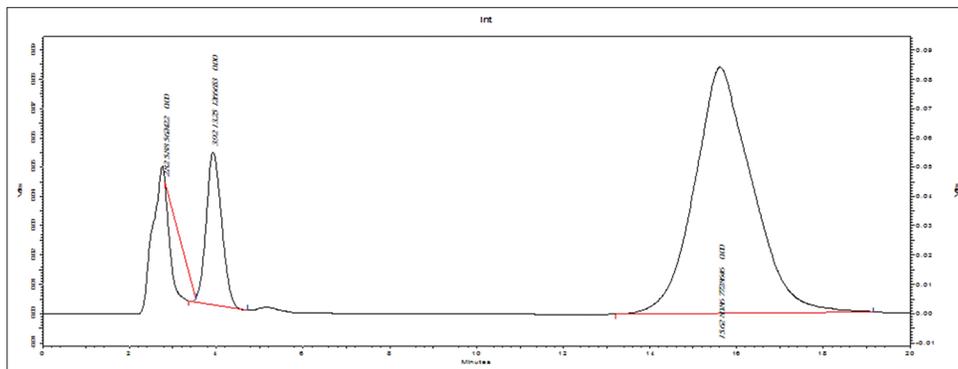
Figure 4: High-performance liquid chromatography of naringenin solution

Table 1: Standard graph of NAR	
Concentration (µg/ml)	Response factor
10	0.23
25	0.65
50	1.35
75	1.91
100	2.46

NAR: Naringenin

of NAR in rabbit plasma ($n = 6$) was 87.08% ± 0.38%, 89.0 ± 0.18%, and 91.01± 0.13% for the concentrations of 10, 50, and 100 µg/mL, respectively.

Evaluation of pharmacokinetic profile

The study was designed to assess the influence of per oral NAR-S and NAR-NS administration on its distribution profiles in rabbit serum. The concentration of NAR-S and
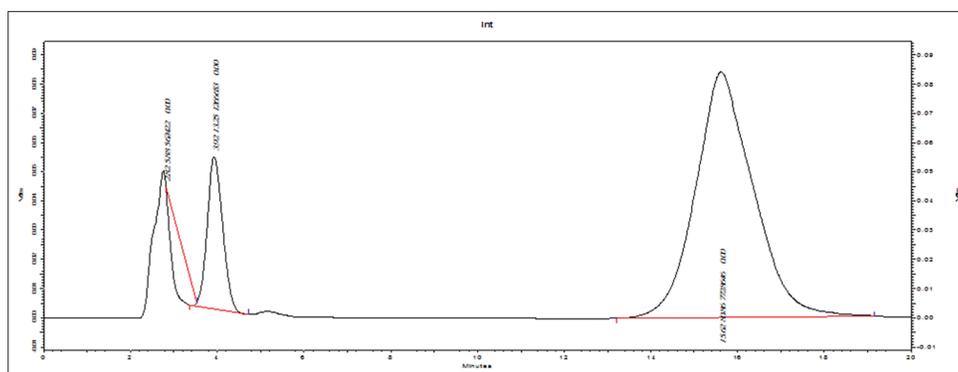
Figure 5: High-performance liquid chromatography of naringenin solution
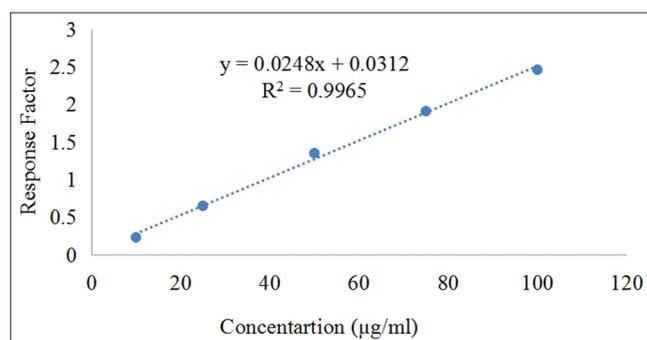
Figure 6: Calibration curve of naringenin

Table 2: Plasma concentration profile after oral administration of NAR-S and NAR-NS

Time (h)	Concentration (ng/ml)	
	NAR-S	**NAR-NS**
0	0	0
0.5	220	430
2	816	1550
4	613	725
8	415	490
12	410	470

NAR: Naringenin solution, NAR-NS: Naringenin nanosuspension

Table 3: Pharmacokinetic parameters of NAR-S and NAR-NS after oral administration

Parameters	NAR-S	NAR-NS
C_{max} (ng/ml)	816±0.93	1550±601.2
t_{max} (h)	1.8±0.88	2.6±0.71
$AUC_{0-12\ h}$ (ng.h/ml)	6047±901.29	8317.5±281.53
Cls (ng.h/ml)	34402±1941.49	42500±1211.20
MRT	5.1±0.94	5.68±0.71

*$P<0.05$, compared with the value of 10 mg/kg dose.
NAR-S: Naringenin solution, NAR-NS: Naringenin nanosuspension

NAR-NS in extracted serum samples was checked. The mean residence time MRT (5.68 ± 0.71 h) for the NAR-NS

formulation was considerably longer than that of NAR-S (5.1 ± 0.94 h). The peak plasma concentration (C_{max}) of NAR-NS (1550 ± 601.2 ng/mL) was significantly greater ($P < 0.01$) than that of NAR-S (816 ± 0.93 ng/mL). AUC is an important pharmacokinetic parameter to assess the exposure and circulating time of a drug; AUC_{0-12} for NAR-NS (8317.5 ± 281.53 ng.h/ml) was approximately 1.3-fold higher than that of NAR-S (6047 ± 901.29 ng.h/ml). NAR-S has low oral bioavailability might be due to its hydrophobic rings structure in contrast to NAR-NS. These results demonstrated that the incorporation of NAR into NS brought about an increased absorption after oral administration. The possible reason was that NS coated with hydrophilic polymer had a reduced opsonization and a longer period in circulation. High mobility of linear poly (ethylene oxide) chains in TPGS and soya lecithin might present to repel approaching proteins from the particle surface because the protein does not have sufficient contact time with the mobile chains which delayed the residence time of NAR in blood. Possible reasons for improvement of the oral bioavailability of NAR are particle size decrease, dissolution rate increase, and membrane permeation enhancement. In our previous research, the particle size of NAR was reduced ($80.52 \pm 0.\ 13$ d.nm) to nanoscale; this significant decrease may provide tremendous surface area and lead to faster dissolution velocity of the drug as elaborated in the Noyes–Whitney equation. Furthermore, reduction in particle size, improved drug uptake not only by a better dissolution time but also by a prolonged transit time which resulted efficient oral bioavailability of the drug. Therefore, the decreased particle size and increased dissolution rates were indeed the determinant reasons for the higher C_{max} and AUC_{0-12} of NAR observed after the oral administration of the NAR-NS. In comparison with NAR-S, NAR-NS exhibited higher C_{max} and faster t_{max}, indicating a higher absorption amount and more rapid absorption rate [Tables 2-4 and Figures 7 and 8].

Tissue distribution studies

Regarding analysis of tissue collected 6 h post-17th dose, quantitation showed NAR-NS as major forms which was consistent compared to NAR-S. After nanoformulation, the

Table 4: Concentrations (nmol/g for tissues) of NAR-S and NAR-NS in various organs at 6 h following 17th dose of NAR (210 mg/kg) in 16 rats

Organs	Concentration (ng/g)							
	0.16 h		1 h		3 h		6 h	
	NAR-S	NAR-NS	NAR-S	NAR-NS	NAR-S	NAR-NS	NAR-S	NAR-NS
Liver	7.9±0.8	9.9±0.7	20.0±0.7	27.1±0.8	67.2±3.5	86.9±6.0	80.9±1.4	93.7±6.3
Spleen	5.2±0.4	7.8±0.6	15.3±0.5	16.0±0.4	61.9±3.8	69.2±5.3	77.1±1.2	92.8±0.9
Kidney	4.9±0.9	6.4±0.2	12.5±0.4	13.1±0.5	51.7±6.2	66.6±1.2	75.8±6.0	92.0±1.4
Lungs	4.1±0.6	5.4±0.2	14.4±0.7	17.4±0.5	30.5±0.4	37.3±0.5	34.1±0.4	42.9±0.8
Heart	-	-	-	-	2.9±0.9	3.4±0.2	5.9±0.9	6.4±0.2
Brain	-	-	-	-	-	-	-	-
Testicle	-	-	2.3±0.3	3.5±0.2	7.2±0.8	9.2±0.3	12.5±0.9	15.4±0.5
Uterus	5.5±0.3	7.3±0.1	12.0±0.3	23.2±0.3	34.38±1.1	47.7±6.2	51.5±5.3	57.7±6.2
Ovary	3.5±0.9	4.4±0.5	8.0±0.1	12.6±0.4	30.16±6.2	36.4±2.7	44.9±5.8	56.9±6.0

NAR-NS: Naringenin nanosuspension
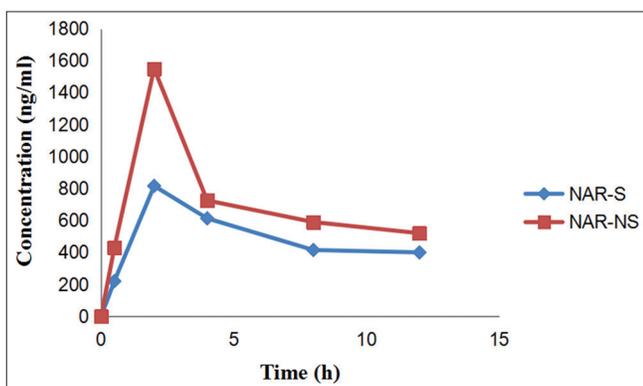
Figure 7: Plasma concentration-time profiles of naringenin solution (NAR-S) and NAR nanosuspension powder. Results were expressed as the mean ± SD ($n = 6$)
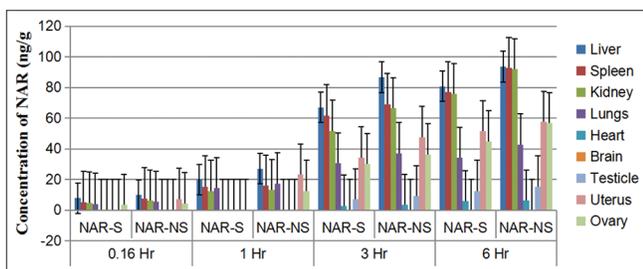
Figure 8: Tissue distribution histograms of NAR to rats (mean, $n = 4$)

concentration and retention time of NAR in these organs were significantly increased. Among the assayed organs, liver homogenate had high concentration of NAR followed by the spleen, kidney, lungs, and heart suggesting, that NAR easily enters these blood-rich tissues and organs after oral administration of the NAR. In the brain, NAR was not detected, which indicated that NAR could not efficiently cross the blood–brain barrier. Furthermore, the concentration level in the uterus was much higher, demonstrating that the gender-related difference of NAR in tissue distribution does exist and the reproductive organs are the main target organs of NAR for female rat. Therefore, NAR could play a long-lasting and effective role in therapeutic hepatocellular carcinoma or uterus, ovary, spleen, lung, and kidney cancer.

CONCLUSION

We have developed a simple, rapid, and sensitive method for the quantitative determination of NAR in biological samples including plasma and tissues. The pharmacokinetic study demonstrated a significantly improved oral bioavailability in rabbits by NAR-NS. In this study, an analytical method for determining NAR and its nano-formulation in different organs was studied. The *in vivo* tissue distribution studies revealed that the NAR-NS is highly biocompatible and showed enhanced therapeutic efficacy compared to NAR-S. The tissue distribution further showed that that the formulation was predominantly present in the liver. However, there exist a few remarkable gender differences, such that in genital organs which have high in female and low in male. These results provide information to help more effectively in employing NAR and to clarify its formulation in therapeutic applications. Hence, the results confirmed that the NS area is a promising approach for NAR delivery with improved dissolution and oral bioavailability.

REFERENCES

1. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 1983;32:1141-8.
2. Rouseff RL, Martin SF, Youtsey CO. Quantitative survey of narirutin, naringin, hesperidin, and neohesperidin in citrus. J Agric Food Chem 1987;35:1027-30.
3. Kanaze FI, Kokkalou E, Niopas I, Georgarakis M, Stergiou A, Bikiaris D. Thermal analysis study of

flavonoid solid dispersions having enhanced solubility. J Therm Anal Calorim 2006;83:283-90.

4. Shulman M, Cohen M, Soto-Gutierrez A, Yagi H, Wang H, Goldwasser J, *et al*. Enhancement of naringenin bioavailability by complexation with hydroxypropyl-β-cyclodextrin. PLoS One 2011;6:e18033.

5. Gera S, Talluri S, Rangaraj N, Sampathi S. Formulation and evaluation of naringenin nanosuspensons for bioavailability enhancement. AAPS PharmSciTech 2017;18:3151-62.

6. Yen FL, Wu TH, Lin LT, Cham TM, Lin CC. Naringenin-loaded nanoparticles improve the physicochemical properties and the hepatoprotective effects of naringenin in orally-administered rats with CCl(4)-induced acute liver failure. Pharm Res 2009;26:893-902.

7. Jawahar N, Meyyanathan SN, Senthil V, Gowthamarajan K, Elango K. Studies on physico-chemical and pharmacokinetic properties of olanzapine through Nanosuspension. J Pharm Sci Res 2013;5:196-202.

8. Krishnakumar N, Sulfikkarali N, Prasad NR, Karthikeyan S. Enhanced anticancer activity of naringenin-loaded nanoparticles in human cervical (HeLa) cancer cells. Biomed Prev Nutr 2011;1:223-31.

9. Sulfikkarali N, Krishnakumar N, Manoharan S, Nirmal RM. Chemopreventive efficacy of naringenin-loaded nanoparticles in 7,12-dimethylbenz(a)anthracene induced experimental oral carcinogenesis. Pathol Oncol Res 2013;19:287-96.

10. Sumathi R, Tamizharasi S, Sivakumar T. Formulation and evaluation of polymeric NS of naringenin. Int J Appl Pharm 2017;9:60-70.

11. Guo Y, Luo J, Tan S, Otieno BO, Zhang Z. The applications of Vitamin E TPGS in drug delivery. Eur J Pharm Sci 2013;49:175-86.

12. Rege BD, Kao JP, Polli JE. Effects of nonionic surfactants on membrane transporters in caco-2 cell monolayers. Eur J Pharm Sci 2002;16:237-46.

13. Lee YS, Reidenberg MM. A method for measuring naringenin in biological fluids and its disposition from grapefruit juice by man. Pharmacology 1998;56:314-7.

14. Ishii K, Furuta T, Kasuya Y. Determination of naringin and naringenin in human plasma by high-performance liquid chromatography. J Chromatogr B Biomed Appl 1996;683:225-9.

15. Islam J, Zaman K, Chakrabarti S, Sharma Bora N, Mandal S, Pratim Pathak M, *et al.* Validated RP-HPLC/DAD method for the quantification of insect repellent ethyl 2-aminobenzoate in membrane-moderated matrix type monolithic polymeric device. J Chromatogr Sci 2017;55:645-53.

16. United States Pharmacopeial. USP 31: General Tests, Chapter 621-Chromatography System Suitability. M. D. Rockville: United States Pharmacopeial Convention (USP); 2009.

17. Gupta V, Srivastava M, Maurya R, Paliwal SK, Dwivedi AK. HPLC Method development for naringenin and its glucoside in rat serum and their bioavailibilty studies. J Bioequiv Availab 2012;S14:1-4.

18. Ni H, Zhang SF, Gao QF, Hu Y, Jiang ZD, Chen F. Development and evaluation of simultaneous quantification of naringin, prunin, naringenin, and limonin in citrus juice. Food Sci Biotechnol 2015;24:1239-47.

19. Kanaze FI, Kokkalou E, Georgarakis M, Niopas I. A validated solid-phase extraction HPLC method for the simultaneous determination of the citrus flavanone aglycones hesperetin and naringenin in urine. J Pharm Biomed Anal 2004;36:175-81.

20. Magiera S, Kwietniowska E. Fast, simple and efficient salting-out assisted liquid-liquid extraction of naringenin from fruit juice samples prior to their enantioselective determination by liquid chromatography. Food Chem 2016;211:227-34.

21. Ishii K, Furuta T, Kasuya Y. Determination of naringin and naringenin in human urine by high-performance liquid chromatography utilizing solid-phase extraction. J Chromatogr B Biomed Sci Appl 1997;704:299-305.

22. Suseela P, Premkumar K, Saraswathy SD. Formulation, characterization and pharmacokinetic evaluation of naringenin-loaded gastroretentivemucoadhesive polymeric nanosystem for oral drug delivery. J Drug Deliv Ther 2015;5:107-14.

23. Lin SP, Hou YC, Tsai SY, Wang MJ, Chao PD. Tissue distribution of naringenin conjugated metabolites following repeated dosing of naringin to rats. Biomedicine (Taipei) 2014;4:16.

24. Xu S, Yu J, Zhan J, Yang L, Guo L, Xu Y, *et al*. Pharmacokinetics, tissue distribution, and metabolism study of icariin in rat. Biomed Res Int 2017;2017:4684962.