PROGRAMME & ABSTRACT BOOK

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ISOCRATIC RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF REACTION RATE IN N-PHENYLBENZAMIDE SYNTHESIS AND ITS INTERMEDIATE COMPOUNDS

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Abstract

Antiviral activity against Hepatitis C virus (HCV) and Enterovirus 71 (EV 71) has been discovered in a sequence of novel N-phenyl benzamide (Benzanilide) derivatives. Recently, such findings inspired researchers to look for more potent antiviral agents through the N-phenyl benzamide synthesis. A high-pressure liquid chromatography method has been developed to observe the rate of N-phenyl benzamide synthesis. This is the first time reporting the approach has been established together for N-phenyl benzamide and aniline or phenylamine in an amide coupling reaction. These compounds were detected on a reversed-phase column (C_{18}) using a 50:50 mobile phase of acetonitrile - 10mM (P^H 5) sodium acetate buffer and UV detection is recorded at 254 nm. At a temperature of 30°C, isocratic elution was employed with a flow rate of 0.7 ml/min and an injection volume of 20 µL. The retention time for aniline and N phenyl benzamide was ±4.10 and ±7.60 min respectively. A linear relationship (r^2=0.9998 for benzanilide and r^2=0.9992 for aniline) over the concentration range 10-100 µg/ml was found when the peak area was plotted against concentration. The percentage of RSD was also 0.23 and 0.85 for benzanilide and aniline respectively. The method was verified in accordance with the International Conference on Harmonization (ICH) guidelines. The precise, linear, and more accurate method was applied to find out the reaction rate of aniline to produce the N-phenyl benzamide.

The 7th International Conference on Advancement in Science and Technology (iCAST2021)

Title

Isocratic RP-HPLC Method for the Simultaneous Determination of Reaction Rate in N-phenylbenzamide Synthesis

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Contents…

Introduction

- Activity of novel N-phenylbenzamide derivatives against HCV and EV 71
- Utilization of hazardous solvents
- Dangerous for health, safety, environment, and makes the problem in drug manufacturing
- Require alternative solvents which consider as safer and green solvent
- The proposal regarding the hazardous solvent replacement by HBD-HBA in synthesis
- Need to develop a HPLC method to observe the reaction rate and yield

Experimental

HPLC instrumentation

HPLC analysis was performed using a Agilent 1200 series (Agilent, USA) consisting of a G1322A-degasser, G1311A-quaternary pump, G1329A autosampler, G1316A-thermostatted column compartment, G1362A-refractive index detector, G1314B-variable wavelength detector, C18 column (150 × 4.6 mm i.d.; 5 µm; Agilent, USA) and a Agilent chemstation software.

Chromatographic conditions

A reverse-phase HPLC assay was carried out using an isocratic system with a flow rate of 0.700 mL/min, a column temperature of 30 °C, a mobile phase (50:50) of acetonitrile and 10mM sodium acetate buffer (pH 5) while acetic acid was used to adjust the pH and a detection wavelength of 254 nm.

The injection volume was 20 µL. Solutions were filtered through a 0.45 µm, 47 mm nylon membrane prior to HPLC injection. The total chromatographic analysis time was 25 min per sample.

Experimental

Preparation of standard solutions

- ✓ Stock solutions of N-PBA and aniline in methanol were prepared separately at concentrations of 100 μg/mL.
- ✓ A combined standard solution was prepared of 100 μg/mL and diluted with methanol to obtain the concentrations of 10, 20, 40, 60 and 80 μg/mL for N-PBA and aniline.

Sample preparation

- ✓ Reactions were observed using the HPLC at 0 hour (approx. 1 min.), 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 24 h.
- ✓ 50 μL sample was collected and diluted with 500 μL in methanol (10 times dilution)

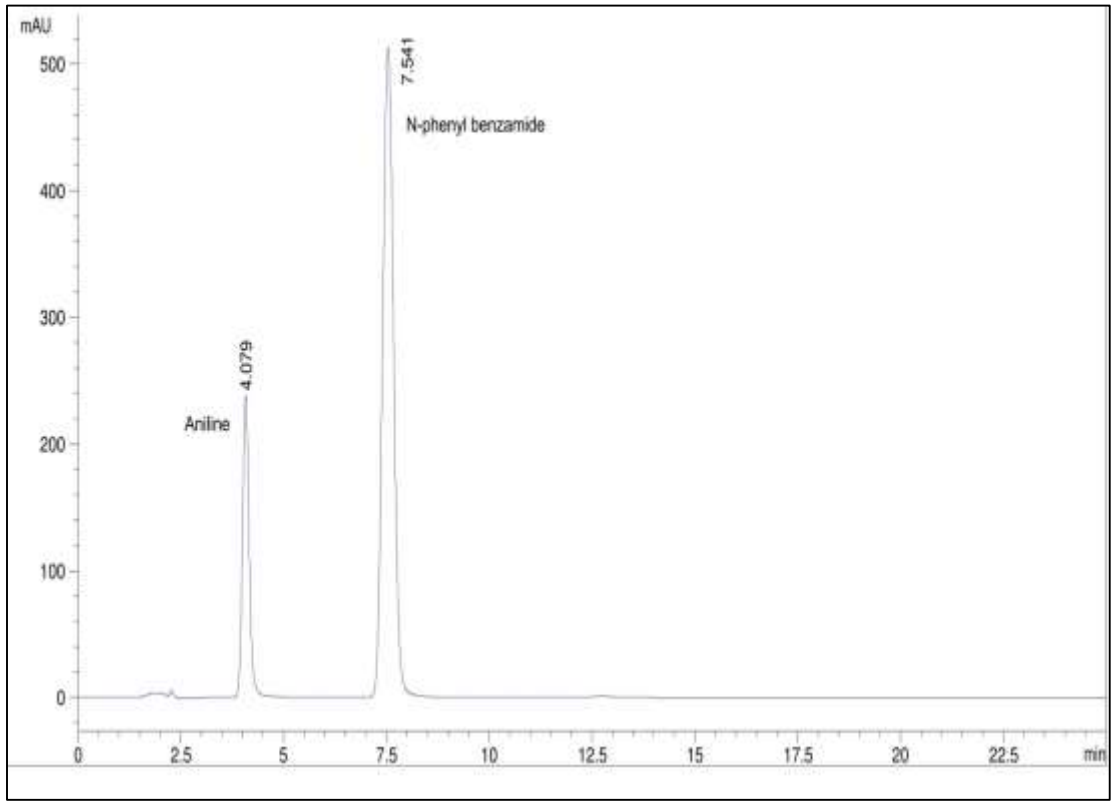
Method Optimization

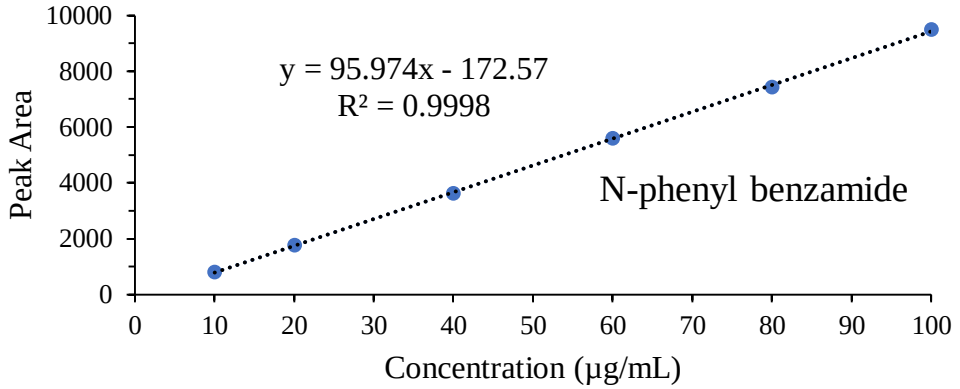
- ✓ To optimize the operating conditions for isocratic RP-HPLC detection of a number of parameters such as the column type, mobile phase composition, pH, effectors (phosphoric or acetic acid), flow rate, wavelength, and temperature were varied. A representative chromatogram is shown in Figure 1.

Table 1: Optimization of operating conditions

	Mobile Phase	Mobile Phase composition	pH	Effector	Flow rate	Temperature	Wavelength	Injection
For detection of N-PBA and aniline	ACN/H_2O	25/75, 40/60, 50/50	pH 3, pH 5	Phosphoric acid	1mL/min 0.7 mL/min	25 °C, 30 °C	254 nm, 210 nm	20 µL, 30 µL
	ACN/Acetate buffer	40/60, 50/50	pH 5	Acetic acid	1mL/min 0.7 mL/min	30 °C	254 nm, 210 nm	20 µL

Method Optimization





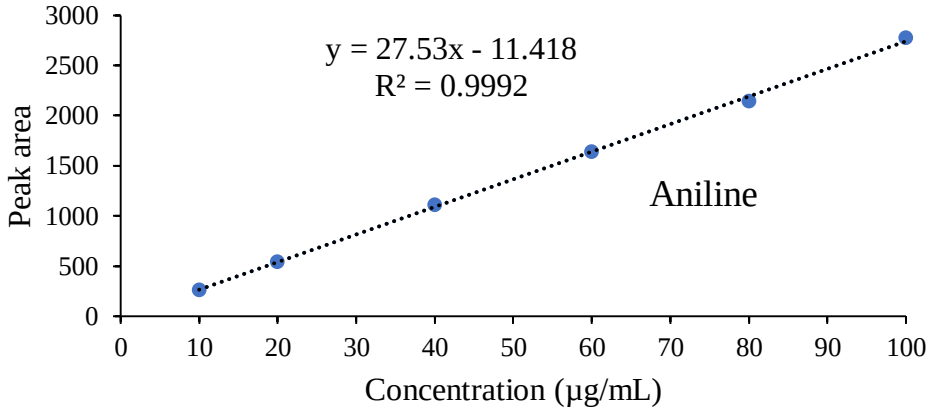


Figure 1. HPLC chromatograms and calibration curve of standard solutions of N-PBA and aniline

Validation of the HPLC method

1. System suitability test
2. Linearity (calibration curve)
3. Limits of detection (LOD) and quantification (LOQ)
4. Precision and accuracy

Validation of the HPLC method

1. System suitability test

RSD%/repeatability, retention time, peak area, tailing factor, theoretical plate/column efficiency, resolution, signal to noise ratio (S/N ratio) **……**

- ✓ Here, system suitability was assessed by six replicate analyses of the system suitability solution. The acceptance criterion was ±2% for the percentage relative standard deviation (%RSD).
- ✓ The retention time, peak area, tailing factor, and theoretical plate were also determined.

Table 2. System suitability study for the determination of N-PBA and aniline (n = 6)

	Retention time %RSD<2	Peak area %RSD<2	Tailing factor NMT 2/1.5	Theoretical Plate NLT 2000
N-PBA	7.567	9501.779	1.009	10447.770
RSD% (NMT 2)	0.19	0.13	0.47	1.36
Aniline	4.090	2745.002	1.010	8569.771
RSD% (NMT 2)	0.21	0.87	0.44	1.06

Validation of the HPLC method

2. Linearity (calibration curve)

Linearity was performed for each standard compound. Six different standard solutions 10–100 µg/mL for N-PBA and aniline were analyzed in triplicate for each concentration. Calibration curves were constructed by plotting peak areas against analyte concentrations. The linearity was assessed by calculating the slope, y-intercept and coefficient of determination (r^2) using least squares regression

Table 3: Linear regression analysis parameters for determination of N-PBA, aniline

Compound	Linearity range	R^2	Slope	Intercept	Recovery%	RSD%
N-PBA	10-100 µg/mL	0.9998	95.974	-172.57	100.01	0.23
Aniline	10-100 µg/mL	0.9992	27.530	-11.418	99.53	0.85

Validation of the HPLC method

3. Limits of detection (LOD) and quantification (LOQ)

The linear regression equation of the calibration curve was used to determine the LOD and LOQ. The limit of detection (LOD) and quantification (LOQ) were determined using the ICH formula: LOD=(3.3σ/s) and LOQ=(10σ/s), where s is the slope of the regression line and σ is the standard deviation of the intercept.

The LOD and LOQ with SD value were found 3.72±1.6 ppm and 11.28±1.2 ppm for N-PBA, and 6.85±1.7 ppm and 16.77±1.4 ppm for aniline, respectively.

Validation of the HPLC method

4. Precision and accuracy

Precision data on the intra- and inter-day variation for four different concentration levels are summarized in Table 4 and 5. Both intra- and inter-day RSD were less than 2%, indicating a sufficient precision. All percentage recoveries were within 99.25–101.25%, indicating the good accuracy of the method.

Table 4: Precision (% RSD) and accuracy (% recovery) for the determination of N-PBA

Given Conc. (µg/mL)	Found Conc. (µg/mL) Mean±SD	%RSD	%Recovery
10	10.0±0.16	1.59	99.89
20	20.1±0.02	0.09	100.28
60	59.9±0.04	0.07	99.86
100	100.7±0.13	0.13	100.70

Table 5: Precision (% RSD) and accuracy (% recovery) for the determination of aniline

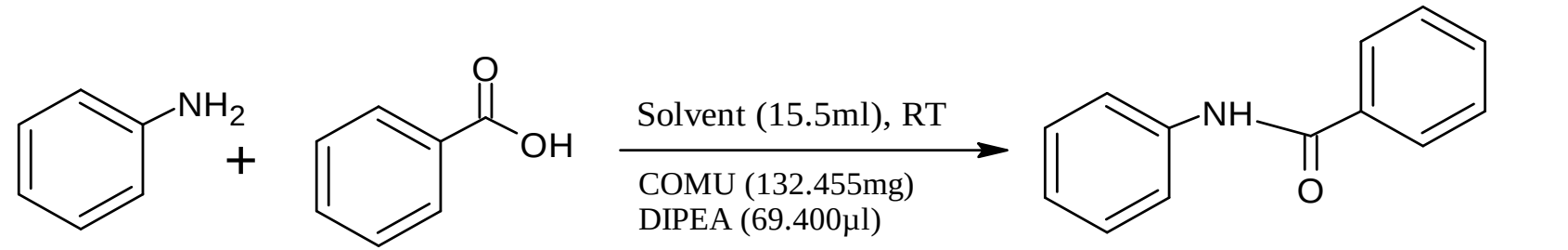
Given Conc. (µg/mL)	Found Conc. (µg/mL) Mean±SD	%RSD	%Recovery
10	10.2±0.11	1.12	102.26
20	19.7±0.19	0.95	98.70
60	60.2±0.24	0.40	100.27
100	100.0±0.72	0.72	100.04

Application of the HPLC method in synthesis

Synthesis procedure of N-phenyl benzamide (N-PBA)

At room temperature, benzoic acid (24.448 mg), a amide coupling reagent (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU, 132.455 mg)) and then a base N,N-Diisopropylethylamine (DIPEA, 69.400 µL) were added in the solvent mixture (1:1, 15.5 mL) respectively. Then, aniline (10.637 µL) was added in the mixture after 15 min proper stirring. Reactions were observed using the HPLC at 0 hour (approx. 1 min.), 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 24 h.

The procedure was applied to the synthesis of N-PBA with 8 solvent-pair mixtures in triplicate



Aniline (10.637µl)

Benzoic acid (24.448mg)

Benzanilide or. N-phenylbenzamide

Solvent= HBD-HBA solvent-pair
Water-THF
Water-DMSO
Water-Ace
Water-DMF
EtOH-DMF
EtOH-DMSO
Water-IPA
Water-EtOH

Application of the HPLC method

Aniline (RT = 4.04), N-PBA (RT = 7.6), Easter (RT=14.9)

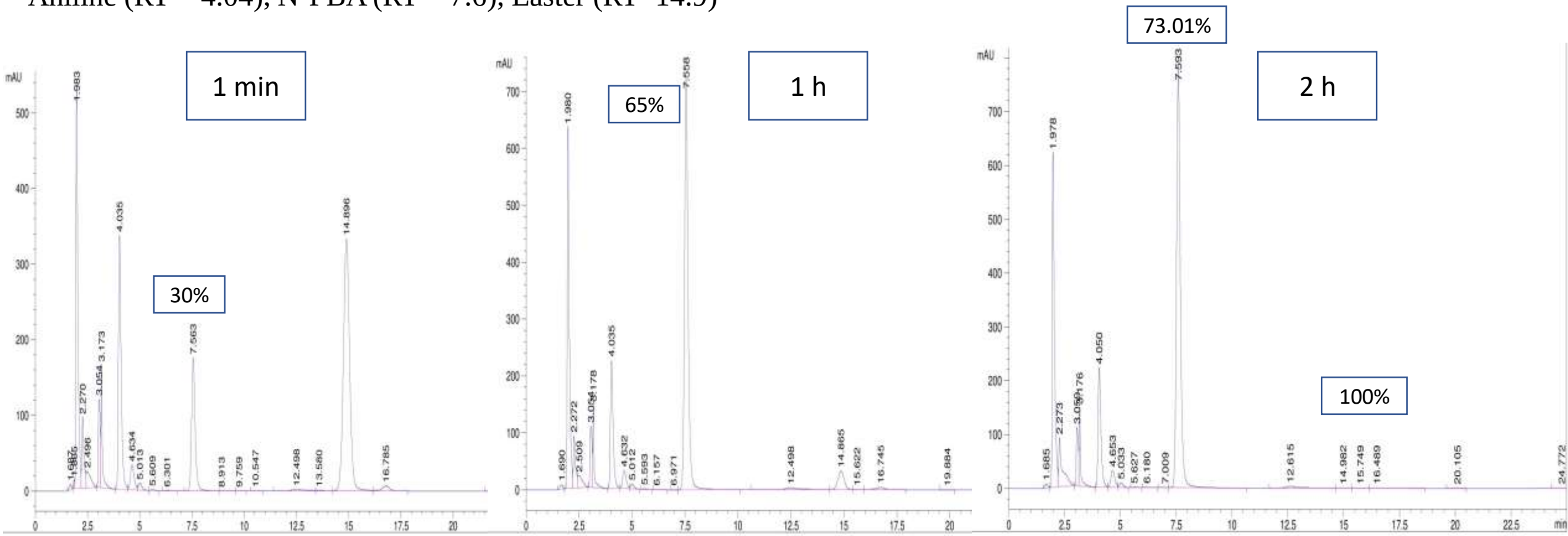
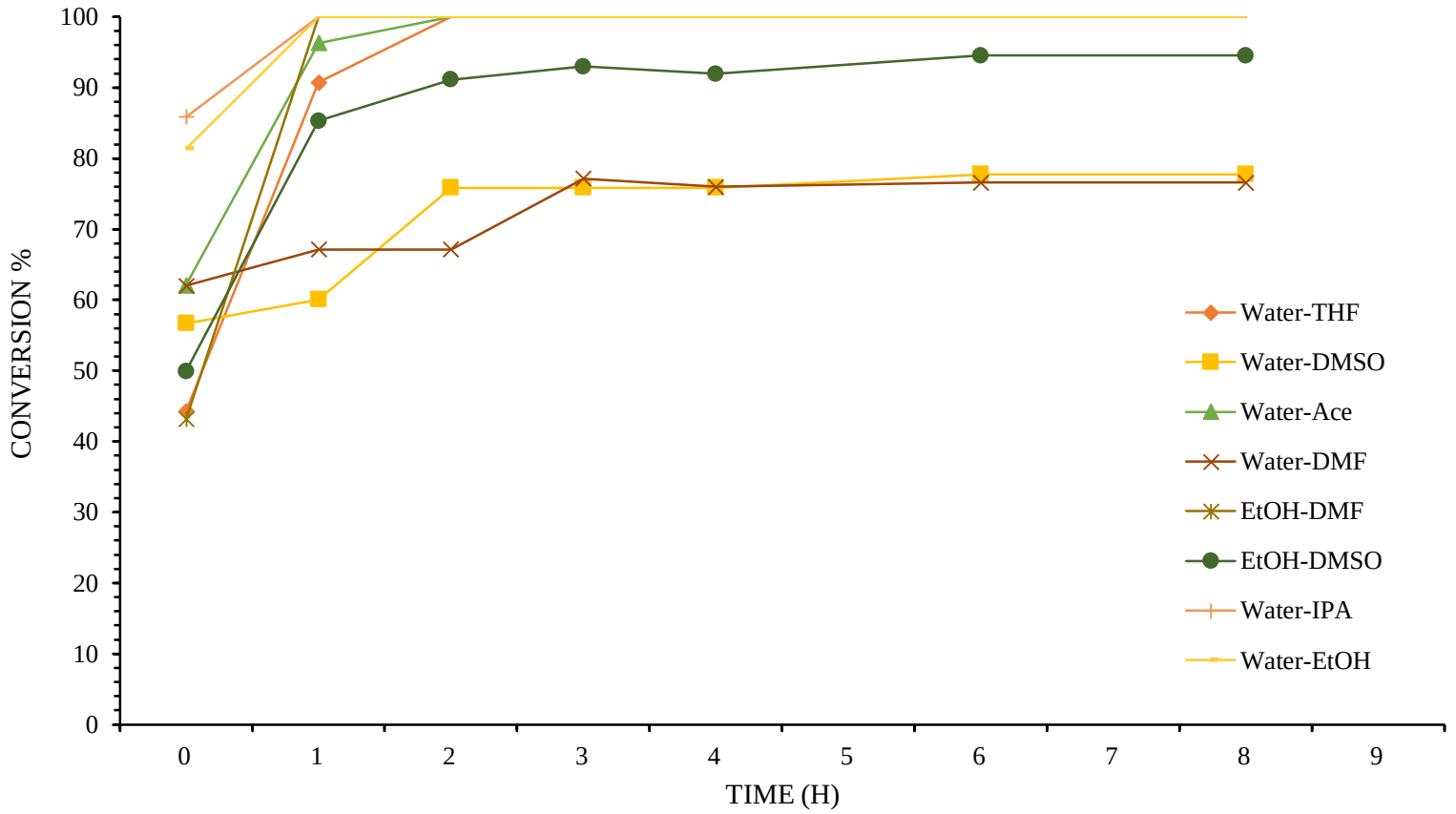


Figure 2. HPLC chromatograms of N-PBA synthesis using the H_2O-THF solvent mixture

Application of the HPLC method



Percent yield of N-PBA	
Solvent/Time	%Yield
Water-THF	73.01
Water-DMSO	58.08
Water-Ace	73.47
Water-DMF	43.41
EtOH-DMF	81.79
EtOH-DMSO	68.10
Water-IPA	66.28
Water-EtOH	47.71

Figure 3: Conversion rate of aniline in N-PBA synthesis using the solvent-pair mixtures