Journal of Chromatography, 225 (1981) 446—449
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 966

Note

Determination of pyridine dinucleotides in cell extracts by high-performance liquid chromatography

DEAN P. JONES

Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322 (U.S.A.)

(Received April 2nd, 1981)

During recent studies of the effects of various respiratory inhibitors and hypoxia on cellular adenylates and pyridine dinucleotides [1], we have found that the high-performance liquid chromatographic method of Schweinsberg and Loo [2] for adenylate determination can be modified in a very simple fashion to obtain simultaneous determination of NAD^+ and $NADP^+$.

Currently, most widely used methods for determination of pyridine dinucleotide concentrations in cell and tissue extracts rely upon changes of the absorbance or fluorescence of the reduced forms following incubation with selected enzymes [3, 4]. High-performance liquid chromatography (HPLC) has also been used in studies of these compounds [5, 6] but has not been optimized for tissue determinations. The following method provides such an application. NADH and NADPH are determined similarly but require alkaline extraction because they are labile in acid.

EXPERIMENTAL

Chemicals used for buffer solutions were at least of reagent grade. Buffer A was 100 m*M* potassium phosphate, pH 6.0, and Buffer B was 100 m*M* potassium phosphate, pH 6.0, containing 5% methanol (v/v). Buffers were filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to use. Distilled water was used for buffer preparation. β-NADH, β-NAD^+, NADPH, $NADP^+$, ATP, ADP, AMP and collagenase (Type IV) were purchased from Sigma (St. Louis, MO, U.S.A.). Calibration of standards was done using the millimolar extinction values as follows: NADH, 6.2 (340 nm); NAD^+, 18.0 (260 nm); NADPH, 6.2 (340 nm); $NADP^+$, 18.0 (340 nm); ATP, 15.4 (259 nm); ADP, 15.4 (260 nm); AMP, 15.4 (260 nm). Isolated liver cells were pre-

pared by the recirculating perfusion method of Moldeus et al. [7], and extractions were performed as described by Tischler et al. [8]. For NADH and NADPH determination, a 1-ml cell suspension (10^6 cells/ml) was treated with 0.1 ml of 0.5 *M* potassium hydroxide containing 50% (v/v) ethanol and 35% (w/v) cesium chloride, immediately cooled on ice, and centrifuged to remove insoluble material. For NAD^+ and $NADP^+$ determinations, cell suspensions (10^6 cells/ml) were treated with 3 *M* perchloric acid (0.5 ml per 1.0-ml incubation), immediately cooled on ice, and centrifuged to remove insoluble material. The oxidized forms were stable at −20°C for several days, but the reduced forms showed about 30% loss after 24 h. Pyridine nucleotides were separated on a μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.) with either a two-pump Waters system with Model 720 system controller and Data Module or a Beckman Model 334 gradient chromatograph. The Waters chromatograph was equipped with a variable-wavelength detector employing an 8-μl flow cell, and the Beckman chromatograph was equipped with a Model 155 variable-wavelength detector employing a 20-μl flow cell. General operating conditions were as follows: flow-rate, 1 ml/min; recycling time between runs, 10 min; temperature, 18—22°C.

RESULTS AND DISCUSSION

Standard mixtures of NADH, NAD^+, NADPH and $NADP^+$ could be readily separated using 3.75% methanol in 100 m*M* phosphate, pH 6.0, and measuring absorbance at 260 nm. Retention times were: $NADP^+$, 4.6 min; NADPH, 6.6 min; NAD^+, 8.0 min; and NADH, 12.4 min. Decreased retention times occur as the C_{18} columns are continually exposed to phosphate buffers. Consequently, new columns show somewhat longer retention times and columns aged for several months show shorter retention times under the conditions illustrated here. Compensation for these changes can be made by increasing or decreasing the methanol content, respectively. Calibration of standards by peak integration was linear over the range of 10—2000 pmol for absorbance at 260 nm (injection volumes 10 μl or 80 μl). Similar calibration of NADH and NADPH at 340 nm was linear over the range of 30—2000 pmol. Calibration by peak height was acceptable above 100 pmol; below this there was an apparent peak broadening that required either integration or a complete standard curve for accurate quantitation.

Simultaneous separation of the adenylates from NAD^+ and $NADP^+$ in the perchloric acid extract of liver cell suspension is shown in Fig. 1. Peaks identified as ATP, ADP and AMP in cell extracts have retention times the same as the corresponding standards and can be selectively manipulated by incubation of cell under anaerobic conditions or with antimycin A, ethionine or carbonyl-cyanide-P-trifluoromethoxyphenylhydrazone (FCCP). The absolute concentrations as well as the ratios of ATP/ADP and ATP/AMP are the same as those obtained by other approaches [9]. Peaks identified as NAD^+ and $NADP^+$ in cell extracts have retention times corresponding to standards and can be eliminated by alkaline treatment or reduction with dithionite. Recoveries of NAD^+ and $NADP^+$ added to cell extracts were 96 ± 3% (n = 5) and 101 ± 4% (n = 5), respectively, for 0.14 nmol per 80-μl additions. Measured values

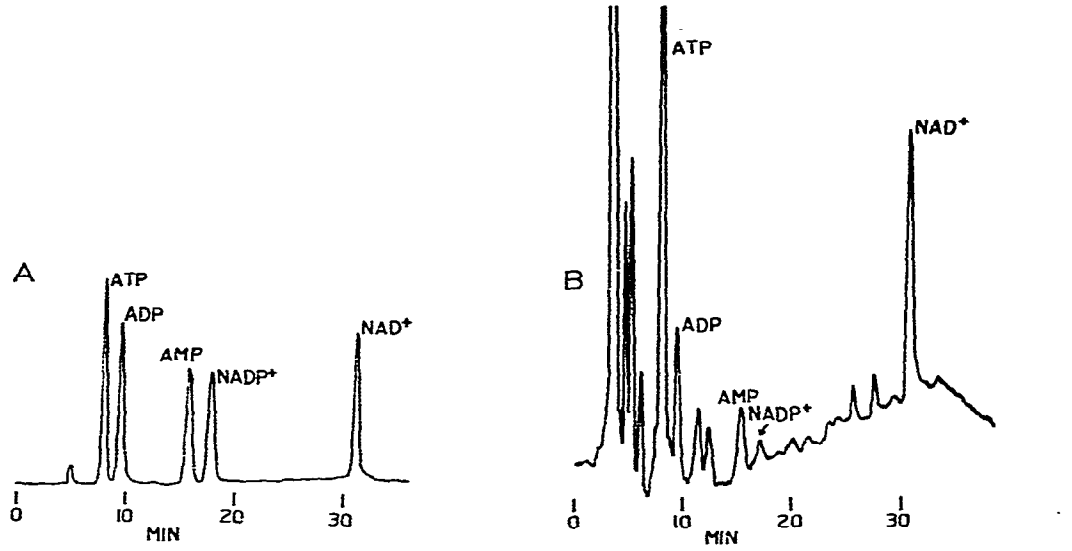

Fig. 1. Separation of NADP+, NAD+, ATP, ADP and AMP in extracts of isolated liver cells. (A) Standard mixture (2 nmol each: NAD+, NADP+; 3 nmol each: ATP, ADP, AMP) was injected on a column run on the Beckman system with the program as follows: 8.5 min at 100% Buffer A, 0.5 min gradient from 100% Buffer A to 80% Buffer A, hold 6 min at 80% Buffer A, 5 min gradient to 0% Buffer A, hold 20 min at 0% Buffer A. Flow-rate was maintained at 1 ml/min; recycle time was 0.5 min to 100% Buffer A and 10 min before next injection. Injection volume was 7 μl. Detector was at 0.1 sensitivity. (B) 20 μl of the perchloric acid extract of 10^6 liver cells per ml (approx. 1.6 mg cell protein per 10^6 cells; 0.5 ml 3 *N* perchloric acid added per ml of cells) was injected and chromatographed as above. Perchloric acid was removed by neutralization of sample with potassium hydroxide and centrifugation immediately before injection. Detector was at 0.01 sensitivity.

TABLE I

COMPARISON OF DETERMINATIONS BY HPLC METHOD TO REPORTED VALUES FOR FLUOROMETRIC METHOD

	HPLC* (nmol/10^6 cells)	*n*	Fluorometric** (nmol/10^6 cells)
NADP+	1.47 ± 0.27	8	1.03
NAD+	11.8 ± 2.2	8	8.7
NADPH	4.12 ± 0.45	7	3.24
NADH	1.49 ± 0.24	7	1.05

*Data obtained by methods as shown in Figs. 1 and 2 for isolated rat liver cells.
**Data from Williamson and Corkey [3] converted from perfused liver data (μmol/g dry weight) using 3.7 g wet weight per g dry weight and 10^6 cells per 10 mg wet weight. Sums of values, NAD+ plus NADH, and NADP+ plus NADPH, measured by the fluorometric method [8] for isolated liver cells were higher than those shown above for perfused liver, presumably due to the elimination of the vascular space in the cell preparation.

for NAD+ and NADP+ contents in isolated liver cells by this method (Table I) correspond well with previously reported values.

Separation of NADH and NADPH in alkaline extract of cell incubations is most readily visualized at 340 nm (Fig. 2) due to lack of interference by

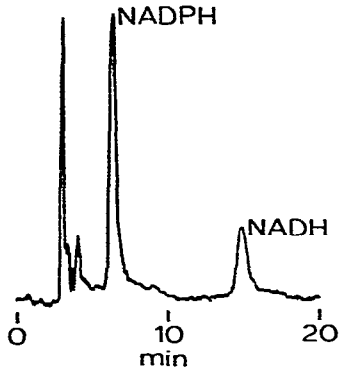

Fig. 2. Separation of NADPH and NADH in extracts of isolated liver cells. An 80-μl aliquot of ethanolic potassium hydroxide extract of cells (10^6 cells/ml) was injected on a column run with the following program: 5 min at 50% Buffer A, 10 min gradient to 25% Buffer A, hold 4 min at 25% Buffer A. Recycle time was 1 min to 50% Buffer A and 10 min before next injection. Detector sensitivity was 0.01.

other chromophores. Detection is more sensitive at 260 nm due to the greater extinction coefficient; however, interference by other chromophores is pronounced so that measurement at 340 nm is preferred. Retention times for these peaks correspond to those for standards, and the peaks are destroyed by acid treatment. Values obtained with this method are comparable to reported values for the fluorometric method (Table I).

The current approach to pyridine nucleotide quantitation in cell extracts offers a simple alternative to the enzyme-coupled fluorometric assays. Total run time for determination of NAD^+, NADH, $NADP^+$, NADPH, ATP, ADP and AMP is slightly more than 1 h, and thus, for large numbers of samples probably offers no time advantage. However, for smaller numbers of samples or under conditions in which compounds may be present that interfere with the fluorometric assays, this HPLC method offers a reliable and sensitive approach.

ACKNOWLEDGEMENTS

This research was supported by NIH grant GM 28176 and by American Heart Association Grant-in-Aid 80-902 with funds contributed in part by the Georgia Affiliate.

REFERENCES

1 T.Y. Aw and D.P. Jones, in preparation.
2 P.D. Schweinsberg and T.L. Loo, J. Chromatogr., 181 (1980) 103—107.
3 J.R. Williamson and B.E. Corkey, Methods Enzymol., 13 (1969) 434—513.
4 H.U. Bergmeyer (Editor), Methods of Enzymatic Analysis, 2nd ed., Academic Press, New York, 1974.
5 E. Nissinen, Anal. Biochem., 106 (1980) 497—505.
6 J.R. Miksic and P.R. Brown, J. Chromatogr., 142 (1977) 641—649.
7 P. Moldeus, J. Hogberg and S. Orrenius, Methods Enzymol., 51 (1978) 60—71.
8 M.E. Tischler, D. Friedrichs, K. Coll and J.R. Williamson, Arch. Biochem. Biophys., 184 (1977) 222—236.
9 D.P. Jones and H.S. Mason, J. Biol. Chem., 253 (1978) 4874—4880.