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HPLC Method Development for Naringenin and its Glucoside in Rat Serum and their Bioavailibilty Studies

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Abstract

This study was aimed at finding a potent derivative of Naringenin (N) with osteogenic action. In CDRI, we have isolated Naringenin-6-**C**-Glucoside (NCG). This was found more active than naringenin. This paper reports a bioanalytical HPLC method for comparative bioavailability studies of N and NCG. In this method, separation was achieved on a Lichrosphere Lichrocart RP_{18} (250 mm, 4 mm, 5 µm, Merck) column, with the mobile phase consisting of a mixture of 0.5% phosphoric acid in triple distilled water & acetonitrile (75:25). The flow rate was kept at 1.5 ml/minute and the column effluents were monitored at 290 nm and 325 nm. The retention time of NCG was about 2.5 min, whereas naringenin eluted at about 14.5 min. There is no interference of serum impurities at these retention times. Validation parameters were checked and were found within limits. The highest serum concentrations (C_{max}) of naringenin (5 mg/kg dose) was recorded at 4 h after dosing and reached 1584 ± 439 ng/ml, followed by a marked decrease between 6 and 24 h. In case of NCG (5 mg/kg dose) highest concentration 738 ± 300 ng/ml was found at 3 hours (C_{max}). These data indicate that N & NCG are efficiently absorbed after feeding to rats and that their bioavailability is related to the glucoside moiety.

Keywords: Naringenin; Naringenin-6-C-Glucoside; Bioavailability; HPLC

Introduction

Variety of botanicals and dietary products contains number of non-steroidal phytoestrogens. Several preclinical studies have confirmed positive effect of flavanones on bone functions. After isoflavones, the flavanones are the subgroup of polyphenols with most in vivo preclinical evidence responsible for improved bone health [1]. When young male rats were fed with a semi-purified diet to which oranges were added at 1 $g.day^{-1}$ dose, significant inhibition of bone resorption was observed [2]. Hesperidin, a citrus flavanone glycoside, slows down femoral bone loss when administrated in Ovariectomized (OVx) mice [3]. Naringenin (N) (Figure 1), a major flavanone present in grapefruit, and its analogs, having disaccharide grouping attached to position 7 of N and which is hydrolyzed to N by gut flora prior to being absorbed, have been implicated in bone health for their oestrogen-'like' effects but low bioavailability impedes clinical potential. Earlier we reported that young male rats receiving N rich nutrients were found to enhance bone formation during alveolar bone development [4]. N has been shown to have positive skeletal effects in OVx mice. The doses of naringin (7-(2-O-(6-deoxy-alpha-L-mannopyranosyl)-beta-D-glucopyranosyloxy)-2, 3-dihydro-4', 5, 7-trihydroxyflavone) used were 0.2 and 0.4 $mg.g^{-1}.day^{-1}$ (200-400 $mg.kg^{-1}.day^{-1}$) that would yield very high doses when translated to humans [5]. However, these reports importantly point to N being a promising pharmacophore model for exploration of more potent osteogenic derivatives.

In the course of our search for more potent analogs of N with positive skeletal effects, we first screened various naturally occurring analogs of N in an assay for osteoblast differentiation. Naringenin-6-C-Glucoside (NCG), isolated from an Indian medicinal plant, *Ulmus wallichiana* (Himalayan Elm), [6] was identified as the most potent member of the N analogs in inducing osteoblast differentiation. Because the bioavailability is vital for any compound to exert biological effects, so we determined oral bioavailability of NCG and N. Subsequently, the effects of NCG on bone properties of OVx mice and in primary osteoblast cultures were studied and compared with N [4]. In this paper we are reporting a new validated method for simultaneous analysis of N and NCG in serum.

Experimental

Materials and reagents

The sample of Naringenin (N) and its glucoside (NCG) were provided by Dr. Naibaidhya Chattopadhya, Endocrinology Division of our Institute. HPLC grade methanol and acetonitrile were purchased from M/S Merck Limited (Mumbai, India). Triple distilled water, from an all glass apparatus, was used. Phosphoric acid and other reagents used were of analytical grade.

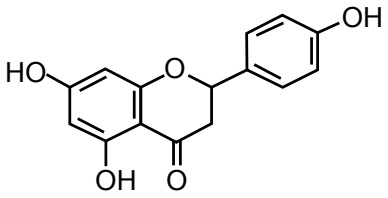

Figure 1: Chemical structure of N.

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HPLC apparatus

HPLC analysis of the sample was done on a Shimadzu HPLC system, equipped with binary gradient pump (10 ATVP), Rheodyne (Cotati, CA, USA) model 7125 injector with a 20 µl loop and diode array detector (10 ATVP). HPLC separation was achieved on a Lichrosphere Lichrocart C_{18} column (250 mm, 4 mm, 5 µm, Merck).

HPLC analysis

For analysis, 20 µl of each sample was injected into a HPLC column. Elution was performed using a mixture of 0.5% phosphoric acid in triple distilled water and acetonitrile (75:25). Both the solutions were filtered and degassed before use. Chromatography was performed at 25 ± 3°C at a flow rate of 1.5 ml/minute. The flavanones (N and NCG) were quantified with isocratic conditions with absorbance monitored at 290 nm and 325 nm.

Preparation of standard stock solution

Stock solution of N & NCG was prepared by dissolving, in methanol, about 5 mg of the compounds in 25 ml volumetric flask to give a final concentration of 208 µg/ml & 206 µg/ml respectively. A series of standard solutions with concentrations in the range of 1.04-10.40 µg/ml for N and 1.03-10.30 µg/ml for NCG were obtained by serial dilution method with methanol. All the solutions were stored at -20°C and were brought to room temperature before use.

Standard calibration samples

To prepare the standard calibration samples, different concentrations of compounds (N and NCG) were spiked in 200 µl of serum, leaving two of them blank without any concentration. The samples spiked with different concentrations of N & NCG were incubated for 30 minutes at 37°C. The final standard serum concentrations were 104-1040 ng/ml (N) & 103-1030 ng/ml (NCG).

Sample processing

To each 0.2 ml of standard calibration samples and serum samples for analysis, 0.4 ml of methanol was added. The resulting mixtures were vortexed for 5 minutes and centrifuged for 10 min at 3,000 rpm at room temperature. Supernatants (500 µl) were taken in other clean tubes and solvents were evaporated under reduced pressure (using HETO system) and reconstituted in 50 µl methanol.

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.

Analyte Stability

The stability of N and NCG in serum was investigated under a variety of storage conditions: performing three cycles of freeze (-20°C)—thaw (room temperature), 24 h storage at room temperature and under -20°C freezer at least one month.

Results and Discussion

Method development and Optimization

Several stationary and mobile phases were tried. Columns like C-18, C-8 and CN were tried with mobile phase consisting of ACN and water (1:1) at a flow rate of 1.5 ml/min. and detection at 290 and 325 nm. As NCG was a highly polar compound it is poorly retained on reverse phase HPLC columns. Its peak gets merged with the peaks of solvent and other serum impurities (Figure 2a-2c). Peak of N also came at about 4 minutes and it is also contaminated with the other impurities. Both compounds N and NCG were phenolic and, therefore, acidic in nature and to make them more lipophilic, we had to reduce their

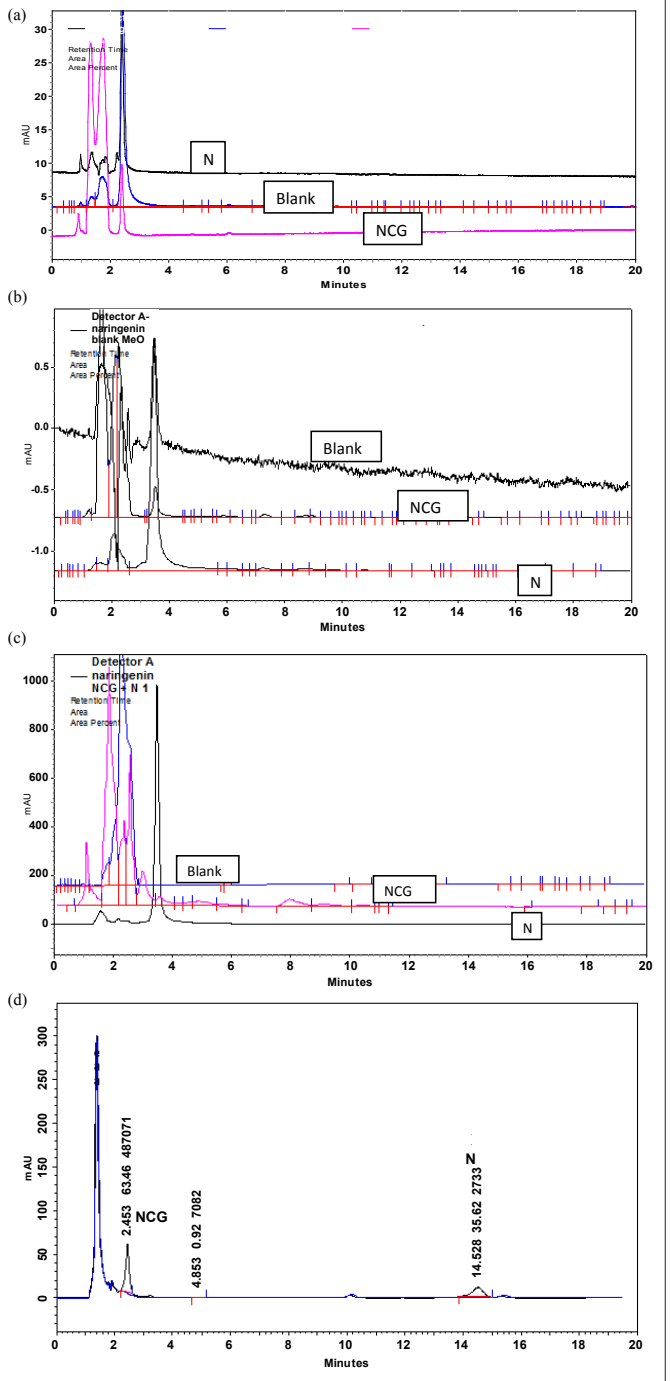

Figure 2: HPLC Chromatogram of N & NCG in serum. Background is blank serum. a: HPLC Chromatogram of N & NCG using C-18 column with mobile phase of ACN: Water (1:1). **b:** HPLC Chromatogram of N & NCG using C-8 column with mobile phase of ACN: Water (1:1). **c:** HPLC Chromatogram of N & NCG using CN column with mobile phase of ACN: Water (1:1). **d:** HPLC Chromatogram of N & NCG in serum.

ionization. This could be achieved by optimization of the pH by adding an acid. Method parameters such as mobile phase composition, its pH, flow rate, etc, (Figure 2a and 2d) were optimized on the basis of peak characteristics and run time. Finally, we used a Lichrocart Lichrosphere C18 column (250 mm, 4 mm, 5 µm) (Merck) using a mixture of 0.5% phosphoric acid in triple distilled water and acetonitrile (75:25) with a flow rate of 1.5 ml/minute as mobile phase The column effluents were monitored at 290 nm and 325 nm (Figure 2d).

Assay specificity

HPLC analysis of standard solutions of N and NCG indicated that, in given chromatographic conditions, NCG had retention time about 2.5 min, whereas N eluted at about 14.5 min. There was no interference of other impurities at these retention times.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Based on the signal to noise ratio (S/N) of three and ten the LOD and LOQ were found to be 104 ng/ml and 208 ng/ml (N) and 103 ng/ml and 206 ng/ml (NCG) respectively.

Linearity and range

Under the above optimized chromatographic conditions, the linearity of the method was evaluated over concentration range of 208-1040 ng/ml (N) and 206-1030 ng/ml (NCG).

Accuracy and precision

Accuracy and precision of the analytical method was determined by analyzing Quality Control samples (QC) at three different concentrations within the calibration range in triplicate (n = 3). The precision (% RSD) of the analytical procedure was evaluated by determining intra- and interday coefficient of variation. Percentage relative standard deviation (% RSD) was found to be less than 15-20 % for intra and inter- day precision of QC standards in serum, which were under acceptable limits [7-10] and proved that method is precise. The results are given in Table 1.

Stability

Stability was assessed by comparing against the freshly thawed samples. The % mean stability for N and NCG were within the acceptance limits of 85 to 115%. The samples were found to be stable for all the parameters studied as per the experimental protocol (Table 2).

Robustness

The method was found to be robust as minor changes in the method parameters did not alter method performance.

Recovery

The % mean recoveries for N and NCG at three concentration levels in serum were 84-89% (Table 3).

Application of the method

Comparative Bioavailability Studies of N and NCG

This study was designed to assess the influence of per oral N and NCG administration on its distribution profiles in rat serum. The concentration of N and NCG in extracted serum samples was checked after storage over 5 days. Both were found to be stable under the storage conditions. In rats fed a single dose of 5mg.kg^{-1}, the rate of appearance of NCG and N in plasma differed. Figure 3 and 4 shows the individual serum pharmacokinetics profile of N and NCG over the 24 h. After rats receiving a single dose of N (5 mg/kg) significant concentration of N

	Inter assay variation			Intra assay variation		
Conc.taken (ng/ml)	**Conc. found (Mean ± S.D.) (ng/ml)**	**D.F.A (%)**	**R.S.D. (%)**	**Conc. found (Mean ± S.D.) (ng/ml)**	**D.F.A (%)**	**R.S.D. (%)**
N						
208	222.54 ± 20.56	9.24	6.99	210.77± 27.67	13.13	1.33
616	560.68± 34.65	6.18	-8.98	577.93± 37.64	6.51	-6.18
1040	1024.22± 57.28	5.59	-1.52	1047.98±30.22	2.88	0.77
NCG						
206	220.88 ± 23.63	10.70	7.22	219.53 ± 25.61	11.67	6.57
618	669.52 ± 25.05	3.74	8.34	670.31 ± 27.13	4.05	8.46
1030	1095.38 ± 71.20	6.50	6.35	1050.25 ± 64.51	6.14	1.97

R.S.D. = Relative standard deviation
D.F.A. = Deviation from actual concentration
S.D. = Standard deviation

Table 1: Intra and Inter assay validation of N and NCG.

Stability studies	% of N remaining	% of NCG remaining
Short-term	97.68 %	98.60 %
Long-term	100.18 %	99.68 %
Freeze-thaw	98.20%	99.33 %

Table 2: Stability Data (Short-term, Long-term, Freeze-thaw).

N			NCG		
Conc. Taken	**Conc. found**	**% Recovery**	**Conc. taken**	**Conc. found**	**% Recovery**
208	181.93	87.47	206	169.9142	82.48
616	548.89	89.11	618	559.3464	90.51
1020	910.39	89.25	1030	843.2761	81.87
	Mean	**88.61**		**Mean**	**84.95**

Table 3: Recovery data.

was recorded in serum after 2 h. It showed three peaks in the serum due to hepatic recirculation. The highest serum concentration of N (C_{max}) was recorded after 4 h of dosing and reached 1584 ± 439 ng/ml, followed by a marked decrease up to 24 h (Figure 3). In case of NCG (5 mg/kg dose) highest concentration 738 ± 300 ng/ml was obtained in 3 hours (C_{max}) (Figure 4). At 8 h, serum concentrations of NCG was again significantly increased and reached to 728 ng/ml. Twenty-four hours after dosing, high levels of NCG (570 ± 97 ng) were present in serum. The bioavailability results of N and NCG are summarized in Table 4. These results suggested that absorption of N and NCG took place early in the digestive tract (stomach or small intestine), as reported before for other flavonoids [11]. The AUC $_{0\to\infty}$ of N and NCG were 46247ng/ml and 71557ng/ml respectively. This indicated that NCG had more bioavailability than N. N has low oral bioavailability might be due to its hydrophobic rings structure [12]. Several reports on N and naringin bioavailability in rat model served as the reference point [12,13]. *C*-glycosylation of N in NCG might have imparted hydrophilicity to N leading to better absorption and higher bioavailability of NCG over N. *C*-glucoside (C-C) bond present in NCG in contrast to *O*-glucoside (C-O) bond in naringin, gave an acid-resistant and largely enzyme-resistant by gut microflora. This might be responsible for a greater metabolic stability and oral bioavailability of NCG [14].

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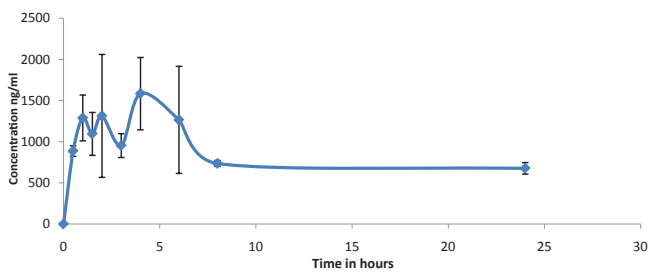

Figure 3: Evolution of the serum concentration of N in rats receiving a single dose of 5 mg/kg values are means ± SE; *n* = 3.

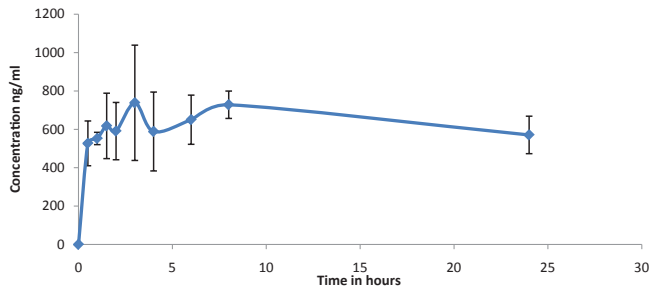

Figure 4: Evolution of the serum concentration of NCG in rats receiving a single dose of 5 mg/kg, values are means ± SE; *n* = 3.

Parameter	N	NCG
Tmax (h)	4.00	3.00
Cmax (ng/ml)	1584±439	738±300
AUC $_{0\to24}$	20515 ng*h/ml	15330 ng*h/ml
AUC $_{0\to\infty}$	46247 ng*h/ml	71557 ng*h/ml

Table 4: Bioavailability parameters of N and NCG.

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