Drug Development and Industrial Pharmacy, 25(8), 929–935 (1999)

RESEARCH PAPER

Validation of an Enzymatic Method for the Determination of Trace Levels of Ethanol

R. V. Alvencar,[1] S. Lam,[1] M. Sidhom,[2] and T. U. Okarter[2,*]

[1]*Barr Laboratories Incorporated, Pomona, NY*
[2]*Division of Pharmaceutics and Industrial Pharmacy, Arnold and Marie Schwartz College of Pharmacy, Long Island University, 75 DeKalb Avenue, Brooklyn, NY 11201*

ABSTRACT

The validation of an enzymatic method for the determination of trace levels of ethanol is described. The method, which involves the use of alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD$^+$), was validated according to USP 23 requirements. The validation parameters, including linearity and range, accuracy (recovery), precision, ruggedness, limit of detection (LOD), and limit of quantitation (LOQ), were established satisfactorily. The method is specific and selective for ethanol except in the presence of propyl, isopropyl, or butyl alcohol.

INTRODUCTION

Ethanol is used in pharmaceutical products as a solvent or cosolvent, a preservative, a vehicle, and a granulating agent and for other purposes. A quick and accurate method of quantifying the ethanol level would be needed if there were a specification, including an in-process limit, for the ethanol content of the product or in-process material.

Ethanol residue in pharmaceutical preparations can be determined rapidly and conveniently by enzymatic reactions involving alcohol dehydrogenase (ADH) (1–4). The equation for this reaction is as follows:

$$CH_3CH_2OH + NAD^+ \overset{ADH}{\rightleftharpoons} CH_3CHO + NADH + H^+$$

In solution, ADH catalyses the oxidation of ethanol to acetaldehyde with the simultaneous reduction of nico-

* To whom correspondence should be addressed.

tinamide adenine dinucleotide (NAD^+) to NADH. The amount of NADH formed is proportional to the ethanol concentration in the sample and is detected spectrophotometrically at 340 nm (1) or by fluorimetry (5), electrochemical detection (5), or bioluminescence (6). The reaction is driven almost completely to the right by the use of excess NAD^+ and ADH and by using an aldehyde-trapping agent such as semicarbazide or tris(hydroxymethyl) aminomethane in alkaline conditions (1).

The enzymatic method described here offers a quick and reliable means of determining trace levels of ethanol in finished and in-process pharmaceuticals and may be used as an alternative to gas chromatography and capillary electrophoresis, two methods usually used for the analysis of ethanol.

The U.S. Pharmacopeia (USP) (7) and the International Conference on Harmonization (ICH) (8) have defined the parameters and the essential data elements needed for a satisfactory method validation. Various authors have described the concept, procedure, and requirements for the validation of chromatographic and nonchromatographic methods in the pharmaceutical and biotechnology industries (9–15).

The purpose of this study was to validate the enzymatic method of ethanol determination according to the USP requirements by demonstrating selectivity and specificity, linearity and range, accuracy and recovery, precision, ruggedness, limit of quantitation (LOQ), limit of detection (LOD), and robustness.

EXPERIMENTAL

Materials

The equipment used included a Hewlett Packard HP-8450A ultraviolet/visible (UV/Vis) diode array spectrophotometer (Hewlett Packard, Wilmington, DE) and an incubator, model BT-23 (American Scientific Products, Princeton, NJ).

The Quantitative Enzymatic Determination Test kit by Sigma Diagnostics (Sigma Chemical Company, St. Louis, MO) was used; it includes NAD^+-ADH vials, glycine buffer reagent, and ethanol standards (%w/v 0.025%, 0.050%, 0.100%, and 0.150%).

Method

Preparation of Standard Vials

The ''standard'' vials were prepared by adding 3.0 ml glycine buffer and 25 μl of ethanol to the NAD-ADH vials. Each vial was stoppered, gently vortexed to mix, and incubated for 10 min at 37°C. The UV absorbance of each solution was determined at 340 nm using the blank as the reference.

The results from the standard solutions were used to plot a calibration curve. The alcohol concentrations for the calibration curves were usually from 0.0125% to 0.2%. As part of the system suitability, the calibration was repeated each time that a test was conducted.

Preparation of Sample Vials

The ''sample'' vials were prepared by adding 3.0 ml glycine buffer and 25 μl of sample solution to the NAD-ADH vials. Each vial was stoppered, gently vortexed to mix, and incubated for 10 min at 37°C. The UV absorbance of each solution was determined at 340 nm using the blank as the reference.

Preparation of Blank Vials

The ''blank'' vials were prepared by adding 3.0 ml glycine buffer and 25 μl deionized water to the NAD-ADH vials. Each vial was stoppered, gently vortexed to mix, and incubated for 10 min at 37°C. The blank solutions were used as reference for the determination of the UV absorbance of the standard and sample solutions.

Preparation of Powder Sample

A powder sample was prepared by blending 402.8 g of lactose with 48.6 g each of microcrystalline cellulose and cornstarch.

Preparation of Sample Solution from Powder Samples

To prepare the sample solution from powder samples, 1 g of the powder blend was first spiked with a known amount of ethanol. The 1-g sample was then extracted with 3.0 ml of deionized water, and the extract was transferred to a centrifuge tube. The centrifuge tube was stoppered, vortexed to mix, and centrifuged at 2500 rpm for 5 min to obtain a clear supernatant. The clear supernatant, 25 μl, was used to prepare the sample vials.

Validation of the Method

The following parameters were validated in accordance with the USP 23 requirements (7):

Selectivity (Specificity)

To determine interference from the powder sample, unspiked 1-g and 2-g powder samples were extracted as

described above for sample solutions. The resultant solutions were tested, as previously described, for UV absorption at 340 nm.

To determine any interference from the glycine buffer and the NAD-ADH reagent, blank solutions were prepared as described above, some having the usual amount of buffer, and others containing an additional 25 μl of the glycine buffer. The UV absorptions of the solutions were determined at 340 nm.

To establish the absence of UV absorbance at 340 nm in the absence of the NAD-ADH reagent, sample solutions were prepared from previously spiked 1-g and 2-g powder samples containing 0.1% and 0.2% of ethanol, respectively. Following the method described above, some samples were tested as described previously, and others were tested without NAD-ADH reagent.

An interference study with other alcohols was performed. The individual alcohol, 25 μl, corresponding to 0.1% alcohol, was added to the sample vial already containing the glycine buffer. The sample was then tested as previously described to determine the absorbance at 340 nm. Using linear regression, the alcohol level corresponding to that absorbance was determined, and the percentage bias in the ethanol content, were this alcohol present together with ethanol, was calculated.

Linearity and Range

Five standard ethanol solutions of concentrations 0.0125%, 0.025%, 0.05%, 0.10%, and 0.20% were prepared and analyzed, in triplicate, as described above. A linear regression analysis was performed using the five coordinates.

Accuracy (Recovery)

The tests were run at two ranges of ethanol content: (*a*) 0.0125–0.1499% and (*b*) 2.08–26.05%. At each ethanol level, duplicate powder samples were spiked with ethanol, and sample solutions were prepared as described previously. The UV absorbance of each recovered solution was tested in duplicate. The recovery at each level, the mean of all the levels, and the relative standard deviations (RSDs) were determined.

For set 1, powder samples were spiked with ethanol at concentrations of 0.0125%, 0.025%, 0.050%, 0.0999%, and 0.1499%. The corresponding sample solutions were prepared and tested as described previously. The mean recovery was determined for each sample.

For set 2, powder samples were spiked with alcohol at concentrations of 2.08%, 6.25%, 9.38%, 20.84%, and 26.05% ethanol. The sample solutions were prepared and tested as described above. The mean recovery was determined.

Precision

Six replicate samples from the same bulk powder sample were tested. Each powder sample was spiked with ethanol and tested as described above. The mean and the RSD of the six replicate assays were determined.

Ruggedness

The six powder samples used in the precision study were analyzed by a second analyst using a different instrument and on a different day. The samples were analyzed as described above. The mean and the RSD of these assays were determined and were compared with the results from the precision study.

Limit of Quantitation

Nine standard ethanol solutions ranging from 0.0031% to 0.25% were prepared and tested as previously described. Their UV absorbances were plotted against the corresponding concentrations. The linear LOQ was established based on the lowest concentration in the linearity range and in the accuracy (recovery) studies.

Limit of Detection

For the LOD, 17 standard ethanol solutions, ranging from 0.0000023% to 0.15%, were tested. The last positive absorbance before a negative absorbance was observed was used to establish the LOD.

Stability of the Enzymatic Reaction

Standard ethanol solutions containing 0.025%, 0.05%, 0.10%, and 0.15% of ethanol were tested as described above, by incubating for 10 min. The UV absorbance of each solution was determined in duplicate. The solutions were let stand for an additional 56 min, for a total period of 66 minutes. Samples were taken and analyzed, in duplicate, at 26, 46, and 66 min from the start of incubation.

Effect of Temperature on the Enzymatic Reaction

Standard solutions of 0.05% and 0.10% ethanol were incubated at 22°C and at 40°C for 10 min. The percentage recoveries were compared with the calibration standards, which were incubated at 37°C for 10 min.

RESULTS AND DISCUSSION

NAD^+ is an important coenzyme physiologically because it furnishes the redox driving force for many enzyme-catalyzed reactions. NAD^+ in the presence of ADH, which acts as the catalyst, oxidizes ethanol to acetaldehyde.

The USP 23 requires the validation of assay methods, has established analytical parameters to be used for such validation (7), and specifies the parameters of selectivity and specificity, linearity and range, accuracy (recovery), precision, ruggedness, LOQ, and LOD.

The ICH also has established similar guidelines for methods validation (8). Current good manufacturing practices mandate that test methods, which are used for assessing the compliance of pharmaceutical products with established specifications, must meet proper standards of accuracy and reliability (16,17). The following acceptance criteria, which are based in part on the USP (7) and other publications (18–23), were set for the validation of this analytical method:

1. Selectivity (specificity): Similar analytical results should be obtained in both the presence and the absence of interfering substances; interfering substances should be identified with a recommendation not to use the test if such substances are present.
2. Linearity and range: Correlation coefficient range 0.997–1.01.
3. Accuracy (recovery): Acceptable accuracy range 95–105%.
4. Precision: Acceptable RSD not more than 5%.
5. Ruggedness: Acceptable RSD range 1–5%.
6. LOQ: Reasonable quantitation limit 0.05%.
7. LOD: Reasonable detection limit 0.01%.

The selectivity (specificity) of an analytical method is the ability of the method to measure the analyte accurately and specifically in the presence of other components that may be expected to be present in the sample matrix.

The NAD-ADH reagent alone or with the glycine buffer showed no UV absorbance at 340 nm. Also, the solutions from unspiked powder had no absorbance in that region. Therefore, the absorbance results obtained would be due to the presence of the ethanol.

Table 1 shows that there would be significant interference in the presence of propyl alcohol (60.8% recovery), butyl alcohol (48.5 % recovery), and isopropyl alcohol (22.6% recovery). Recovery in the presence of ethanol alone was 97%. This method, therefore, is not recommended for the determination of alcohol in the presence of propyl, isopropyl, or tertiary butyl alcohol.

Linearity was demonstrated in the range of about 0.0125% to 0.2% (Fig. 1; Table 2). The correlation coefficient of 0.99995 meets the acceptance criterion for this linearity in the specified range. The method was found to be accurate and reproducible in the range 0.0125% to 0.2%. Tables 2 and 3 show that recovery is satisfactory for concentrations below 2% ethanol. Above 2%, recov-

Table 1

Selectivity of the Method with Relation to Various Alcohols

Sample	Theoretical Concentration (%)	Determined Concentration[a] (%)	Bias Relative to Ethanol Recovery (%)
Ethanol	0.1095	0.1056	97.0 (ethanol recovery)
Butyl alcohol	0.1005	0.0489	48.5
Secondary butyl alcohol	0.0880	0.0036	3.4
Tertiary butyl alcohol	0.0765	−0.0001	[b]
Ethylene glycol	0.1011	0.0043	4.1
Methanol	0.0959	−0.0005	[b]
Isobutyl alcohol	0.1028	0.0003	0.3
Propyl alcohol	0.0978	0.0614	60.8[c]
Isopropyl alcohol	0.1052	0.0245	22.6

[a] Absorbance calculated as equivalent to ethanol.
[b] Not entered as results are negative.
[c] Not generally used in pharmaceutical preparations.

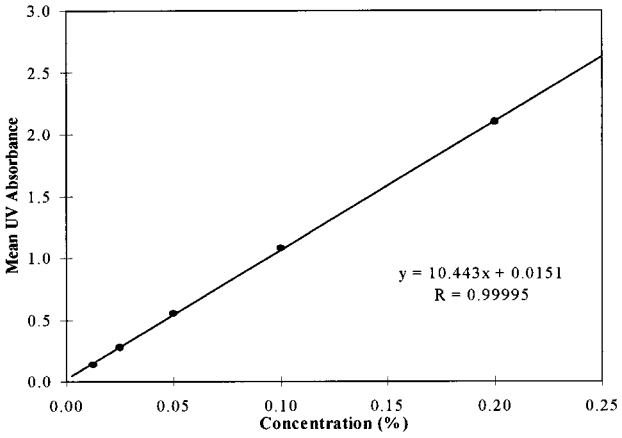

Figure 1. Ethanol standard linearity plot.

Table 2

Recovery of Ethanol from Powder Samples with Varying Concentrations of Ethanol (0.0125% to 0.1499%)

Actual Concentration (%)	% Recovery 1	% Recovery 2	Mean Recovery (%)
0.0125	97.7	97.2	97.4
0.0250	96.5	95.9	96.2
0.0500	97.6	97.8	97.7
0.0999	100.6	102.8	101.7
0.1499	99.3	98.8	99.0

Mean of all recoveries = 98.4%.
RSD (%) = 2.1.

Table 3

Recovery of Ethanol from Powder Samples Spiked with Varying Concentrations of Ethanol (2.08% to 26.05%)

Actual Concentration (%)	% Recovery 1	% Recovery 2	Mean Recovery (%)
2.08	103.9	101.4	102.7
6.25	53.9	53.9	53.9
9.38	35.9	35.7	35.8
20.84	16.2	16.2	16.2
26.05	12.9	13.0	13.0

ery is significantly lower with increasing levels of ethanol, decreasing to 13% recovery at 26% ethanol concentration.

The precision and ruggedness of the method were satisfactorily established (Table 4). The means of six replicate assays obtained from two different chemists were 0.0410 and 0.0406, with RSDs of 0.76% and 3.45%, respectively. The difference between the means was 0.0004%. The ruggedness of the assay procedure was demonstrated by the closeness of the precision and ruggedness results. These results met the acceptance criteria of not more than 5% RSD and not more than 1% difference between the means.

The LOQ is the lowest concentration of the analyte in a sample that can be determined with acceptable accuracy in the linear range and the accuracy (recovery) range under the stated experimental conditions.

The mean UV absorbances of each of the standard solutions in the linearity study were plotted against their corresponding concentrations (Fig. 2; Table 5), and a linear regression analysis was performed on the eight coordinates. The resulting plot was found to be linear in the range 0.0031% to 0.25% with a correlation coefficient of 0.9995. This meets the acceptance criterion of not less than 0.997 and not more than 1.01 set for this study.

The relative response factors were calculated relative to the mean response factor of all eight levels of the linearity study. The percentage relative response factors at 0.0061% and 0.25% concentrations of ethanol did not meet the acceptance criterion of 95.0% to 105.0% set for this study, so the results at levels 0.0031%, 0.0061%, and 0.25% were not accepted (Table 5). Based on the valid results of the percentage relative response factors, the linearity range was redefined as 0.0125% to 0.20% with a correlation coefficient of 0.99995 (Fig. 1, Table 4).

Table 4

Comparison of the Results from the Precision and Ruggedness Studies

Sample	Precision % Ethanol	Ruggedness % Ethanol
1	0.0409	0.0393
2	0.0408	0.0397
3	0.0405	0.0415
4	0.0414	0.0429
5	0.0411	0.0395
6	0.0412	0.0407
Mean	0.0410	0.0406
RSD (%)	0.76	3.45

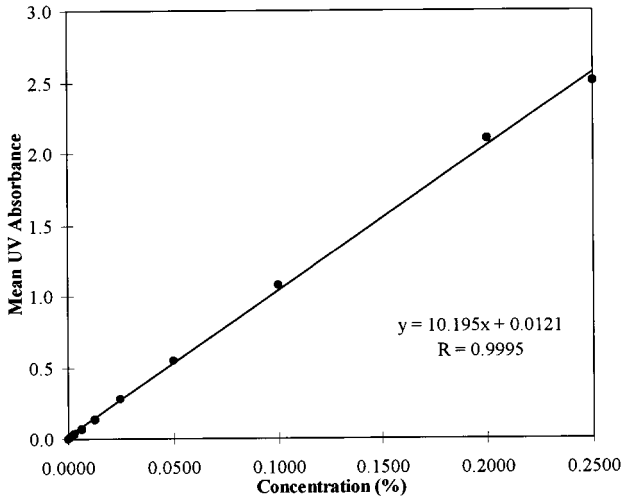

Figure 2. Determination of limit of quantitation.

Therefore, based on the lowest levels in the linearity range and the accuracy (recovery) studies, the linear LOQ was set at 0.0125%. This met the acceptance criteria for this study. Any result below 0.0125% should be reported as less than the linear LOQ.

The LOD was also established satisfactorily. Since the lowest positive UV absorbance was observed at the corresponding concentration of 0.0000046% of ethanol (Table 6), the LOD was set at that value. This shows that this method is sensitive and detects very low concentrations of ethanol.

Table 6

Determination of the Limit of Detection

Concentration of Ethanol (%)	UV Absorbance
0.0000023	−0.036
0.0000046	0.002
0.0000090	0.002
0.000018	0.003
0.000037	0.004
0.000073	0.006
0.00015	0.006
0.00029	0.006
0.00059	0.011
0.0012	0.018
0.0023	0.027
0.0047	0.052
0.0094	0.098
0.0190	0.216
0.0375	0.371
0.075	0.671
0.150	1.316

The ruggedness and robustness of the method were demonstrated by the reproducibility in the method even when different analysts, using different instruments on different days, performed the test independently. The test results were also found to be unaffected by incubation time and temperature as satisfactory results were obtained at 37°C and at 22°C–40°C for 10 min, attesting to the robustness of the method. Incubation at 37°C for

Table 5

Linearity of Ultraviolet Response and Percentage Relative Response Factor

Standard Linearity Solution Concentration (%)	Mean UV Absorbance	Response Factor (Absorbance/Concentration)	% Relative Response Factor[a]
0.0031	0.034	11.28	104.0
0.0061	0.071	11.59	106.9
0.0125	0.135	10.82	99.8
0.025	0.275	11.01	101.5
0.05	0.543	10.85	100.0
0.10	1.072	10.72	98.9
0.20	2.097	10.48	96.6
0.25	2.504	10.02	92.5

Mean of all eight response factors = 10.846.

[a] % Relative response factors relative to the mean of all the eight response factors.

10 min is recommended. Based on the results and the discussions above, this method is validated for the parameters such as selectivity (specificity), linearity and range, accuracy (recovery), precision, ruggedness, LOQ, and LOD. The validation criteria for all the parameters in the above trace-level study were met. This enhances the test's usefulness and possible application in pharmaceutical products and in-process samples.

CONCLUSION

Trace levels of ethanol in pharmaceutical samples can be analyzed by the enzymatic method involving the oxidation of ethanol to acetaldehyde by ADH with the simultaneous reduction of NAD^+ to NADH. The concentration of NADH is detected by the UV spectrophotometer at 340 nm. The method was satisfactorily validated in accordance with USP 23 specifications for methods validation. The method is not specific and selective for ethanol in the presence of propyl, isopropyl, and butyl alcohols. Based on its probable use, the method described here would be useful as a category II test for impurities or degradation products and for limit tests (7).

REFERENCES

1. G. Maeder, M. Pelletier, and W. Haerdi, in *Analytical Applications of Immobilized Enzyme Reactors* (S. Lam and G. Malikin, Eds.), Blackie Academic and Professional, New York, 1994, p. 243.
2. J. Ruz, M. D. Luque de Castro, and M. Valcarcel, Analyst, 112, 259 (1987).
3. S. Black, Arch. Biochem. Biophys., 34, 86 (1951).
4. Sigma Chemical Company, *Sigma Technical Bulletin No.332-UV*, Author, St. Louis, MO, 1990.
5. J. Ruz, A. Fernandez, M. D. Luque de Castro, and M. Valcarcel, J. Pharm. Biomed. Anal., 4(5), 545 (1986).
6. A. Roda, S. Girotti, and G. Carrea, Meth. Enzymol., 133, 238 (1986).
7. U.S. Pharmacopeial Convention, *United States Pharmacopeia 23/National Formulary 18*, Author, Rockville, MD, 1995, ⟨1225⟩, p. 1982.
8. International Conference on Harmonization, *International Conference on Harmonization Guideline on Method Validation Methodology*, Author, Geneva, 1996.
9. L. Huber, Pharm. Technol.—Analytical Validation, 6 (1998).
10. I. Krul and M. Swartz, LC/GC, 15(9), 842 (1997).
11. M. E. Swartz and Ira S. Krull, Pharm. Technol., 3, 104 (1998).
12. H. G. Brittain, Pharm. Technol., 3, 82 (1998).
13. E. W. Ciurczak, Pharm. Technol., 4, 44 (1998).
14. J. Hammond, D. Irish, and S. Hartwell, Spectroscopy, 13(2), 64 (1998).
15. I. Krull and M. Swartz, LC/GC, 16(3), 258 (1998).
16. 21 CFR 211.165 (e), U.S. Government Printing Office, Washington, DC, 1988.
17. 21 CFR 211.194(a)(2), U.S. Government Printing Office, Washington, DC, 1988.
18. U.S. Pharmacopeial Convention, *United States Pharmacopeia*, Author, Rockville, MD, 1990, 1710–1712.
19. D. L. Masart, A. Dijkstra, and L. Kaufman, *Evaluation and Optimization of Laboratory Methods and Analytical Procedures*, Elsevier Scientific, 1978.
20. A. L. Wilson, The performance characteristics of analytical methods, Talanta, 17, 21–29, 31–44 (1970).
21. A. L. Wilson, The performance characteristics of analytical methods, Talanta, 20, 725–732 (1971).
22. A. L. Wilson, The performance characteristics of analytical methods, Talanta, 21, 1109–1121 (1974).
23. W. L. Paul, Pharm. Technol., 15, 130–141 (1991).