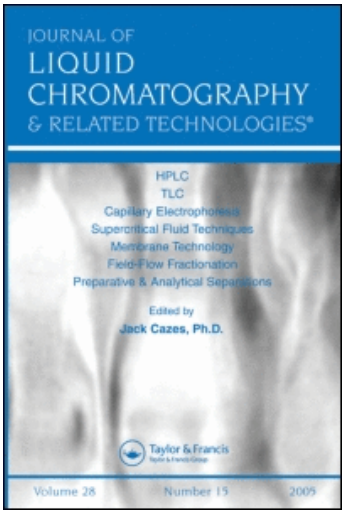

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Development and Validation of a HPLC Method for NAD: Application to Stability Studies in Buffered Solutions and Dry Test Strips

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Development and Validation of a HPLC Method for NAD: Application to Stability Studies in Buffered Solutions and Dry Test Strips

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Abstract: A reversed-phase high performance liquid chromatographic (RP-HPLC) method for nicotinamide adenine dinucleotide (NAD) has been developed and validated. The method was then applied to stability studies of NAD in aqueous buffered solutions and commercial dry test strips (Optium Plus™, Abbott Laboratories) for assaying blood glucose. NAD was resolved from its decomposition products on a XTerra C_{18} column (150 mm × 4.6 mm, 5 μm) using a mobile phase composed of a mixture of methanol and 20 mM potassium phosphate buffer pH 8.0 (5:95, v/v), at a flow rate of 1 mL/min with UV detection at 260 nm. The chief decomposition products were identified as ADP-ribose and nicotinamide, the amount of which depended upon the identity of the buffer. The solid state stability of NAD formulated in Optium Plus™ test strips containing BES buffer at pH 7.0 was determined to be acceptable; >80% residual NAD after 3 months at 50°C.

Keywords: HPLC, Method development, Method validation, NAD, Stability studies

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is an essential cofactor for a large number of dehydrogenase enzymes, many of which have

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diagnostically important substrates such as glucose, lactate, hydroxybutyrate, ethanol, cholesterol, etc. As such, the stability of NAD in solution and solid preparations for assay kits and test strips is of great interest to the diagnostic industry. There are many examples in the patent literature[1–8] of stabilised NAD solution formulations attesting to the high level of attention given to this area. Cofactors have been stabilised in solution through the use of NAD derivatives[1] and various additives/buffers such as zwitterionic buffers,[2] thiols,[3] sulfite,[4] hydroxylamines,[5] heavy metal ions,[6] polyalcohols,[7] proteins.[8] In addition, the stability of NAD has been enhanced by coupling to dextran,[9,10] and polyethylene glycol.[9] One stability study for NAD in solution pharmaceutical preparations has been conducted.[11] However, the majority of these studies have relied upon UV absorbance at 340 nm as an indirect measure of NAD stability since most are connected with spectroscopic kinetic assays involving the determination of NADH formation rates. The specific method of UV detection of NAD following HPLC has seen no use for stability studies, as far as we are aware, but has been applied in other areas such as the analysis of body fluids and tissues.[12–15] The question of cofactor stability also has some significance for the use of NAD dependent enzymes in organic synthesis; this has been discussed in a review.[16]

Early work[17] established that the first step in the decomposition of NAD is the loss of nicotinamide followed by slower cleavage of ADP-ribose to form adenosine monophosphate (AMP) and, in some cases,[18] adenosine diphosphate (ADP). The hydrolysis of NAD is dependent on pH, the molecule being relatively stable in acid media but not alkaline solutions.[16] In contrast, its reduced counterpart NADH displays the reverse pH stability profile.[16] Control of pH via the use of buffers is therefore important in formulating stable assay reagent solutions containing NAD. However, NAD is known to be reactive towards nucleophiles such as sulfite,[19] dithionite,[20] cyanide,[21] thiols,[22] carbonyl compounds,[23] and hydroxylamine[24] as well as buffers.[25,26] Furthermore, it has been reported that adducts of NAD with nucleophiles can act as enzyme inhibitors. The decomposition profile of NAD under various conditions was the subject of much discussion[27] during the 1960s and 1970s due to the formation of several species,[27,28] which inhibited lactate dehydrogenase (LDH) and thereby led to inaccurate results in diagnostic assays. Beillmann et al.[27] characterised one as an adduct of phosphate and NAD while Gallati[26] found that the identity of the buffer played an important role in the degree of LDH inhibition.

Our interest in NAD relates to the development of enzyme based biosensor electrodes containing NAD dependent dehydrogenases, such as glucose dehydrogenase (GDH) and D-3-hydroxybutyrate dehydrogenase (HBDH), and a redox mediator, such as 1,10-phenanthroline-5,6-dione,

capable of oxidising NADH.[29] Here, the dry test strips must maintain their response for a minimum of 18 months. Stabilisation of the NAD cofactor together with the enzyme and mediator in the electrode are important in achieving this target. In this article, we report the development of a validated HPLC assay for NAD and apply it to stability studies of NAD in buffered solution and dry test strips.

EXPERIMENTAL

Materials

Methanol (HPLC-grade), potassium dihydrogen phosphate (KH_2PO_4), and sodium hydroxide (NaOH) were obtained from VWR International (Poole, UK). Buffer salts and ADP-ribose were purchased from Sigma Aldrich Chemicals (Gillingham, UK). The sodium salt of NAD was obtained from Oriental Yeast Co. (Tokyo, Japan). Nicotinamide, adenosine, adenine, AMP, and ADP were purchased from Alfa Aesar (Heysham, UK). Deionised distilled water was used throughout the experiment. All other reagents were of analytical grade.

The dry test strips containing NAD and GDH were Optium Plus™ glucose electrodes from Abbott Diabetes Care (Maidenhead, UK). The test strips were supplied, packaged in foil at low humidity.

HPLC Instrumentation and Conditions

A PerkinElmer (Norwalk, CT) HPLC system equipped with a module series 200 UV-vis detector, series 200 LC pump, series 200 autosampler, and series 200 peltier LC column oven were used in this work. The data were acquired via PE TurboChrom Workstation data acquisition software using PE Nelson series 600 LINK interfaces. Chromatographic separation was achieved with Xterra RP 18, 5 μm (150 × 4.6 mm), which has a stationary phase based on silica gel where methyl groups are incorporated to reduce the number of surface silanol groups free to interact with basic compounds during chromatography. The column temperature was set at 35°C and UV detection is set in the range of 200–400 nm. Mobile phase consisting of 20 mM potassium dihydrogen orthophosphate pH 8.0–methanol (95:5, v/v) was used at a flow rate of 1.0 mL/min. Injection volume was 10 μL. The mobile phase was filtered through 0.45 μm membrane filter and continuously degassed online. The second HPLC system used to measure the UV spectra consisted of a Waters Alliance 2690 Separations Module to a 996 Waters photodiode array (PDA) Detector (Waters, Elstree, UK).

Preparation of Solutions and Samples

Solution Preparation

An accurately weighed amount (30 mg) of NAD was placed in a 100 mL volumetric flask and dissolved in the mobile phase to produce a standard solution. Aliquots of this solution were diluted with the mobile phase to produce solutions with NAD concentrations in the range 0.01–0.7 mg/mL.

For solution stability studies, a 10 mM solution of NAD (0.686 g) in the relevant buffer solution (100 mL, 100 mM, pH 7.0, and 8.0) was prepared.

Preparation of Dry Test Strips

For solid state stability studies, dry test strips (Optium Plus^{TM}) were stored as supplied in foil packets at temperatures of 4, 30, 40, and 50°C. At the relevant time point, ten test strips for each storage temperature were removed from their foil packets and prepared for NAD extraction. This involved peeling off a covering tape on each test strip then cutting off the end of each strip containing the dried active reagents including NAD. The ten strip pieces were added to a test tube containing 1.0 mL of water then vortex mixed for 40 s. The extract was finally transferred to a vial for HPLC analysis.

RESULTS AND DISCUSSION

Chromatographic Separation

The chromatographic analysis of NAD was carried out in the isocratic mode using a mixture of phosphate buffer methanol (95:5, v/v) as mobile phase. The column was equilibrated with the mobile phase flowing at 1.0 mL/min for 30 min prior to injection. Sample solutions of 10 μL were injected automatically into the column. The optimal wavelength for NAD detection was established using two UV absorbance scans over the range of 200 to 400 nm, one scan of the mobile phase, and the second of the analyte in the mobile phase. As expected, 260 nm was shown to be the optimal wavelength to maximise the signal (Figure 1). Chromatograms of the NAD gave good peak shape. The retention time for NAD was 2.0 min. To evaluate the quantitative nature of the analytical method, a series of samples with different amounts of NAD were run to investigate the best assay concentration. Using a C_{18} column, the best concentration

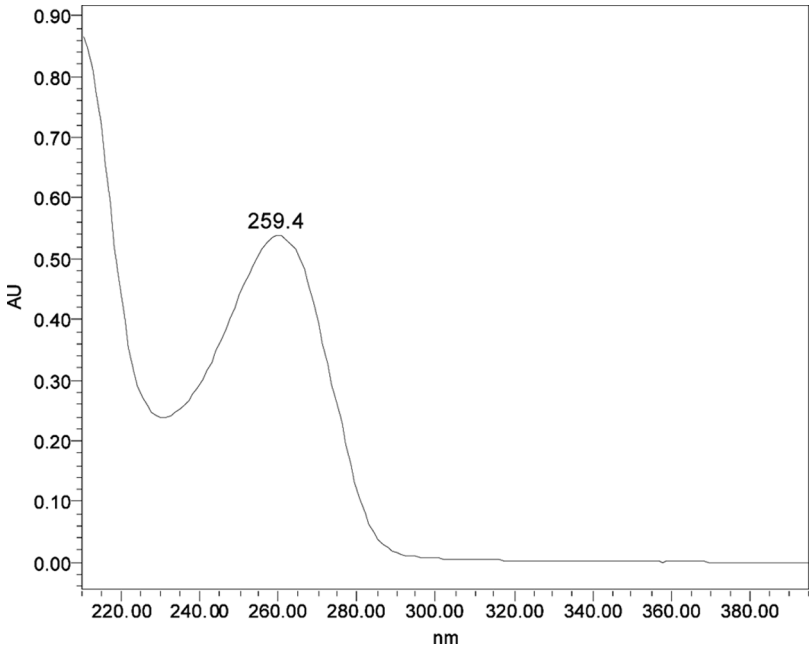


Figure 1. UV spectrum of a standard solution of NAD measured by HPLC-PDA.

was assessed by injecting six reference standard solutions of NAD in the range of 0.01–0.7 mg/mL. The integrated peak areas were plotted versus amount injected. The calibration curve was found to be linear from the concentration range 0.1–0.5 mg/mL with a correlation coefficient of 1.000. On the bases of these data, 0.3 mg/mL was chosen as a working concentration for the assay.

System suitability testing was performed to determine the accuracy and precision of the system from six replicate injections of a solution containing 0.30 mg NAD/mL. The percent relative standard deviation (% RSD) of the retention time (min) and peak area were found to be less than 0.40%. The retention factor (also called capacity factor, k) was calculated using the equation $k = (t_r/t_0) - 1$, where t_r is the retention time of the analyte and t_0 is the retention time of an unretained compound; in this study, t_0 was calculated from the first disturbance of the baseline after injection and the capacity factor value obtained was 8.38 for the NAD peak. The separation factor (α) was calculated using the equation, $\alpha = k_2/k_1$ where k_1 and k_2 are the retention factors for the first and last eluted peaks, respectively. The separation factor for the NAD peak

Table 1. Experimental design for robustness study

Test parameter	1	2	3	4	5	6	7	8
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	c	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g
Results	s	t	u	v	w	x	y	z

was 2.16. The plate number (also known as column efficiency, N) was calculated as $N = 5.54\ (t_r/w_{0.5})^2$ where $w_{0.5}$ is the peak width at half peak height. In this study, the theoretical plate number was 2674. Resolution is calculated from the equation $R_s = 2(t_2 - t_1)/(t_{w1} + t_{w2})$, where t_1 and t_2 are retention times of the first and second eluted peaks, respectively, and t_{w1} and t_{w2} are the peak widths. The resolution for the NAD peak was >2.0. The asymmetry factor (A_s) was calculated using the US Pharmacopeia (USP) method. The peak asymmetry value for each NAD peak was 1.02.

Robustness studies were also performed in the method development phase applying the experimental design as shown in Table 1. A sample of NAD was prepared at the working concentration (0.3 mg/mL) and assayed using the experimental design with eight test combinations for seven different chromatographic parameters as shown in Table 2. For each parameter, four combinations of (AAAA) and four combinations of (aaaa) were studied. The actual value of each parameter ($V_A - V_G$) (Table 2) shows which parameter has a dominant influence on the developed analytical method. In all cases, good separations of NAD were always achieved, indicating that the analytical method remained selective for the NAD component under the optimized conditions.

Method Validation

According to the best practice,[30–32] the newly developed analytical method was validated in terms of linearity, assay range, precision (repeatability and intermediate precision), accuracy (recovery), specificity, limits of detection, and quantification.

Table 2. Chromatographic parameter for robustness study

Parameter	Test conditions 1	Test conditions 2	Differences
Analytical column	A = column C-18	a = column C-18	$V_A = \frac{1}{4}(s+t+u+v) - \frac{1}{4}(w+x+y+z) = A-a$
Sample solvent	B = Buffer/water	b = Mobile phase	$V_B = \frac{1}{4}(s+t+w+x) - \frac{1}{4}(u+v+y+z) = B-b$
Temperature	C = 30°C	c = 40°C	$V_C = \frac{1}{4}(s+u+w+y) - \frac{1}{4}(t+v+x+z) = C-c$
Flow rate	D = 0.8 mL/min	d = 1.3 mL/min	$V_D = \frac{1}{4}(s+t+y+z) - \frac{1}{4}(u+v+w+x) = D-d$
Wavelength	E = 250 nm	e = 270 nm	$V_E = \frac{1}{4}(s+u+x+z) - \frac{1}{4}(t+v+w+y) = E-e$
Mobile phase	F = 3% methanol	f = 7% methanol	$V_F = \frac{1}{4}(s+v+w+z) - \frac{1}{4}(t+u+x+y) = F-f$
Solubility stability	G = 1 h	g = 8 h	$V_G = \frac{1}{4}(s+v+x+y) - \frac{1}{4}(t+u+w+z) = G-g$

Table 3. Validation results obtained for the HPLC assay of NAD

Validation criterion	Concentration range (mg/mL)	Results
Linearity ($n = 3$; $k = 5$)	0.10 to 0.50	$y = 12058264.90x + 67726.33$ $R^2 = 1.000$
Precision		
(A) Intra-day (%RSD; $n = 6$)	0.1, 0.2, 0.3, 0.4, 0.5	0.21, 0.32, 0.20, 0.27, 0.35
(B) Inter-day (%RSD; 2 days; $n = 6$)	0.1, 0.3, 0.5	0.26, 0.22, 0.38
LOD		(s/n = 3.3), 8.8 μg/mL
LOQ		(s/n = 10.0), 26 μg/mL

Linearity and Range

The linearity of peak area response versus concentration for NAD was studied from approximately 0.1 to 0.5 mg/mL. Five solutions were prepared corresponding to 10, 25, 75, 100, and 150% of the nominal analytical concentration (0.3 mg/mL) and each one was injected in triplicate. The calibration curve was linear in the range of 0.1 to 0.5 mg/mL for this assay, with a correlation coefficient (R^2) for NAD (Table 3). A typical calibration curve has the regression equation of $y = 12058264.90x + 67726.33$.

Precision (Repeatability and Intermediate Precision)

The precision of the analytical method was evaluated in terms of repeatability and intermediate precision. Repeatability was assessed on the same day by means of the repetitive application of the NAD procedure to two samples (experimental replicates) at each concentration level (0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL), and each one was injected in triplicate (instrumental replicates). Intermediate precision was assessed on six replicate injections at three different concentrations (0.1, 0.3, and 0.5 mg/mL) for two consecutive days. The precision data and RSD values presented in Table 3 were less than 0.40% in all cases and illustrated excellent precision for the analytical method.

Accuracy/Recovery Study

In order to test the efficiency of the analytical method, recovery studies at three known added concentration levels (0.2, 0.3, and 0.4 mg/mL, 75%,

Table 4. Recovery studies of NAD from samples with known concentration

Concentration (mg/mL)	Percent of nominal	Amount of NAD (mg)		Recovery (%)	RSD (%)	*t*-test
		Added	Recovered			
0.2	75	0.102	0.097	97.46	0.012	0.041
0.3	100	0.301	0.302	100.78	0.003	0.071
0.4	150	0.501	0.494	98.87	0.161	0.024
Mean				99.04		

100%, and 150% of nominal) were carried out. Three replicates were injected at each concentration level. Mean recoveries higher than 97.5% were obtained in all cases with RSD less than 0.17%. The results are shown in Table 4.

Limits of Detection and Quantitation

The limit of detection (LOD) was considered as the minimum analyte concentration yielding a signal to noise ratio equal to three. The limit of quantitation (LOQ) was adopted at the lowest analyte concentration yielding a signal 10 times greater than the noise. The LOD and LOQ values for NAD were found to be 8.8 μg/mL (S/N = 3.3) and 26 μg/mL (S/N = 10.0), with RSD 0.88% for six injections, respectively (Table 3).

Specificity

Forced degradation studies were performed to evaluate the specificity of NAD under four stress conditions (heat, UV light, acid, base). Solutions of NAD were exposed to 50°C for 1 h, UV light using a Mineralight UVGL-58 light for 24 h, acid (1 M HCl) for 24 h, and base (1 M NaOH) for 4 h. A summary of the stress results is shown in Table 5. The method was capable of separating NAD from various degradation products such as AMP, ADP, AD-ribose, nicotinamide, adenine, and adenosine.

Table 5. Specificity results of NAD under stress conditions

Stress conditions	Sample treatment	t_R (min)	Peak area (μVs)	Assay (%)
Reference	Fresh solution	2.048	3694298	95.77
Acid	1 M HCl for 24 h	2.046	3705876	95.73
Base	1 M NaOH for 4 h	2.059	3714595	95.82
Heat	50°C for 1 h	2.062	3715813	95.82
Light	UV Light for 24 h	2.046	3711822	95.71

Method Application

The validated HPLC method was applied to the determination of the stability of NAD under various conditions. Aqueous solutions of NAD containing a wide range of different buffers were investigated at neutral and alkaline pH. These included inorganic anions and zwitterionic aminosulfonic acids, the so called Good buffers.[33–35] In a second application of the HPLC method, the stability of NAD in a dry test strip incorporating a single selected buffer salt was assessed at temperatures in the range 4–50°C.

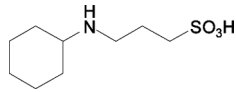
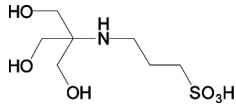
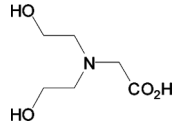
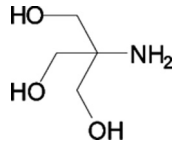
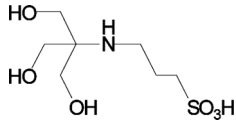
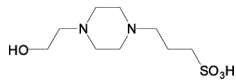
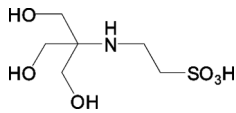
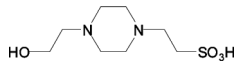
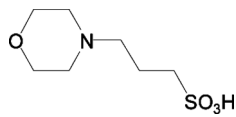
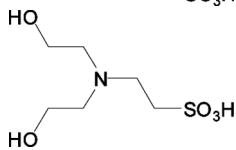
Stability of NAD in Aqueous Buffered Solution

A range of buffers with different structural features was evaluated in terms of the amount of NAD remaining after heating at 50°C for 24 h (Table 6). The buffered aqueous solutions of NAD were assessed against a control comprising a simple solution of NAD in water, i.e., no buffer. In general, the zwitterionic Good buffers tended to have little effect on the stability of NAD in solution at pH 7. The level of residual NAD varied over a small range from 66.8% for MES to 70.4% for CAPS compared to 69.2% for the water control. However, there was a small but definite trend of increasing residual NAD with increasing buffer pK_a for these zwitterionic buffers. This is believed to be simply due to greater protonation of the amine group in the buffers with high pK_a at pH 7, leading to less nucleophilic attack on NAD. In practice, one would not use the high pK_a buffers such as CAPS and TAPS at pH 7, since this lies outside their effective buffering range.

The correlation between buffer pK_a and residual NAD breaks down when one attempts to include buffers of a different structural class to the zwitterionic Good ones. Thus, the 1° amine TRIS has a relatively high residual NAD level of 69.9% for its pK_a while the 2° diamine bis-TRIS-propane gives a low level of 59.6%. The inorganic anions, carbonate and borate, are clearly very reactive towards NAD yielding very low residual NAD results of 2.2% and 26.2%, respectively.

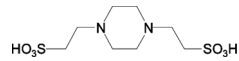
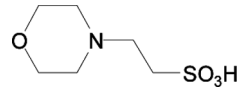
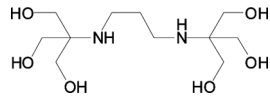
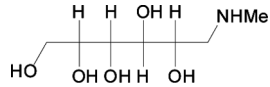
It is interesting to note that the profile of NAD decomposition products depends on the buffer. Both BES and borate gave almost exclusively ADP-ribose and nicotinamide, i.e., the first step in the decomposition of NAD,[17] when solutions at pH 8.0 were heated at 60°C for 24 h (Figures 2a and c). In contrast, carbonate and TRIS also gave AMP and ADP, presumably derived from the decomposition of ADP-ribose;[17,18] especially high levels of these two nucleotides were seen in the former case (Figures 2b and d). In addition, the NAD

Table 6. Stability of 10 mM NAD solutions in 100 mM buffer (or additive) pH 7.0: residual NAD by HPLC after heating at 50°C for 24 h

Buffer/additive	Structure	Buffer pKa (effective pH range)	Residual NAD (%)
Water control	–	–	69.2
CAPS		10.4 (9.7–11.1)	70.4
TAPS		8.4 (7.7–9.1)	70.1
Bicine		8.3 (7.6–9.0)	68.7
TRIS		8.1 (7.0–9.0)	69.9
Tricine		8.1 (7.4–8.8)	68.6
EPPS (or HEPPS)		8.0 (7.3–8.7)	68.4
TES		7.5 (6.8–8.2)	68.4
HEPES		7.5 (6.8–8.2)	67.9
MOPS		7.2 (6.5–7.9)	67.5
BES		7.1 (6.4–7.8)	67.5

(*Continued*)

Table 6. Continued

Buffer/additive	Structure	Buffer pKa (effective pH range)	Residual NAD (%)
PIPES		6.8 (6.1–7.5)	67.7
MES		6.1 (5.5–6.7)	66.8
Bis-TRIS-propane		6.8, 9.0 (6.3–9.5)	59.6
Carbonate	–	6.4	2.2
N-Methyl-D-glucamine		–	50.8
Borate	–	9.1, 12.7, 13.8	26.2

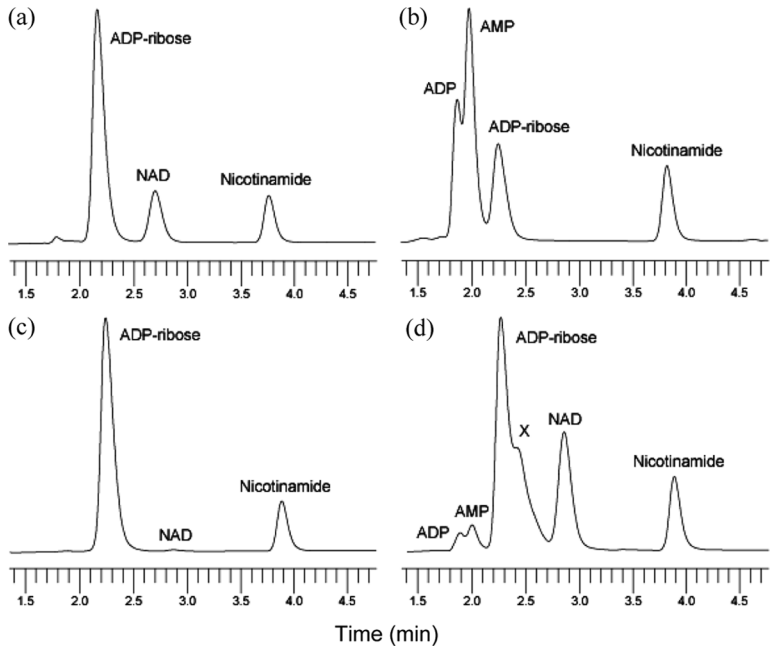


Figure 2. Chromatograms of NAD solutions heated at 60°C for 24 h in the presence of various buffer salts at pH 8.0: (a) 0.1 M BES, (b) 0.1 M carbonate, (c) 0.1 M borate, and (d) 0.1 M TRIS where X is an unidentified species.

decomposition profile in TRIS displayed an unidentified shoulder on the ADP-ribose peak.

Stability of NAD in Dry Test Strips

As a result of the solution studies in stability of NAD in aqueous buffered solution section, BES was selected as the buffer for use in a dry test strip for blood glucose (Optium Plus^{TM}) containing the enzyme GDH and NAD. A high proportion of NAD was found to be retained in BES buffer solutions at elevated temperatures while any decomposition of NAD resulted in the clean formation of nicotinamide and ADP-ribose, species that were not anticipated to interfere with the functioning of the test strip. The active reagents including NAD on the test strip were buffered at pH 7.0, close to physiological pH, which is within the effective buffering range of BES (Table 6).

Five lots of dry glucose test strips (Optium Plus^{TM}), containing NAD and BES buffer, were heated at 4°C, 30°C, 40°C, and 50°C for three months. NAD was readily extracted from the dry strip into water and then analysed by HPLC using the validated method. The stability of NAD in test strips at 30°C, 40°C, and 50°C was assessed by determining the residual NAD at each temperature versus control test strips stored at 4°C. Mean residual NAD levels for the five test strip lots were 96.6%, 92.8%, and 82.6% at 30°C, 40°C, and 50°C, respectively (Table 7, Figure 3). Nicotinamide and ADP-ribose were the only detected decomposition products of NAD. These results show that NAD is very stable in dry test strips (Optium Plus^{TM}) packaged in foil under low humidity and stored at temperatures in the range 30–50°C. Currently, the test strips are

Table 7. Stability of NAD in five lots of glucose test strips: residual NAD by HPLC after storage at 30°C, 40°C, and 50°C for 3 months

	Residual NAD (%) versus 4°C		
Test strip lot no.	30°C	40°C	50°C
1	96.9	94.4	85.1
2	100.3	93.4	82.1
3	95.0	93.4	82.9
4	94.5	91.1	83.3
5	96.1	91.5	79.8
Mean	96.6	92.8	82.6

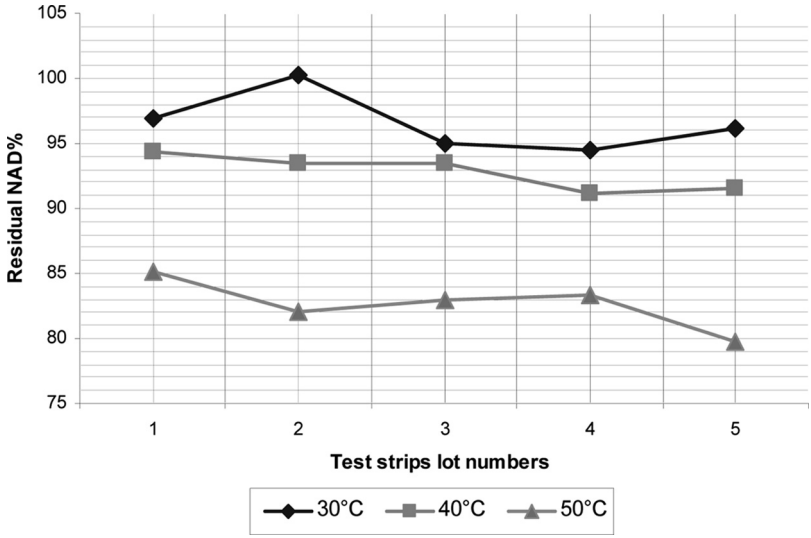


Figure 3. Plot for stability of NAD in five lots of glucose test strips: residual NAD by HPLC after storage at 30°C, 40°C, and 50°C for 3 months.

marketed successfully with a shelf life of 18 months at a maximum storage temperature of 30°C.

CONCLUSION

A new, simple and robust reversed phase HPLC method for the determination of NAD and the identification of its decomposition products in solution and solid phase stability studies was developed and validated. Experiments in solution in the presence of a number of zwitterionic Good buffers demonstrated that there was a small trend of increasing residual NAD with increasing buffer pK_a. The decomposition profile of NAD in solution depended on the identity of the buffer. Following these studies, BES was selected as an ideal buffer for incorporation into dry test strips to maintain a high residual NAD level on storage while also restricting the products of any decomposition to nicotinamide and ADP-ribose as demonstrated by HPLC analysis. The resulting commercial test strips (Optium Plus^{TM}) for glucose have a long shelf life.

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