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Short communication

High-performance liquid chromatographic determination of denatonium benzoate in ethanol with 5% polyvinylpyrrolidone

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Abstract

An analytical method for the determination of denatonium benzoate in ethanol with 5% polyvinylpyrrolidone has been developed using reversed-phase ion-pair high-performance liquid chromatography with ultraviolet (210 nm) detection. The procedure is linear and accurate over the range 1.0–20.0 ppm with a limit of detection of 0.25 ppm (at a signal-to-noise ratio of 3).

1. Introduction

A bitter tasting substance can be added to a product to avoid unwanted ingestion or as a denaturant. Denatonium benzoate (Fig. 1), marketed as Bitrex, is commonly used for this purpose. Bitrex is used in ethanol as a denaturant with typical concentrations in the range 2–10 ppm. The determination of denatonium benzoate has been the subject of several publications. Techniques utilized include colorimetric assay [1,2], thin-layer chromatography (TLC) [3], high-performance liquid chromatography (HPLC) [4,5], and ion-selective potentiometry [6].

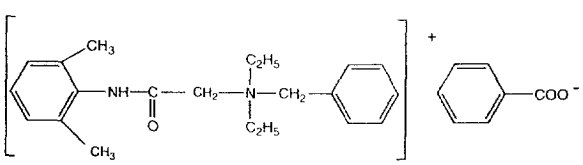

Fig. 1. Structure of denatonium benzoate (Bitrex).

HPLC procedures are especially useful because they provide the required separation to achieve good sensitivity and selectivity for the determination of low-level analytes. This was found to be the case for Bitrex in ethanol with 5% polyvinylpyrrolidone (PVP). Both previously reported HPLC methods were found to be inapplicable due to the lack of resolution between PVP and Bitrex. Therefore, a new HPLC method was required to determine Bitrex at low ppm levels. This paper describes the use of reversed-phase ion-pair chromatography to achieve this goal along with the required validation data to demonstrate the accuracy, precision, and linearity of the method.

2. Experimental

2.1. Instrumentation

The liquid chromatograph consisted of a Shimadzu (Kyoto, Japan) LC-600 pump, a

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Shimadzu SIL-6B injector, a SYS-TEC (Minneapolis, MN, USA) column heater, a Shimadzu SPD-6AV Detector, and a Shimadzu CR501 Integrator. The photodiode-array detector was a Hewlett-Packard (Waldbronn, Germany) Model HP 1040M. The chromatographic column was a Waters (Milford, MA, USA) Symmetry C_{18}, 100 Å, 5 μm, 150 × 3.9 mm I.D.

2.2. Reagents and drugs

HPLC-grade acetonitrile and water were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Electrophoresis-grade sodium dodecyl sulfate, reagent-grade sodium phosphate monobasic, and 2 *M* hydrochloric acid were also obtained from Fisher Scientific. Ethyl alcohol U.S.P., 190 proof, was obtained from Quantum Chemical (Tuscola, IL, USA). The denatonium benzoate was obtained from Macfarlan Smith (Edinburgh, UK). Reagent-grade benzoic acid was obtained from Aldrich (Milwaukee, WI, USA). Denatonium saccharide (min. 95%) was obtained from Sigma (St. Louis, MO, USA). Polyvinylpyrrolidone (Kollidon 30) was obtained from BASF (Mount Olive, NJ, USA).

2.3. Procedure for HPLC

A stock solution of 0.1 *M* phosphate buffer was prepared by dissolving 13.8 g sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) in 900 ml HPLC-grade water. The pH was adjusted to 3.0 using 2 *M* HCl and the solution was diluted to 1000 ml with HPLC-grade water. The 0.01 *M* phosphate buffer solution was prepared by diluting 100 ml of the 0.1 *M* stock solution to 1000 ml with HPLC-grade water. The mobile phase consisted of acetonitrile–0.01 *M* phosphate buffer pH 3 (50:50, v/v) containing 25 m*M* sodium dodecyl sulfate pumped at 1.2 ml/min. The column was thermostated at 35°C and UV detection at 210 nm was used (0.08 AUFS). The injection volume was 20 μl.

2.4. Standard preparation

Accurately weigh 100 mg of denatonium benzoate into a 100-ml volumetric flask. Add about 50 ml ethanol and sonicate until dissolved. Dilute to volume with ethanol and shake well. Pipet 10.0 ml of this solution into a 100-ml volumetric flask and dilute to volume with ethanol. Pipet 10.0 ml of this solution into a 100-ml volumetric flask and dilute to volume with ethanol. Pipet 10.0 ml of this solution into a 100-ml volumetric flask and dilute to volume with acetonitrile–0.01 *M* phosphate buffer pH 3 (50:50). This is the working standard solution (concentration, 1 μg/ml). Filter this solution through a 0.45-μm acrodisc filter and inject.

2.5. Preparation of samples

The sample solutions should be diluted with acetonitrile–0.01 *M* phosphate buffer pH 3 (50:50) to give a desired final concentration of about 1 μg/ml denatonium benzoate. For a 10-ppm Bitrex sample, pipet 1.0 ml of the ethanol solution containing 5% PVP into a 10-ml volumetric flask. Dilute to volume with acetonitrile–0.01 *M* phosphate buffer pH 3 (50:50) and shake well (concentration, 1 μg/ml). Filter this solution through a 0.45-μm acrodisc filter and inject.

3. Results and discussion

During pre-formulation studies, Bitrex (denatonium benzoate) was being evaluated as a deterrent for ingestion of a formulation consisting mainly of ethanol and polyvinylpyrrolidone (5%). Low ppm levels were being tested. Therefore, a sensitive analytical method was needed to determine Bitrex in this solution. Attempts to determine Bitrex were first made using a published HPLC method [4,5]. Results were found to be unsatisfactory due mainly to the presence of PVP, which elutes as a very broad peak overlapping with the Bitrex peak. Using these two methods, the capacity factor for Bitrex was 2.5 and 2.3, respectively, and resolution from PVP was not possible. Thus, further method development was required to obtain the needed separation.

3.1. Physico-chemical characteristics of Bitrex

To optimize the separation and sensitivity for Bitrex, the physico-chemical characteristics of this compound were examined. Because Bitrex is a quaternary ammonium (denatonium) salt of benzoic acid, its hydrophilic nature will not change as a function of pH. However, silanophilic interactions with the bonded stationary phase of the reversed-phase column would be a problem at elevated pH. Neutral-pH mobile phases were found to be inadequate, with a long retention time of denatonium, unless the organic content was increased significantly. However, high organic content was found to give a very broad peak shape for PVP, preventing adequate selectivity to be achieved for the determination of Bitrex. The effect of increasing the ionic strength by using a higher concentration of phosphate buffer was also found to be unsatisfactory. At low pH, denatonium was unretained ($k' = 0.77$) by the reversed-phase column when using a mobile phase of acetonitrile–0.01 *M* phosphate buffer pH 3 (50:50). This would be expected since residual silanols are protonated at low pH, significantly reducing ionic interactions. Although the denatonium molecule contains hydrophobic groups, the positively charged quaternary amine causes low retention under reversed-phase conditions. Consequently, ion-pair chromatography was evaluated with a low-pH mobile phase to increase the retention of denatonium while achieving separation from PVP.

3.2. Selection of an ion-pair reagent

The mechanism of retention in ion-pair chromatography can be described by two models [7]. In the ion-pair model, a complex is formed between the analyte and the ion-pair reagent, and this complex interacts with the stationary phase in the column. In the dynamic ion-exchange model, the ion-pair reagent coats the stationary phase in the column, resulting in a charged layer that the analyte will interact with. The mechanism of separation can be a combination of these models. These models predict that the capacity factor of the analyte will increase with higher ion-pair reagent concentration to a limiting value.

Short hydrocarbon chain ion-pairing reagents were first evaluated for use in the mobile phase. With octane sulfonic acid sodium salt for example, denatonium retention was found to be too short and resolution from PVP was not adequate. The relatively high organic content of the mobile phase was needed to prevent the PVP peak from becoming excessively broad. As the organic content of the mobile phase increases, an ion-pair reagent with a longer hydrocarbon chain is needed to achieve an optimal separation. With this mobile phase, sodium dodecyl sulfate (SDS) would be expected to provide the most selectivity based on a compilation of adsorption isotherm and retention data [8]. SDS consists of a very long hydrocarbon chain that provides improved hydrophobic character, thereby increasing concentration of the ion-pair reagent at the surface of the stationary phase.

Experiments were performed to determine the most desirable concentration of the ion-pair reagent. The mobile phase of acetonitrile–0.01 *M* phosphate buffer pH 3 (50:50) was prepared with various concentrations of sodium dodecyl sulfate. The results are presented in Fig. 2. With the Symmetry column, the capacity factor was found to increase with ion-pair reagent concentration, reaching a plateau at about 25 m*M*. Theoretical treatment of ion-pair HPLC predicts that selectivity will approach a maximum as the

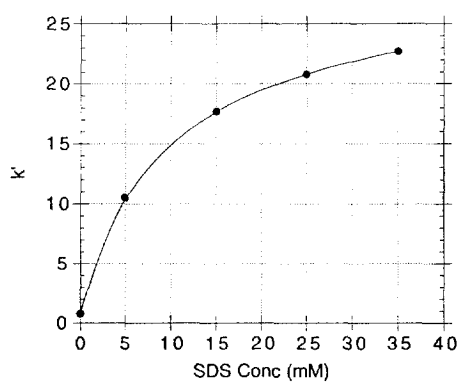

Fig. 2. Capacity factor of Bitrex versus concentration of sodium dodecyl sulfate in the mobile phase.

surface concentration of the ion-pair reagent increases. The curve in Fig. 2 is in good agreement with this prediction [7,8]. The mobile phase was buffered at low pH (3) to protonate residual silanol groups in the stationary phase and to ensure dissociation of denatonium benzoate (benzoic acid $pK_a = 4.19$). The molarity of the phosphate buffer was found to have no effect on the retention of Bitrex when changed from 0.01 to 0.05 *M*.

3.3. Optimization of the separation

Following the selection of a low-pH mobile phase and ion-pair chromatography to achieve the required separation for the determination of Bitrex, separation of the two ionic species of denatonium benzoate had to be demonstrated. Because a low-pH mobile phase would protonate the benzoate anion to give benzoic acid with increased retention by reversed-phase, two separate solutions were prepared. One solution consisted of benzoic acid and the other was a solution of denatonium saccharide, which is a salt of saccharin in lieu of benzoic acid. HPLC photodiode-array spectra along with the chromatogram of denatonium benzoate are shown in Fig. 3. Clearly, both ionic species were found to be well separated using a mobile phase consisting of acetonitrile–0.01 *M* phosphate buffer, pH 3 (50:50) containing 25 m*M* sodium dodecyl sulfate. Benzoic acid was found to elute at 1.4 min and denatonium eluted at 15.2 min. Since denatonium is responsible for bitter taste, there was no need to quantitate benzoic acid; however, the lack of interference was clearly demonstrated. Detection at 210 nm was preferred to other wavelengths to achieve adequate sensitivity for the determination of denatonium at low ppm levels.

The solution of denatonium saccharide was analyzed and compared with a solution of denatonium benzoate. When corrected for the difference in molecular mass, the denatonium peak had an equivalent response in both cases, thus demonstrating complete dissociation of the analyte under the HPLC conditions. Typical chromatograms of a Bitrex standard and an ethanol with 5% PVP sample containing 10.0 ppm Bitrex are shown in Fig. 4. Baseline separation of PVP and denatonium is illustrated.

3.4. Analytical method validation

Validation of the method was conducted by spiking known amounts of Bitrex into ethanol with 5% PVP. Solutions were prepared containing 1, 5, 10, and 20 ppm Bitrex. Results of replicate analyses and recovery are presented in Table 1. Recovery of Bitrex ranged from 95 to

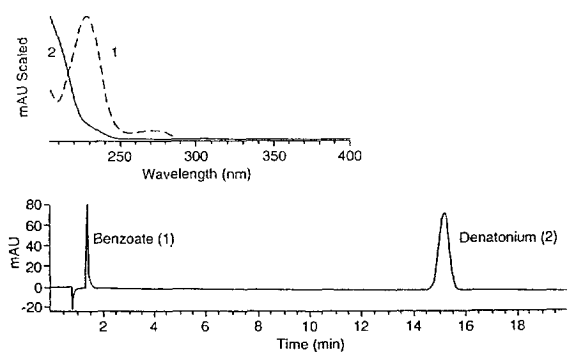

Fig. 3. Diode-array spectra (top) and chromatogram (bottom) of denatonium benzoate (1.0 mg/ml).

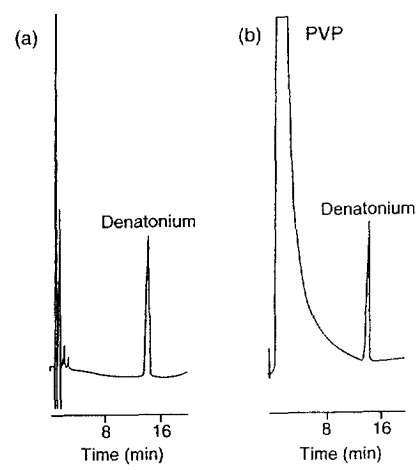

Fig. 4. Chromatogram of (a) Bitrex standard (1 μg/ml) and (b) 10.0 ppm Bitrex in ethanol with 5% PVP.

Table 1
Linearity and recovery results for Bitrex in PVP–ethanol solution

Bitrex added (ppm)	Bitrex found (ppm)	Recovery (%)	Mean recovery (%)	R.S.D. recovery (%)
1.03	1.03	99.9	96.7	2.85
1.03	0.980	94.8		($n = 3$)
1.03	0.987	95.5		
5.17	5.06	97.8	98.6	0.72
5.17	5.11	98.9		($n = 3$)
5.17	5.13	99.2		
10.3	10.3	99.7	99.1	0.88
10.3	10.3	99.5		($n = 5$)
10.3	10.2	98.9		
10.3	10.3	99.8		
10.3	10.1	97.7		
20.7	20.5	99.1	99.3	0.19
20.7	20.6	99.4		($n = 3$)
20.7	20.6	99.4		

Mean R.S.D. recovery	1.16
Slope (standard error)	4201 (±9)
Intercept (standard error)	−164 (±103)
Correlation coefficient	0.9999
Number of data points	14
Linear range studied (ppm)	1–20

100%, thus demonstrating that the method is quantitative. The standard deviation of the results is larger at the lower concentration, as expected. However, the R.S.D. of less than 3% for 1.0 ppm is acceptable at that level. At the target concentration of 10 ppm, the recovery (99.1%) and the precision (0.88%) demonstrate the reliability of the method. Linearity of the method is demonstrated by the correlation coefficient of 0.9999 ($n = 14$), based on the linear regression of response versus concentration of Bitrex from the accuracy data. The intercept is only 0.4% of the response of 10 ppm Bitrex, further supporting the linearity of the method.

Lack of interference was demonstrated by assaying ethanol with 5% PVP. There were no peaks present that would interfere with the quantitation of Bitrex. Based on a signal-to-noise ratio (S/N) of 3, the limit of detection for this method is 0.25 ppm. In ethanol solution without PVP present, where injection without dilution is possible, the limit of detection is 0.025 ppm ($S/N = 3$). The limit of quantitation for this method is 1.0 ppm ($S/N = 12$).

4. Conclusions

A rapid and sensitive analytical procedure for Bitrex in ethanol with 5% PVP was developed. The separation offers improved resolution compared to previously reported methods [4,5]. Also with this method, the retention of Bitrex can be readily optimized to achieve resolution from various components of a formulation by adjusting the concentration of the ion-pair reagent in the mobile phase. The sensitivity is appropriate for most applications in which Bitrex is used as a denaturant.

References

[1] National Formulary XVII, American Pharmaceutical Association, Washington, DC, 17th ed., 1990, p. 1924.
[2] K. Helrich (Editor), Official Methods of Analysis of the Association of Official Analytical Chemists, Association of Official Analytical Chemists, Arlington, 15th ed., 1990, pp. 1152–1154.
[3] M.J. Glover and A.J. Blake, Analyst, 97 (1972) 891.
[4] K. Sugden, T.G. Mayne and C.R. Loscombe, Analyst, 103 (1978) 653.
[5] C.E. Damon and B.C. Pettitt, Jr., J. Chromatogr., 195 (1980) 243.
[6] O.G.B. Nambiar, K. Gosavi and T. Ravindranathan, Analyst, 116 (1991) 1011.
[7] M.T.W. Hearn (Editor), Ion-Pair Chromatography, Marcel Dekker, New York, 1985, Ch. 2.
[8] A. Bartha, G. Vigh and Z. Varga-Puchony, J. Chromatogr., 499 (1990) 423.