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Short communication

Development of ultra fast liquid chromatographic method for simultaneous determination of nitrendipine and carvone in skin diffusate samples

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ABSTRACT

A simple and sensitive reverse phase ultra fast liquid chromatographic (UFLC) method for simultaneous determination of nitrendipine and carvone in skin diffusate samples and microemulsions was developed and validated. The separation was achieved using a gradient mobile phase, on an Onyx column. The eluents were monitored by photodiode array detection. The linearity ranges of proposed method were 0.125–50 μg mL^{-1} and 0.125–30 μg mL^{-1} for nitrendipine and carvone respectively. The intra-day and inter-day coefficient of variation and percent error values of the assay method were less than 10%. The method was found to be precise, accurate, and specific during the study. The method was successfully applied for simultaneous estimation of nitrendipine and carvone in *ex vivo* skin diffusate samples and microemulsions.

1. Introduction

Nitrendipine, NTDP (Fig. 1a) [1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylic acid ethyl methyl ester] is a calcium channel blocker used in the treatment of hypertension. NTDP is available in tablet form (10 and 20 mg), and is administered once or twice daily to treat mild to moderate hypertension. After its oral administration it undergoes first-pass hepatic metabolism resulting in low oral bioavailability of 16–20% [1]. To improve the bioavailability alternate routes, transdermal [2], buccal [3] or parenteral are preferred. Carvone (Fig. 1b) [(*RS*)-5-isopropenyl-2-methylcyclohex-2-en-1-one], a terpenoid is most abundant in the oils from seeds of caraway (*Carum carvi*) and dill. It is available as two enantiomers: *S*-(+)-carvone and *R*-(−)-carvone. Both carvones are used in food and flavor industry [4]. *R*-(−)-carvone (previously referred as l-carvone) is also used for air freshening products, in aromatherapy, in alternative medicine and is reported as penetration enhancer in transdermal drug delivery systems [3]. There are no analytical methods for simultaneous estimation of these two analytes. However some reports on the estimation of NTDP by high performance liquid chromatography (HPLC) [5–13] are available. Till date no report has been published based on HPLC for the estimation of carvone, however one report based on thin layer chromatography [14] and gas chromatography [15] are seen in literature.

The aim of present work was to develop and validate a method for simultaneous estimation of NTDP and carvone by ultra fast liquid chromatography (UFLC). The method was also applied for the estimation of NTDP and carvone in skin diffusate samples and in microemulsions. Microemulsions (composed of oil, surfactant, co-surfactant and water) were developed to enhance the skin permeation of NTDP. The advantages of present method include sensitive, simple sample preparation procedure using inexpensive chemicals, less organic solvent consumption and short run time.

2. Experimental

2.1. Materials

Nitrendipine was a gift sample from US Vitamines, Mumbai, India. *R*-(−)-carvone (98% purity) was purchased from Alfa Aesar (Ward Hill, MA, USA). Acetonitrile and methanol (HPLC grade) were purchased from Merck, Mumbai, India. All other chemicals used were of analytical grade. Double distilled water was used during the entire UFLC procedure.

2.2. Chromatographic conditions

The UFLC system (Shimadzu, Kyoto, Japan) consisted of two LC-20AD Prominence liquid chromatograph pumps, SPD M20A

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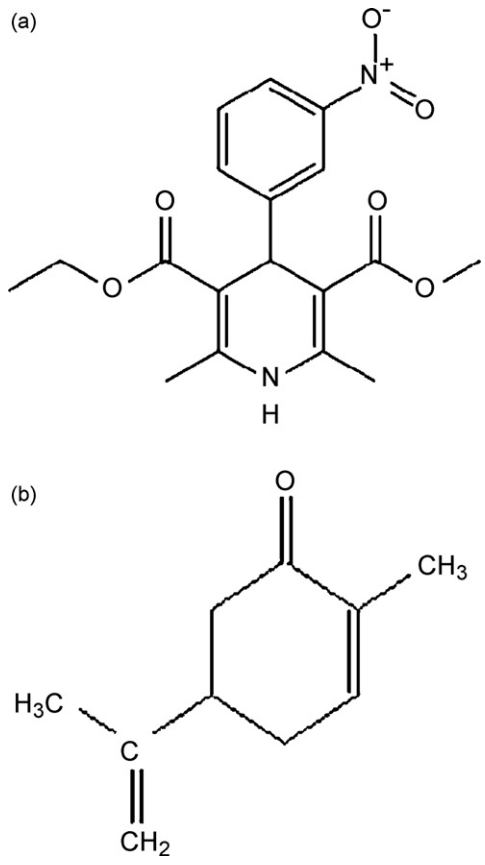

Fig. 1. Structure of (a) nitrendipine and (b) carvone.

Prominence Diode Array detector, CTO-20AC Prominence column oven with Lab solutions (LC solutions) software. The analytical column used was Onyx monolithic C18 column (Phenomenex, 100 mm × 4.6 mm i.d, particle size 5 μm) at ambient temperature. The mobile phase was a gradient elution of double distilled water (filtered through 0.45 μm membrane filter) (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL min^{-1}. The gradient program of solvent A in B (v/v) was as follows: 0–4 min 60% A; 4–5.5 min 50% A and 5.5–7 min 60% A and returned to the initial condition in 1 min and re-equilibrated for 2 min.

2.3. Preparation of the calibration standards and quality control (QC) samples

Primary stock solution of 1 mg mL^{-1} of each NTDP and *R*-(−)-carvone were prepared in methanol. Standard solutions and QC samples were prepared by serial dilution of primary stock solutions using phosphate buffer saline pH 7.4 (PBS) containing 40% v/v polyethylene glycol 400 (PEG400). The calibration standards, 0.15, 0.5, 1, 2.5, 5, 10, 25 and 50 μg mL^{-1}; 0.125, 0.5, 1, 2.5, 5, 10, 20 and 30 μg mL^{-1} were prepared for NTDP and carvone respectively. QC samples at three different levels (0.40, 20.0 and 40.0 μg mL^{-1} for NTDP; 0.30, 15.0 and 25.0 μg mL^{-1} for carvone) were prepared daily from corresponding stock solutions. All the stock solutions and QC samples were refrigerated (4 °C) when not in use.

2.4. Sample preparation

Ex vivo skin diffusate samples were filtered through 0.45 μm membrane filter, diluted suitably with PBS containing 40% v/v PEG400 and injected into UFLC. For the estimation of NTDP and carvone from microemulsion (Nitrendipine 0.5% w/v; isopropyl myristate 10.5% v/v; surfactant mixture, 1:2 of cremophor: propylene glycol 28.5% v/v; carvone 6% w/v and water 55% v/v), 2 mL of microemulsion was diluted with PBS containing 40% v/v PEG400. The solution was adjusted to volume and the resulting solution was filtered through 0.45 μm membrane filter and injected into UFLC.

2.5. Method validation

The UFLC method was validated in terms of precision, accuracy and linearity according to ICH guidelines [16].

2.5.1. System suitability testing

System suitability standard solution containing 5 μg mL^{-1} each of NTDP and carvone were prepared by appropriately diluting and mixing the corresponding stock standard solutions. System suitability was determined from five replicate injections of the system suitability standard before sample analysis.

2.5.2. Linearity and range

Calibration curves were prepared with eight calibration standards over a concentration range of 0.15–50 μg mL^{-1} for NTDP and 0.125–30 μg mL^{-1} for carvone. The data of peak area versus concentration were treated by linear least square regression analysis.

2.5.3. Accuracy and precision

To study the reliability and suitability of the developed method, QC samples at three levels were tested by spiking NTDP (0.40, 20.0 and 40.0 μg mL^{-1}) and carvone (0.30, 15.0 and 25.0 μg mL^{-1}). Measured values were compared with the theoretical concentration. The precision of the method was assessed in terms of repeatability and intermediate precision by analyzing five replicates of QC samples at three levels for each analyte. The % coefficient variation (CV) values of the results corresponding to peak area were expressed for intra-day precision and on 3 days for intermediate (inter-day) precision.

2.5.4. Specificity

Injections of the PBS containing 40% v/v PEG400 and from NTDP and carvone free microemulsions were performed to demonstrate the absence of interference with the elution of the NTDP and carvone. All chromatograms were examined to determine if compounds of interest co-eluted with each other or with any additional peaks.

2.5.5. Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ of NTDP and carvone were determined based on standard deviation of response and slope [16]. NTDP and carvone were prepared in the range of 0.05–5 and 0.01–2.5 μg mL^{-1} respectively and injected in triplicate. Average peak area of three analyses was plotted against concentration. LOD and LOQ were calculated by using following equations.

$$\mathrm{LOD} = (3.3\sigma)/S$$
$$\mathrm{LOQ} = (10\sigma)/S$$

where σ is standard deviation and S is slope. LOD and LOQ for NTDP and carvone respectively.

2.5.6. Robustness

To determine the robustness of method, the final experimental conditions were purposely altered and the results were examined. The flow rate was varied by 1 ± 0.1 mL min^{-1}. The percentage of organic modifier was varied by standard programme ± 5% and column temperature was varied by 30 ± 5 °C.

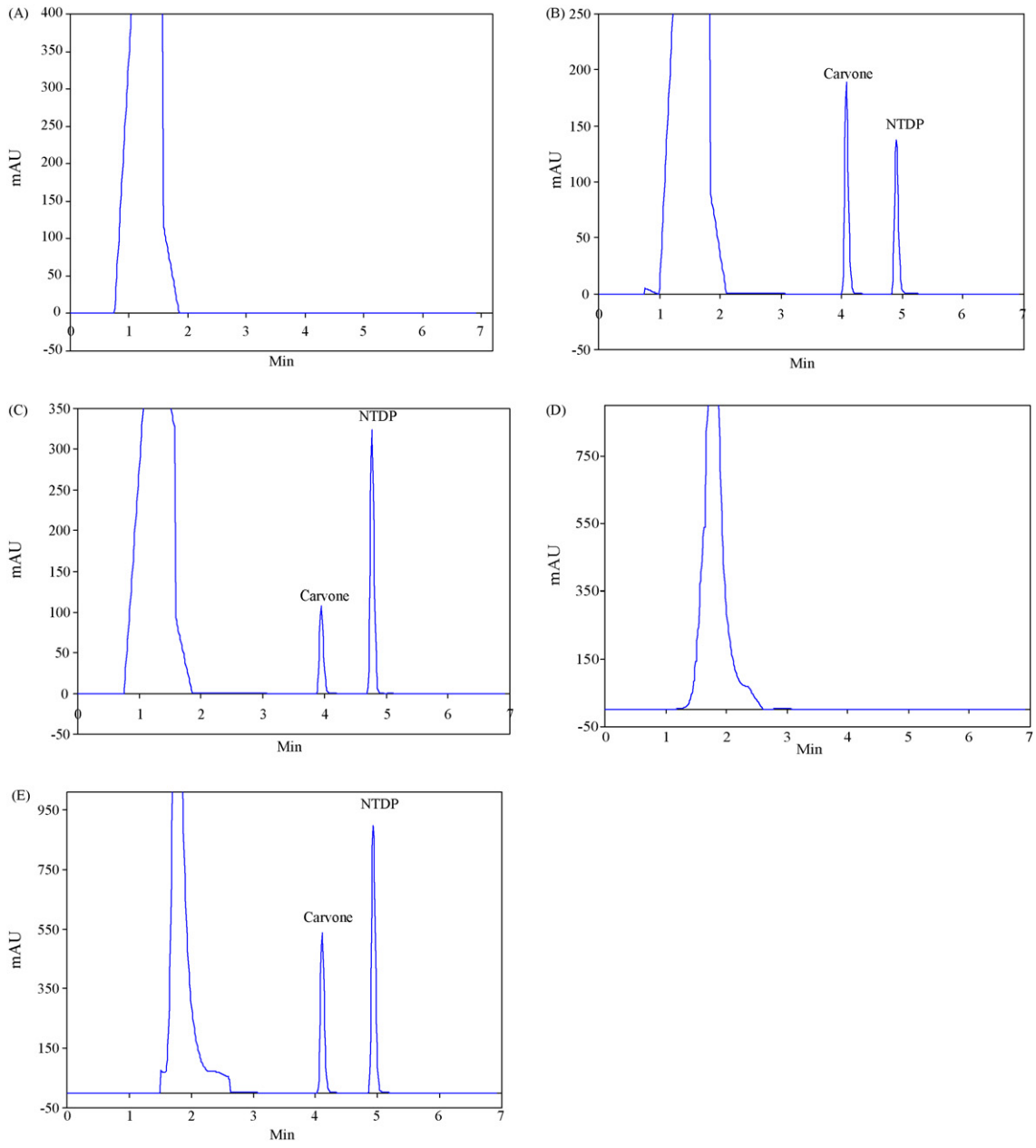

Fig. 2. UFLC chromatograms of (A) PBS with 40 % v/v PEG400 (B) PBS containing 40% v/v PEG400 spiked with 5 μg mL^{-1} of each NTDP and carvone (C) sample collected from *ex vivo* study at 3 h, after application of 2 mL of microemulsion (D) NTDP and carvone free microemulsion and (E) NTDP and carvone loaded microemulsion. The retention times of carvone and NTDP were 4.1 and 4.9 min respectively.

2.6. Application to ex vivo skin diffusate samples

The *ex vivo* permeation study of NTDP microemulsions across excised rat (Albino rats) abdominal skin was conducted with the permission from the institutional ethical committee, University College of Pharmaceutical Sciences, India. Permeation study was conducted using Franz diffusion cell with a diffusional surface area of 3.56 cm^2. The rat abdominal skin was mounted between the compartments of diffusion cell with stratum corneum facing donor compartment and clamped into position. NTDP microemulsion (10 mg of NTDP in 2 mL) was placed in donor compartment and 12 mL of PBS containing 40% v/v PEG 400 was placed in receptor compartment. The diffusion cells were placed on magnetic stirrer and contents were mixed with stirring bars at a speed of 400 rpm. The study was conducted at a temperature of 37 °C. Samples of 1 mL were collected from receptor compartment at preset time points. The samples were filtered through 0.25 μm membrane filter, diluted suitably and injected into UFLC. The permeation parameters, cumulative amounts of NTDP and carvone permeated across skin were calculated.

3. Results and discussion

3.1. Method development and optimization

Typically, method development focuses on identifying mobile phase composition (gradient programme), organic solvent and implementing small changes to optimize selectivity and enhance resolution. Initially, NTDP was found to be eluted with long run time and co-eluted with carvone by using different stationary phases such as C_8 and C_{18} with different lengths. The change of mobile phase programme, temperature and organic solvents like methanol

and acetonitrile also showed similar results. At the first stage, a C_{18} column and water were used with methanol, in subsequent cases acetonitrile was used as organic solvent. The retention time of two eluents was more. An acceptable peak shape and resolution were achieved with increasing acetonitrile by 65–70% throughout the programme. The retention times with this mobile phase programme were about 10 and 14 min respectively for carvone and NTDP with a run time of 17 min. In order to reduce run time; short C_{18} (15 cm and subsequently 10 cm) columns were evaluated. The change of column to 15 and 10 cm resulted in short run time for about 15 and 12 min respectively. The use of C_8 column also showed similar results. The peaks resulted in all the trials were with poor symmetry and resolution and resulted in high back pressure. A typical silica-based monolithic column was examined. The retention time of carvone and NTDP reduced to about 4 and 5 min, respectively at an organic content of 40% ACN at a flow rate of 1 mL min^{-1} and oven temperature of 30 °C. The use of monolithic column shortened the analysis time and reduced the back pressure, which is important to extending the column life time. The optimum wavelength was established experimentally after scanning over a range of 190–400 nm. The absorption maxima observed for NTDP and carvone were 236 and 238 nm, respectively; it was shown that 238 nm is the optimal wave length to maximize the sensitivity and has no interference with other components of the formulation.

3.2. Chromatography

The chromatographic conditions and sample preparation for the proposed method were optimized. Fig. 2 shows typical UFLC chromatograms of (A) PBS containing 40% v/v PEG400 (B) PBS containing 40% v/v PEG400 spiked with 5 μg mL^{-1} of each NTDP and carvone (C) sample collected from *ex vivo* study at 3 h, after application of 2 mL of microemulsion (D) NTDP and carvone free microemulsion and (E) NTDP and carvone loaded microemulsion. The retention times of carvone and NTDP were 4.1 and 4.9 min respectively, with a total run time of less than 7 min. The analytical process of NTDP and carvone were resolved with good symmetry. System suitability parameters for the method were as follows: theoretical plates for carvone and NTDP were 2332 and 3331 respectively. Tailing factor was less than 1.1 for both NTDP and carvone and resolution between NTDP and carvone was 2.1.

3.3. Validation of method

3.3.1. Specificity

The specificity of UFLC method is illustrated in Fig. 2 where complete separation of NTDP and carvone was seen in the presence of *ex vivo* sample or components of microemulsion. The peaks of analytes were clear and there were no endogenous peaks at the retention times of NTDP and carvone. The components of *ex vivo* samples or microemulsion did not interfere with analytes thereby confirming the specificity of the analytical method.

Table 1
Linearity parameters for the simultaneous estimation of NTDP and carvone.

Parameter	Nitrendipine	Carvone
Linearity range (μg mL^{-1})	0.125–50	0.1–30
Slope	32,448 ± 2,044.4	95,391 ± 496.4
Intercept	1,729.3 ± 180.3	21,273 ± 126.7
Correlation coefficient (r)	0.9963 ± 0.002	0.9996 ± 0.001

Values presented are mean ± S.D. of three calibration curves generated on three consecutive days (n = 3). Eight concentrations in the linearity range were evenly distributed.

3.3.2. Linearity and range

The calibration curves were linear over the concentration range of 0.125–50 μg mL^{-1} for NTDP and 0.1–30 μg mL^{-1} for carvone. The results are presented in Table 1 and show a good correlation between the peak area of analytes and concentration with r > 0.996.

3.3.3. Accuracy and precision

The % CVs were found to be less than 5 and 3 for intra-day and inter day precision respectively indicating that the method is reliable (Table 2). The inter-day precision was demonstrated on different days at three QC standards that cover the assay method range. For determining accuracy, PBS containing 40% v/v PEG400 spiked with reference standards were used. The recovery was 100 ± 3% for all samples with % CV less than 5% and % relative error less than ± 6%

3.3.4. LOD and LOQ

LOQ of the method was found to be 0.125 μg mL^{-1} for NTDP and 0.1 μg mL^{-1} for carvone with CV less than 10% and an accuracy of 90–105%. The LOD was determined to be 0.1 μg mL^{-1} for NTDP and 0.075 μg mL^{-1} for carvone respectively.

3.3.5. Robustness

It can be seen that every employed condition, the chromatographic parameters are in accordance with established value [17]. A change of mobile phase composition, flow rate and temperature had no impact on chromatographic performance. The tailing factor for NTDP and carvone was found to be less than 1.5 and both analytes were well separated under all the changes carried out. The resolution ranged between NTDP and carvone was 1.73–3.03 (Table 3). Considering the result of modifications in the system suitability parameters and the specificity of the method, it would be concluded that the method conditions are robust.

3.4. Application to ex vivo skin permeation study and drug content estimation in microemulsions

The method was applied for simultaneous estimation of NTDP and carvone in skin diffusate samples and in microemulsions. Fig. 3 depicts the cumulative amounts of NTDP and carvone permeated across skin. About 62.36 μmol (9368 μg) and 28.3 μmol (4250 μg) of NTDP and carvone were permeated in 24 h with a

Table 2
Intra-day and inter-day precision and accuracy data for assay of nitrendipine and carvone (n = 5).

Added conc. (μg mL^{-1})	Calculated concentration (μg mL^{-1})		%CV		% Relative error		Accuracy (%)	Range (min–max)
	Intra day	Inter day	Intra day	Inter day	Intra day	Inter day		
Nitrendipine								
0.4	0.42 ± 0.01	0.41 ± 0.01	2.12	2.58	5	2.5	102.5 ± 2.5	98.1–102.6
20	20.4 ± 0.94	20.2 ± 0.45	4.61	2.21	2	1	101.5 ± 0.5	96.9–105.1
40	40.2 ± 0.57	39.9 ± 0.93	1.43	2.33	0.5	−0.1	100.5 ± 0.6	99.0–101.6
Carvone								
0.3	0.29 ± 0.01	0.3 ± 0.004	1.84	1.51	−3.3	0.33	99.1 ± 1.5	97.8–101.2
15	15.8 ± 0.75	15.6 ± 0.05	4.79	0.34	5.3	4	103.0 ± 2.1	98.6–100.5
25	24.9 ± 0.38	25.4 ± 0.27	1.53	1.05	−0.4	1.6	100.2 ± 0.8	98.2–101.7

Table 3
The robustness data of the developed UFLC method.

Parameter	Modification	Retention time (min)		Tailing factor		Plates		Resolution
		NTDP	Carvone	NTDP	Carvone	NTDP	Carvone	
Acetonitrile (ACN) (v/v)	Up to 4 min 35%; 4–5.5 min 45% and 5.5–7 min 35% ACN	4.43	5.64	1.2	1.15	1815	2207	3.03
	Up to 4 min 40%; 4–5.5 min 50% and 5.5–7 min 40% ACN	4.12	4.95	0.94	1.1	2355	2266	2.08
	Up to 4 min 45%; 4–5.5 min 55% and 5.5–7 min 45% ACN	3.86	4.57	0.86	1	2067	1932	1.78
Flow rate (mL min^{-1})	0.9	4.23	5.2	1.15	1.1	1655	2501	2.43
	1.0	4.15	4.94	0.98	1.14	2390	2257	1.98
	1.1	3.88	4.68	0.85	0.96	2089	2431	2.00
Temperature (°C)	25	4.5	5.48	1.2	1.14	1873	2083	2.45
	30	4.2	4.96	1	0.98	2448	2276	1.90
	35	3.92	4.61	0.94	0.98	2132	2359	1.73

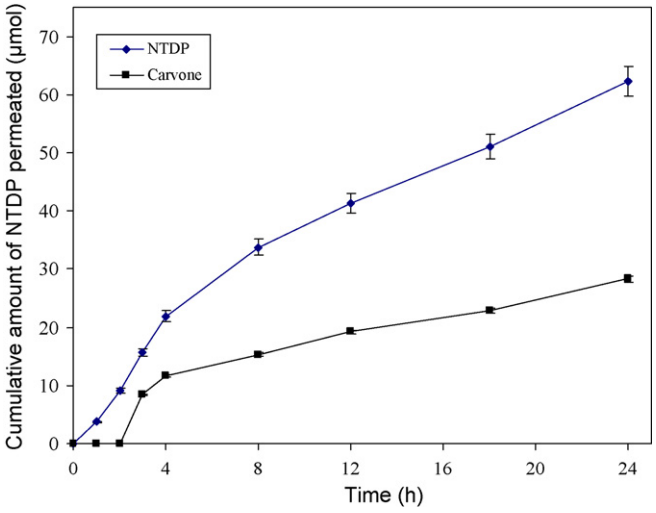

Fig. 3. *Ex vivo* permeation profile of nitrendipine and carvone, results presented are mean ± S.D ($n = 3$).

flux of 102.3 and 37.2 µg h^{-1} cm^{-2} respectively. The drug content in the microemulsions was found to be 10.2 ± 0.45 and 119.6 ± 3.7 mg respectively.

4. Conclusions

A simple, sensitive and reliable method for simultaneous determination of NTDP and carvone in skin diffusate samples by UFLC was developed and validated. No interfering peaks were observed at elution times of NTDP and carvone. The method was accurate, reproducible, specific and applicable to the evaluation of permeation parameters of NTDP and carvone across rat abdominal skin. The developed UFLC method was found to be suitable for the analysis of NTDP and carvone in skin diffusate samples and microemulsions.

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