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Short communication

Separation of positional isomers of nitrobenzoic acid by reversed-phase liquid chromatography with 2-propanol–water–acetic acid as eluent

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Abstract

A method for the separation of *o*-, *m*- and *p*-nitrobenzoic acid was developed using reversed-phase liquid chromatography. A C_{18} bonded column (150 mm×4.6 mm I.D.) was used as stationary phase and 2-propanol–water–acetic acid (20:80:0.4, v/v/v, pH 2.99) at a flow-rate of 1.2 ml/min as eluent; the detection wavelength was 254 nm. The nitrobenzoic acid isomers are separated with $R_s \geq 1.5$, the detection limits (at 254 nm) of the *o*-, *m*- and *p*-isomers are 4, 7 and 5 μg/ml, respectively. This method is very simple and reproducible.

Keywords: Positional isomers; Pharmaceutical analysis; Nitrobenzoic acids

1. Introduction

p-Nitrobenzoic acid (NBA) is a very important intermediate in the preparation of pharmaceutical compounds. For example, *p*-NBA produces *p*-aminobenzoic acid through a reduction reaction, and *p*-aminobenzoic acid can be further esterified to obtain anasthesin. The purity of *p*-NBA is important with respect to the yield of the products. The content of *p*-NBA must be above 99.5% in intermediate products. Unfortunately, *o*-NBA and *m*-NBA are always present in *p*-NBA as impurities (about 0.5%). An effective analytical method is therefore needed for separating the NBA isomers and determining the quality of *p*-NBA. Analytical methods such as paper chromatography, thin-layer chromatography and column [with β-$Ni(NCS)_2(4\text{-}MePy)_4$] liquid chromatography [1,2] have been used for NBA isomer separation. The disadvantages of the reported HPLC methods were primarily the requirements of either using a special column such as a β-cyclodextrin bonded phase column [3], or a rather complicated eluent such as one modified with α-cyclodextrin [4].

In this work, a new method is reported for the separation of *o*-, *m*- and *p*-NBA isomers by reversed-phase HPLC with 2-propanol–water–acetic acid as eluent and a C_{18} bonded stationary phase. It is also noted that the method is advantageous by its easy application and good reproducibility.

2. Experimental

2.1. Apparatus

The chromatograph used in this work is a Varian

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5060 with a gradient elution system. The system was equipped with a single-piston reciprocating pump, a microprocessor controlled proportioning valves, a manual loop sample injector, a low pressure solvent mixing chamber, a variable temperature control of maximum 140°C and a Model 100 UV–Vis variable-wavelength absorbency detector (190~750 nm) with a deuterium lamp. System control and data handling were carried out by a Model 401 computer.

2.2. Column

A 5 μm Micropak C_{18} column (150 mm×4.6 mm I.D.) was used to separate the three isomers in this work.

2.3. Chemicals

2-Propanol, acetic acid, *o*-, *m*- and *p*-NBA isomers (all analytical reagent) from Beijing Chemical Plant (Beijing, China) and methanol (HPLC grade) from Siyou Chemical Plant (Tianjin, China) were used for the study.

2.4. Detection wavelength

The UV spectrum of *o*-, *m*- and *p*-NBA in ethanol solution was determined with ethanol as reference on a Shimadzu Model UV-2100S spectrophotometer. In this work, 254 nm was selected as detection wavelength for the three isomers.

3. Results and discussion

3.1. Selection of eluent

The most common eluents used in reversed-phase HPLC are methanol–water and acetonitrile–water systems [5]. When various ratios of these two eluents were used to separate *o*-, *m*- and *p*-NBA, only a single peak was observed. This was mainly because NBA as an organic acid produced anionic ions when water was present. The small differences in the pK_a values made the three isomers elute at almost the same time, especially *m*- and *p*-NBA. The separation of *o*-, *m*- and *p*-NBA was significantly improved when acetic acid was added to the methanol–water eluent.

The shortcoming of this methanol–water system containing acetic acid was a longer analysis time. Retention times were 4.18, 27.7, 32.2 min for *o*-, *m*- and *p*-NBA, respectively (methanol–water–acetic acid, 20:80:0.4, v/v/v). In order to guarantee an effective separation and obtain appropriate retention times, the polarity of eluent must be adjusted. Weak polar solvents such as ethanol and 2-propanol have proved themselves useful in this respect. A better eluent composition was obtained with 2-propanol instead of methanol in the above eluent. The retention times for the three isomers were shortened considerably.

3.2. Effect of acetic acid

The influence of acetic acid on retention and selectivity of separation was investigated. Experiments were conducted with 2-propanol–water–acetic acid ratios of 20:80:0.4, 20:80:0.8, 20:80:1.2 and 20:80:1.6 (v/v/v). The relation of pH to amount of acetic acid and chromatography parameters for the three isomers are shown in Table 1.

As shown in Table 1, k' changed with the amount of acetic acid in the eluent. The change in k' is only small when the 2-propanol–water–acetic acid ratio changes from 20:80:0.4 to 20:80:1.2 (v/v/v), while there is a significant difference with the ratio 20:80:1.6 (v/v/v). The best range for the pH value was between 2.75 and 2.99.

When the 2-propanol–water–acetic acid ratio was 20:80:0.4 the retention times of *o*-, *m*- and *p*-NBA were 1.45, 8.85 and 9.91 min, respectively and the resolutions of *o*- and *m*-NBA and *m*- and *p*-NBA are 9.67 and 1.52, respectively. The chromatograms of the standard sample and a real *p*-NBA product are

Table 1
The effects of acetic acid on the k' of NBA

2-Propanol–water–acetic acid (v/v/v)	pH	k' of NBA		
		o-	*m*-	*p*-
20:80:0.4	2.99	1.18	6.67	7.79
20:80:0.8	2.84	1.20	6.80	7.89
20:80:1.2	2.75	1.19	6.65	7.64
20:80:1.6	2.68	1.12	5.66	6.32

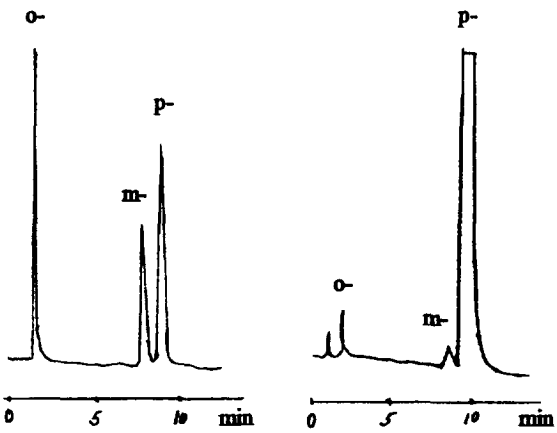

Fig. 1. Chromatograms for (left) standard mixture of *o*-, *m*- and *p*-nitrobenzoic acid and (right) real sample. Chromatographic conditions: C_{18} column, 2-propanol–water–acetic acid (20:80:0.4, v/v/v), pH 2.99, flow-rate 1.2 ml/min, injection amount: 10 μl, detection wavelength 254 nm.

shown in Fig. 1. As a matter of fact the maximum toleration of the *o*- and *m*-isomers was below 0.5%. The relative levels of *o*-, *m*- and *p*-NBA were 0.05%, 0.1% and 99.85%, respectively.

3.3. Method evaluation

Seven different concentrations of the standard NBA isomers in ethanol solution were prepared, in the range 31~1100 μg/ml. The experiments were conducted under the above chromatographic conditions. The three regression equations obtained were, $y=2790.65x-16523.04$, $y=3992.00x-13176.12$ and $y=6642.48x-97661.60$ for *o*-, *m*- and *p*-NBA, respectively (y=peak area, x=concentration, $n=6$).

The accuracy of the method was measured by the recovery rate which was obtained by comparing the experimental results to the calculated theoretical concentrations. The precision of the method was determined by relative standard deviation (R.S.D.) which was calculated statistically based on a group of reproducible experiments with certain concentrations. The detection limit (C) was calculated using $C=3S/b$ [6], where S was the standard deviation of the detected value by the instrument for the sample with lower concentration and b was the slope of regression line. The results are listed in Table 2.

4. Conclusion

In this paper, a method for the separation of *o*-, *m*- and *p*-NBA isomers using reversed-phase HPLC was established. The eluent was 2-propanol–water–acetic acid and the stationary phase was a C_{18} bonded phase. The separation was excellent, especially for *m*- and *p*-NBA isomers. The method was simple and highly reproducibile. The separation method has been used to check *p*-NBA products for quality in many chemical plants successfully.

References

[1] W. Kemula, D. Sybilska, J. Lipkowski, K. Duszczyk, J. Chromatogr. 204 (1981) 23.
[2] W. Kemula, D. Sybilska, J. Lipkowski, J. Chromatogr. 218 (1981) 465.
[3] C.A. Chang, Q. Wu, L. Tan, J. Chromatogr. 361 (1986) 199.
[4] D. Sybilska, J. Lipkowski, J. Woycikowski, J. Chromatogr. 253 (1982) 95.
[5] A.M. Krstulovic and P.R. Brown, Reversed-Phase High-Performance Liquid Chromatography, Wiley, New York, 1982.
[6] D. Bo, Fen Xi Ce Shi Shu Ju De Tong Ji Chu Li Fang Fa, Tsinghua University, Beijing, 1995.

Table 2
Method evaluation

NBA	Recovery (%)[a]	R.S.D. (%)[b]	Detection limit (μg/ml)[a]
o-	99.9	3.38	4
m-	100.0	0.56	7
p-	99.2	0.88	5

[a] $n=5$.
[b] $n=8$.