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ARTICLE *in* ANALYTICAL BIOCHEMISTRY · JULY 2004

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Critical study of preanalytical and analytical phases of adenine and pyridine nucleotide assay in human whole blood

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Received 10 November 2003
Available online 10 May 2004

Abstract

Intracellular redox and energetic status play a crucial role in cardiovascular diseases and metabolic disorders. The physiological status of reducing agents, such as NADPH and NADH, and of high-energy molecules, such as ATP, is required for antioxidant system activity. For these reasons, an accurate measurement of adenine and pyridine nucleotides is fundamental. In this study we examined the preanalytical phase of reduced pyridine (RPN) and adenine and oxidized pyridine (AOPN) nucleotide assay in human whole blood. Different experimental conditions were applied to RPN alkaline and AOPN acid extracts to find the best analytical performance. Our results show that a good RPN and AOPN linearity (r from 0.994 to 0.999), recovery (near to 100%), and precision (coefficient of variation $<5\%$) were obtained when supernatant from acid and ultrafiltrate from alkaline extracts were neutralized, frozen, and thawed just before HPLC injection. Since NADH decays rapidly at $-80\,^{\circ}C$, RPN levels must be assayed within 72 h while AOPN can be stored for 1 month at the same temperature. An accurate and quantitative method for nucleotide determination can be obtained by applying the preanalytical conditions proposed in this study.

Keywords: ATP; ADP; AMP; NADPH; NADP; NADH; NAD; Reduced pyridine nucleotides; Adenine and oxidized pyridine nucleotides

Recently the role of nucleotides in the cellular antioxidant system has been greatly emphasized [1,2]; intracellular pyridine nucleotides play an important role in energy transduction, signaling pathways, and the antioxidant system [3–5], particularly in many cardiovascular diseases in which oxidative stress is involved, such as in heart failure, myocardial ischemia, unstable angina [6–8], and ischemia reperfusion injury [9,10]. Nucleotide status provides biochemical and clinical information on cardiovascular diseases [1,6–13], cellular energetic alterations, and redox metabolism disorders [4,14,15]. Furthermore, alteration of the nucleotide level characterizes different enzymatic deficiencies in human erythrocytes [14].

NAD(P) may serve as a substrate for the synthesis of pyridine nucleotide derivates in the regulation of cytosolic calcium affecting cardiomyocyte contractility [3]. The physiopathological involvement of these molecules makes analytical measurement necessary.

In the last decade several methods were proposed for pyridine and adenine nucleotide determination, particularly HPLC methods, with single or double biological sample extraction as the preanalytical phase [1,3,9,10,16–19]. More recently, other methods were suggested, such as a spectrophotometric method to measure intracellular NAD(P)H [20], capillary zone electrophoresis to determine ATP whole blood [21], and multinuclear high-resolution NMR spectroscopy to evaluate purine and pyrimidine nucleoside ditriphosphates [22]. However, the preanalytical phase of nucleotide assay from whole blood or homogenized tissue has not been sufficiently analyzed. The preanalytical phase is a critical point for assessing the in vivo concentration of a biochemical molecule and a correct sample management is required to achieve nucleotide stability before analytical assays. To this purpose, we analyzed sampling modalities, extraction,

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doi:10.1016/j.ab.2004.03.063

storage, and nucleotide determination in human whole blood under different experimental conditions.

Materials and methods

All chemicals were purchased from Merck (Darmstadt, Germany), while nucleotide and coenzyme standards of the highest available grade were purchased from Sigma Chemical (St. Louis, MO). Bidistilled and deionized water (18 MΩ of conductance) was obtained by MilliQ system (Millipore; Molsheim, France), HPLC-grade methanol from JT Baker (Deventer, Holland); mobile phases for chromatographic analysis were degassed by ultrasonication. The instrument used consisted of a HPLC pump (Varian ProStar 240, Palo Alto, CA), coupled to an autosampler (Varian ProStar 410) with a Peltière temperature control and a variable-wavelength UV-VIS detector (Varian ProStar 310, 15-μL flow cell). Chromatograms were automatically integrated by a dedicated software (Varian Star) and the concentrations were calculated by generated calibration curves.

Determination of adenine and oxidized pyridine nucleotides in whole blood

Acid extraction of whole blood

Evaluation of AOPN[1] [ATP, ADP, AMP, and NAD(P)] was obtained by acid extraction from whole blood. A volume of K^+/EDTA-treated whole blood was added to 2 vol of 7.2% $HClO_4$ (acid extract) and immediately mixed and centrifuged at 14,000*g* for 10 min at 4 °C. The supernatant was neutralized at pH 6.5 by adding a 75% volume of 1 M borate buffer at pH 11 containing 4 mM EDTA.

Chromatographic condition

The mobile phase consisted of two eluants: 0.1 M KH_2PO_4 solution at pH 6.0 (Buffer A) and a 0.1 M KH_2PO_4 solution at pH 6.0, containing 10% (v/v) of CH_3OH (Buffer B). All buffer solutions, after preparation and pH adjustment, were filtered through a 0.22-μm Millipore filter. The chromatographic conditions were as follows: 9 min at 100% of Buffer A, 6 min at up to 25% of buffer B, 2.5 min at up to 90% of Buffer B, 2 min at up to 100% of Buffer B, and held for 6 min. The initial condition was then restored and held for 9.5 min before the next injection. The flow rate was 1 ml/min and detection was performed at 254 nm. Separation was performed on a 5 μm Discovery C18 analytical column (250 × 4.6 mm i.d., Supelco, Sigma–Aldrich) equipped with a Chromolith Performance RP-18e as a guard column (100 × 4.6 mm i.d., Merck). The samples before injection into the column were maintained at 4 °C by the autosampler temperature control system (sample volume was 40 μL).

Recovery

The analytical recovery was performed by adding a known amount of AOPN [standard solution consisted of ATP, ADP, AMP, NADP, and NAD at three different concentrations (0.05, 0.5, or 1.0 mM) in 0.1 M KH_2PO_4 at pH 6.5] or a blank solution (0.1 M KH_2PO_4 at pH 6.5) to pooled whole blood (PWB). The samples were treated as described above. Analytical recovery was calculated as

$$\begin{aligned}&\text{Recovery}\,\% \\ &= 100 \times \{[\text{nucleotide}\,(\text{PWB} + \text{nucleotide standard})] \\ &\quad - \text{nucleotide PWB}\}\,(\text{nucleotide standard})^{-1},\end{aligned}$$

where nucleotide PWB is the basal nucleotide sample concentration and nucleotide (PWB + nucleotide standard) is the measured nucleotide concentration of the sample, spiked with three different nucleotide standard levels.

Linearity

In order to test the linearity of the method we injected increasing concentrations of standard solutions treated with the same procedure as whole blood. Standard solutions of AOPN in 0.1 M KH_2PO_4, pH 6.5, at different concentrations ranging from 0 to 2 mM were processed as described above (acid extraction).

Detection limit

The absolute detection limit was determined as three times the baseline noise level.

Precision

The within-run precision ($N = 10$ aliquots of clear supernatant immediately frozen in liquid nitrogen and stored at −80 °C) was evaluated under four experimental conditions (α, β, γ, and δ): (α) aliquots of the neutralized supernatants were thawed, placed simultaneously into the refrigerated autosampler, and injected into the HPLC system in the following 5–6 h; (β) aliquots of the neutralized supernatants were thawed and placed into the autosampler just before injection into a HPLC system; (γ) aliquots of the supernatants were thawed and neutralized just before injection into a HPLC system; (δ) aliquots of the supernatants were thawed and simultaneously placed into an autosampler maintained at 4 °C and then neutralized just before injection.

The between-day precision was evaluated both on acid extracts and on supernatants. Aliquots of acid extract from the same blood sample, immediately frozen in liquid nitrogen and stored at −20 and at −80 °C, were assayed to measure nucleotides at 0, 1, 4, 7, 14 days, and

[1] *Abbreviations used:* RPN, reduced pyridine nucleotides; AOPN, adenine and oxidized pyridine nucleotides; CV, coefficient of variation; PWB, pooled whole blood.

1 month after blood sampling. Neutralized clear supernatant aliquots, from the same acid extract, immediately frozen and stored at −20 °C and at −80 °C, were assayed to measure nucleotides at 0, 1, 2, 6, 13, and 20 days from blood sampling.

Determination of reduced pyridine nucleotides in whole blood

Alkaline extraction of whole blood

Evaluation of RPN [NAD(P)H] was obtained by alkaline extraction from whole blood. A volume of K^+/EDTA-treated whole blood from healthy volunteers was mixed with a volume of cold 0.5 M KOH (alkaline extract). The mixture, treated with 2 vol of cold distilled water and mixed for 2 min, was divided into aliquots (50 or 100 μL) which were loaded on Amicon ultrafiltration membranes and centrifuged at 14,000*g* for 40 min at 4 °C. Ultrafiltrates were mixed and neutralized with 10% volume of 1 M KH_2PO_4 at pH 6.5.

Chromatographic condition

Only gradient condition (5 min at 100% of Buffer A, 6 min at up to 25% of buffer B, 2.5 min at up to 60% of Buffer B, 5 min at up to 100% of Buffer B, and held for 7.5 min) and spectrophotometric detection (340 nm) were different from AOPN determination. Sample volume injected was 50 μL.

Recovery: influence of ultrafiltration time

Whole blood samples from healthy volunteers were spiked with NAD(P)H standard solutions at fixed concentrations to reach a final concentration of 55 μM of NAD(P)H or with blank solution (0.1 M KH_2PO_4, pH 6.5) and were submitted to an alkaline extraction procedure as described above. The mixture was divided into sets of aliquots (eight 100-μL aliquots for each set) which were loaded on CF 10A, CF 30A, and CF 50A Amicon membranes and centrifuged at 14,000*g* at 4 °C for 10, 25, and 40 min. Ultrafiltrates were neutralized before injection, as reported above. The effect of ultrafiltration phase duration was evaluated comparing the recovery obtained from the sets of aliquots loaded on the same ultrafiltration membrane with a standard solution at the same NAD(P)H final concentration. Recovery was calculated as previously described for AOPN recovery.

Recovery: influence of different ultrafiltration conditions

Reduced nucleotide recovery values of the ultrafiltration phase were considered comparing (a) different ultrafiltration membrane pore sizes, (b) different RPN final concentration values, and (c) different loading volumes of alkaline extract. Whole blood samples were spiked with NAD(P)H standard solutions at different concentrations (110 and 1100 μM NAD(P)H in 0.1 M KH_2PO_4, pH 6.5) or with blank solution (0.1 M KH_2PO_4, pH 6.5). Blood samples were then submitted to alkaline extraction with 1 vol of 0.5 M KOH and 2 vol of cold distilled water. Fifty and 100 μL mixtures were loaded on 10, 30, and 50 kDa Amicon membranes and centrifuged at 14,000*g* for 40 min at 4 °C. Ultrafiltrates were neutralized with 1 M KH_2PO_4 (10%) at pH 6.5 before HPLC injection [10 and 100 μM NAD(P)H, fc]. Recovery was calculated as reported above.

Linearity

In order to test the linearity of the method, increasing concentrations of standard solutions treated with the same procedure as whole blood were injected into a HPLC system. Standard solutions of RPN in 0.1 M KH_2PO_4, pH 6.5, at different concentrations ranging from 0 to 1 mM were processed as described above (alkaline extraction).

Detection limit

The absolute detection limit was determined as described above.

Precision

RPN within-run precision was evaluated as described above for AOPN. Between-day precision was evaluated measuring RPN levels at different time points for 1 month both in alkaline extract and in ultrafiltrate. A volume of whole blood from two healthy subjects was treated with a volume of cold 0.5 M KOH, mixed and divided into aliquots, which were frozen in liquid nitrogen, and stored at −80 °C. All the aliquots were thawed, ultrafiltered on CF 10A Amicon membranes for 40 min at 4 °C, neutralized, and injected into HPLC at 0, 1, 4, 7, 14, 21, and 30 days from blood sampling. Ultrafiltrate aliquots, obtained from the same blood sample, neutralized both before (A) and after −80 °C storage (B), were thawed and injected at 0, 1, 3, 4, and 7 days from blood sampling.

Statistical analysis

Data were reported as means ± SD. Linear regression analysis of means was tested to evaluate the regression, slope, and constant standard error in linearity experiments. The statistical significance of the means of RPN recovery values was tested with Mann–Whitney and Wilcoxon tests for unpaired and paired data, respectively. P values <0.05 were considered statistically significant.

Results

Typical chromatograms of AOPN and RPN from standard solution (panel A) or from blood sample (panel B)

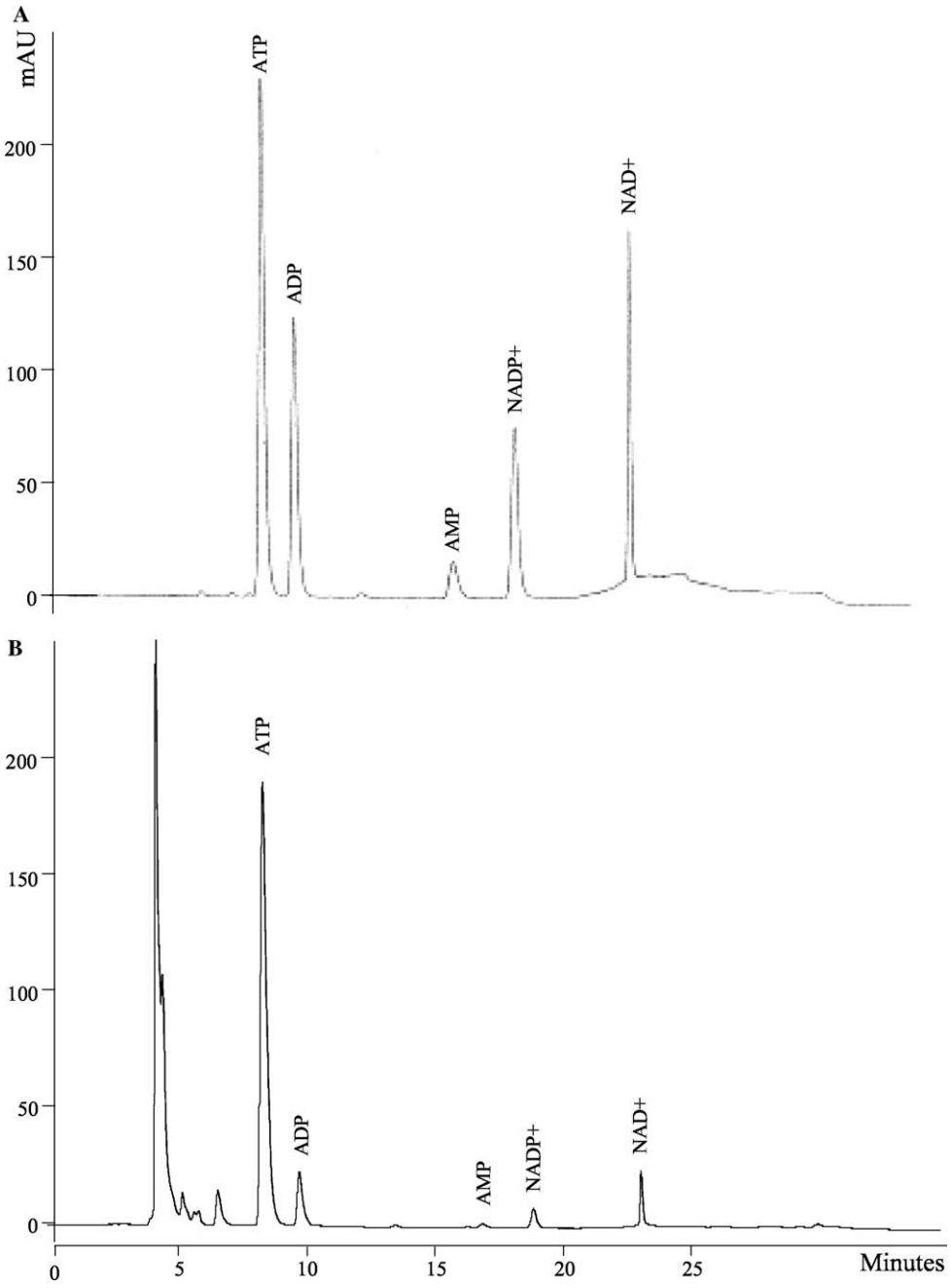

Fig. 1. HPLC separation of adenine and oxidized pyridine nucleotides from standard solution (A) and whole blood sample (B).

are shown in Figs. 1 and 2, respectively. Peaks were well separated within 23–24 min without interfering compounds.

AOPN assay performance: preanalytical procedure

The acid extraction procedure on whole blood sample shows a good AOPN recovery ($\geqslant$90%, Table 1). AOPN linearity was optimal from 5 to 2000 μM (Table 2), except for NADP (from 5 to 500 μM). Under this condition the standard errors (SE) of regression, slope, and intercept values were negligible (SE < 1%, Table 2). AOPN detection limit was 0.5 μM for all nucleotides.

Within-run precision

The AOPN CVs were <5% in treatments α [except for AMP (CV > 10%)], β, and γ, and only for ATP and NADP in treatment δ (Table 3). In treatment δ, an increase in ADP and AMP levels (84.5 and 46.7%, respectively) and a decrease in NADP and NAD concentrations (13.5 and 15.5%, respectively) were observed after 5 h at 4 °C.

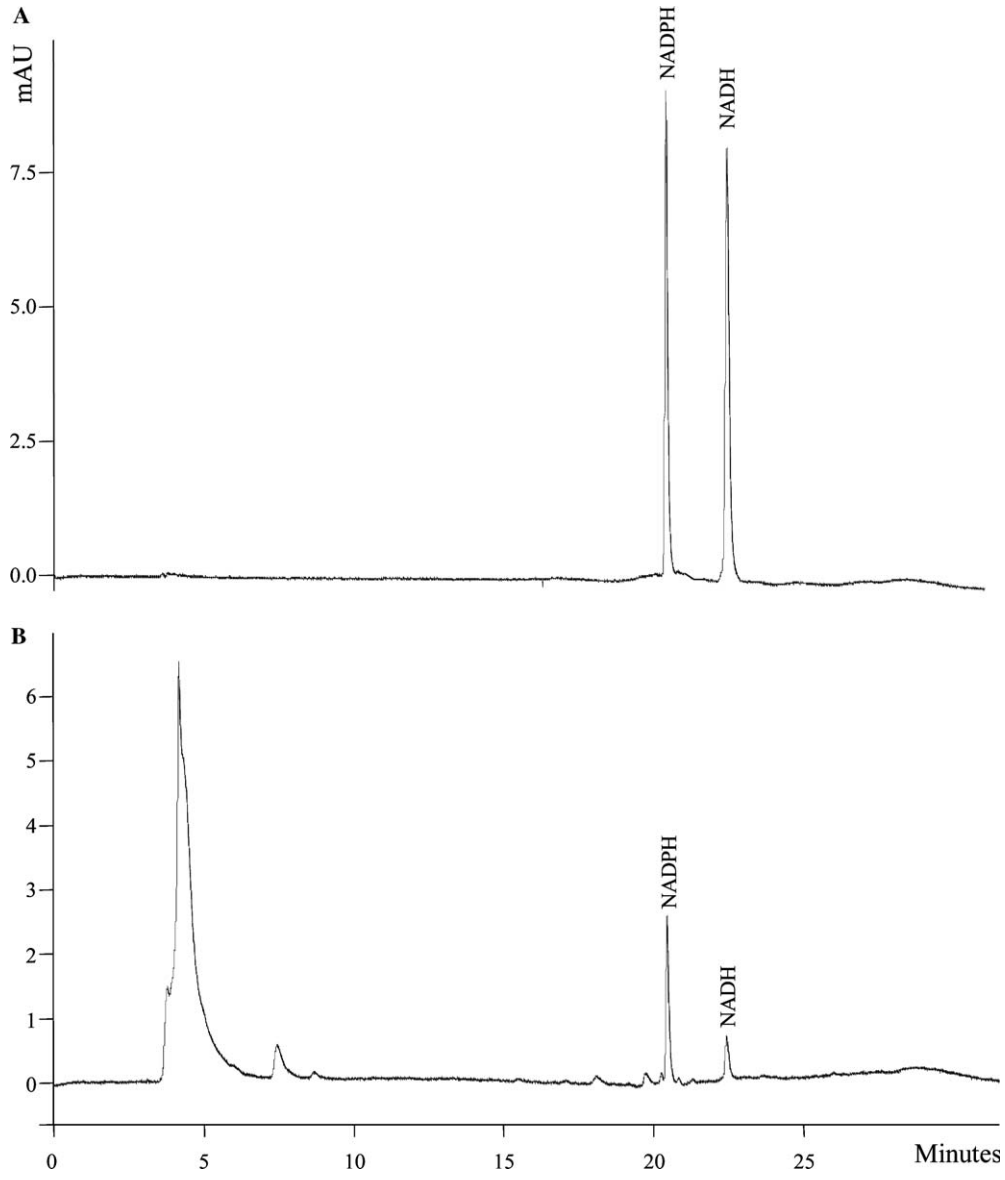

Fig. 2. HPLC separation of reduced pyridine nucleotides from standard solution (A) and whole blood sample (B).

Table 1
Recovery of adenine and oxidized pyridine nucleotides from human whole blood

Nucleotide	AOPN levels[a]		
	A	B	C
ATP	93.4 ± 2.0	95.5 ± 2.2	93.4 ± 2.3
ADP	100.8 ± 1.1	99.1 ± 1.1	97.4 ± 1.6
AMP	100.1 ± 0.9	98.9 ± 1.4	96.0 ± 1.8
NADP	89.2 ± 12.7	96.9 ± 2.6	95.6 ± 1.4
NAD	100.4 ± 6.5	99.3 ± 0.8	97.5 ± 2.2

Mean ± SD of variation coefficient (%) of three experiments.
[a] AOPN levels: A, 0.05 mM; B, 0.5 mM; C, 1.0 mM.

Between-day precision

ATP level was stable (CV < 5%, Table 4) in acid extract for 30 days when the sample was stored at −80 °C, but it decayed just after a few days when stored at −20 °C. ADP and AMP levels increased progressively in acid extract during the first day after blood sampling, while NADP and NAD values were preserved (CV < 10%) for 4 days both at −20 and at −80 °C.

The AOPN levels in neutralized supernatant were stable for 13 days at low temperatures (set A and B, Table 5), except for AMP, the levels of which changed just after blood sampling (CV > 10%).

RPN assay performance: preanalytical procedure

Reduced adenine nucleotide recoveries, obtained under different conditions of ultrafiltration, were higher than 100% (Table 6) and not significantly affected by RPN sample concentrations. NAD(P)H recoveries were affected by ultrafiltration membrane pore sizes only if

Table 2
Linearity of nucleotides HPLC assay

Nucleotide	R	Standard error (%)			R	Standard error (%)		
		Regression	Slope (10^{-7})	Constant		Regression	Slope (10^{-7})	Constant
	Range: 0–500 μM				Range: 0–2000 μM			
ATP	0.999	0.92	5.80	0.25	0.999	1.27	1.93	0.28
ADP	0.999	0.71	3.72	0.20	0.999	2.36	2.94	0.52
AMP	0.999	0.63	2.80	0.17	0.999	1.83	1.99	0.41
NADP	0.999	0.96	4.21	0.27	0.935	30.68	3.74	7.14
NAD	0.999	0.60	2.31	0.17	0.999	1.61	1.51	0.36
	Range: 0–250 μM				Range: 0–1000 μM			
NADPH	0.994	2.21	44.99	0.64	0.992	9.28	36.53	2.23
NADH	0.997	1.50	27.49	0.42	0.991	9.71	36.74	2.20

Table 3
Nucleotide within-run precision

Nucleotide	Treatment of supernatant[a]			
	α	β	γ	δ
ATP	1.7	1.1	2.4	2.4
ADP	3.9	3.9	2.0	20.4
AMP	18.6	2.7	2.6	28.6
NADPH	14.7	1.3	4.9	7.2
NADP	1.9	1.0	2.2	4.2
NADH	24.9	1.4	4.1	6.3
NAD	3.9	1.5	2.2	11.2

Data are expressed as coefficient of variation (%).
[a] Treatment of supernatant is detailed under Precision.

Table 4
Nucleotide between-day precision in extract

Nucleotide	Storage[c]	Time of storage (day)					
		1	4	7	14	21	30
ATP[a]	A	4.4	12.1	20.2	30.6		34.8
	B	3.3	2.9	3.5	3.1		3.8
ADP[a]	A	19.5	52.6	56.0	54.4		149.7
	B	3.3	11.7	16.2	21.9		28.4
AMP[a]	A	14.0	75.2	91.7	98.8		204.1
	B	8.1	23.2	19.1	16.7		31.8
NADP[a]	A	3.8	9.4	13.2	52.3		77.4
	B	1.8	2.2	8.2	16.2		18.0
NAD[a]	A	2.3	3.4	4.5	13.6		100.2
	B	2.3	2.4	4.5	5.5		14.9
NADPH[b]	B	4.0	6.5	6.5	8.5	8.0	7.5
NADH[b]	B	5.0	9.5	11.0	12.5	14.0	22.0

Data are representative of coefficient of variation (%) of two duplicate experiments.
[a] Nucleotides in acid extract.
[b] Nucleotides in alkaline extract.
[c] Storage: A, −20 °C; B, −80 °C.

samples were ultrafiltrated for 10 min, but improved by increasing time from 10 to 40 min (Table 7). A good NAD(P)H linearity was observed from 0.8 to 250 μM (Fig. 3). Under this condition lower standard error values were obtained (Table 2). NADPH and NADH minimal concentrations detected were 1.3 and 0.9 μM, respectively.

Table 5
Adenine and oxidized pyridine nucleotide between-day precision in neutralized supernatant

Nucleotide	Storage[a]	Time of storage (day)				
		1	2	6	13	20
ATP	A	0.5	2.9	2.4	1.9	3.1
	B	0.6	2.9	2.2	1.9	2.6
ADP	A	1.8	2.5	9.9	11.6	19.2
	B	4.5	4.1	7.0	4.0	12.7
AMP	A	32.4	38.8	39.2	31.0	63.9
	B	32.4	26.8	32.0	33.3	43.3
NADP	A	0.0	5.3	7.4	8.9	17.2
	B	2.2	3.7	8.7	9.5	17.6
NAD	A	0.9	3.6	5.1	6.4	13.2
	B	5.8	4.8	6.3	7.4	13.0

Data are expressed as coefficient of variation (%) of two duplicate experiment.
[a] Storage: A, −20 °C; B, −80 °C.

Within-run precision

The RPN CVs were >10% in treatment α, <5% in treatments β and γ, and <10% in treatment δ (Table 3). For treatment α, a decrease in NADPH and NADH (34 and 52%, respectively) was observed after 5 h at 4 °C.

Between-day precision

NADPH between-day precision showed a CV < 10% up to 30 days when blood samples were treated and stored in alkaline extract, but decreased after 4 days when samples were stored at −80 °C as ultrafiltrate. NADH between-day precision progressively decreased after 4 days in alkaline extract (Table 4), while it decreased very rapidly just after 1 day, if samples were stored both as neutralized ultrafiltrate (CV = 15.7%) and as ultrafiltrate (CV = 13.7%).

Table 6
Recovery of reduced pyridine nucleotides under different ultrafiltration phase conditions from human whole blood

Ultrafiltration membrane pore sizes (kDa)							
Final concentration (μM)	Loading volume (μL)	Recovery (%)					
		10		30		50	
		NADPH	NADH	NADPH	NADH	NADPH	NADH
10	50	115.6 ± 34.5	101.8 ± 22.8	125.1 ± 10.4	116.8 ± 8.6	113.5 ± 14.6	115.5 ± 7.5
	100	114.9 ± 5.6	107.4 ± 3.7	114.9 ± 14.1	114.1 ± 17.7	111.5 ± 8.4	104.5 ± 2.2
100	50	134.9 ± 24.0	119.7 ± 16.8	134.8 ± 11.5	122.8 ± 12.7	122.1 ± 5.5	116.7 ± 10.3
	100	126.9 ± 18.9	118.3 ± 11.7	124.6 ± 1.4	115.6 ± 1.1	120.0 ± 8.4	112.1 ± 7.1

Mean ± SD of variation coefficient (%) of three duplicate experiments.

Table 7
Recovery of reduced pyridine nucleotides at different ultrafiltration times from human whole blood

Time (min)	Ultrafiltration membrane pore sizes (kDa)					
	10		30		50	
	NADPH	NADH	NADPH	NADH	NADPH	NADH
10	81.8 ± 4.7*	83.3 ± 1.3*	71.5 ± 8.5*	71.9 ± 5.5*	95.7 ± 1.1	92.7 ± 1.2**
25	93.2 ± 5.4**	88.8 ± 1.4**	99.8 ± 11.8	94.9 ± 7.3	103.0 ± 2.1	96.3 ± 1.4
40	104.5 ± 6.0	98.8 ± 1.6	97.3 ± 11.5	92.5 ± 7.1**	117.0 ± 6.1**	105.7 ± 4.8

Mean ± SD of variation coefficient (%) of three experiments.
* $P < 0.01$ vs time 0 min.
** $P < 0.05$ vs time 0 min.

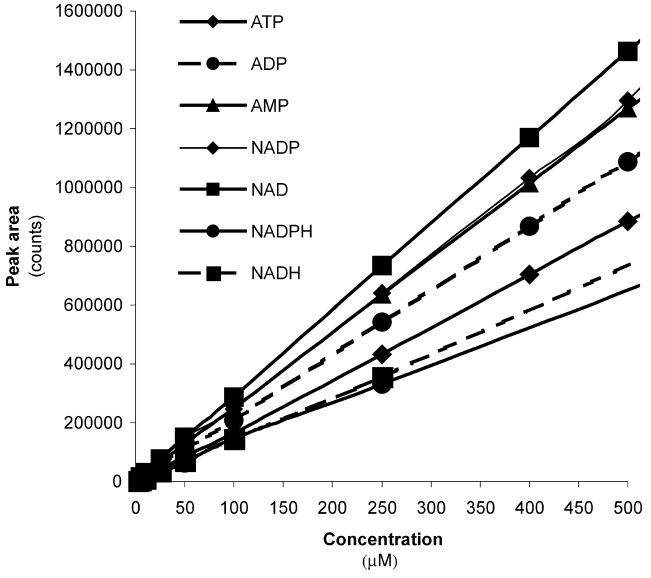

Fig. 3. Linearity of both adenine and oxidized pyridine nucleotides in standard solution at different final concentrations ranging from 5.0 to 500.0 μM (5, 10, 25, 50, 100, 250, and 500 μM of AOPN were tested) and reduced pyridine nucleotides in standard solution at different final concentrations ranging from 0.8 to 250.0 μM (0.8, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0, and 250.0 μM of RPN were tested).

Discussion

We investigated the preanalytical phase of blood adenine and pyridine nucleotide determination, a crucial point for their quantitative assay. Unlike other studies that proposed a single extraction procedure followed by chromatography [15,16,23], we chose to apply a double extraction procedure and two different chromatographic runs (in agreement with other authors), using a modified method [23]. This double extraction procedure allowed us to obtain better nucleotide chromatographic separation than the single one [16,17], without interference peaks. Different neutralizing agents were tested to evaluate pH stability of AOPN supernatant and RPN ultrafiltrate before HPLC analysis. The 1 M borate buffer makes stable the final 6.5 pH of AOPN supernatant, while other neutralizing agents, such as carbonate buffer [14,23] or KOH [17], appear to be inadequate for this purpose. In fact, the carbonate buffer, even in a cold environment, produces CO_2 which causes pH instability while KOH makes it difficult to reach the optimal pH. For RPN ultrafiltrate, we used 1 M KH_2PO_4, pH 6.5, as neutralizing agent [14].

Treatments β and γ were the most accurate preanalytical phases to obtain a within-run precision CVs < 5% both for AOPN and for RPN. Treatments α and δ instead were more suitable for routine use. In fact, the AOPN within-run precision obtained in treatment α shows a CV < 5%, except for AMP (CV = 18.6%). This behavior is probably due to minimal decay of ATP and ADP that strongly increases the physiologically low levels of AMP. Within-run precision CV of RPN was <10% only in treatment δ because a neutral environment (treatment α) probably causes NAD(P)H instability. These different preanalytical conditions (α and δ)

make double extraction and chromatography necessary, unlike what is reported in other studies [15,16,23].

With regard to nucleotide between-day precision, an ATP CV < 5% was obtained either with frozen acid extract or with neutralized supernatant, stored either at −20 or at −80 °C. ADP and AMP CVs increased after 30 days in acid extract; this behavior, which started just after 24 h [19], is probably due to a minimal ATP decay (3.2%) caused by γ-phosphate hydrolysis which produces a substantial increase in ADP and AMP concentrations. In healthy subjects, in fact, ADP and AMP blood levels are, respectively, 10 and 100 times lower than ATP blood levels.

All AOPN levels were stable when supernatant was neutralized and stored at −20 or at −80 °C. By contrast, RPN showed a better between-day precision in alkaline extract than in ultrafiltrate. Under this condition, NADPH levels were constant up to 1 month at −80 °C, while NADH levels were stable only for 4 days. Unlike Kalhorn et al. [24], we observed that NADH concentrations fell by 48.8% in 30 days. NADH instability is, therefore, the main limiting factor for long sample storage (not more than 72 h before HPLC analysis).

As regards RPN evaluation, the alkaline extract ultrafiltration procedure allowed us to obtain clear samples, without interfering compounds, suitable for HPLC analysis. Unlike other studies [16,23], we observed that RPN recovery increases by increasing ultrafiltration time and membrane pore size: 40 min and 10, 30, or 50 kDa membrane pore size were needed to obtain full recovery and to ultrafiltrate the maximum sample volume, but 10 min was enough if a 50 kDa membrane pore size was used. Different loading volumes of alkaline extract or different RPN levels did not influence the recovery. NAD(P)H concentrations were determined using standard increasing levels spiked in whole blood and processed as RPN alkaline extraction. This device allowed us to minimize RPN overestimation found under some ultrafiltration conditions, but it was not necessary for AOPN determination.

The choice of the preanalytical phase depends on the goal of the study: the best performance (recovery, linearity, and precision) was obtained with treatments β and γ, suitable for RPN and AOPN accurate analysis, but more time consuming. Treatment α for AOPN and treatment δ for RPN are instead indicated for routine use. For this purpose, it is advisable to ultrafiltrate RPN alkaline extract on 50-kDa membrane pore size for 10 min and store AOPN as neutralized supernatant at −20 °C.

In conclusion, the correct execution and optimization of the preanalytical phase is crucial to the accuracy and standardization of results, obtained using the procedure proposed above. Moreover, although specifically designed for a HPLC assay, this procedure may be used also for other analytical methods.

Acknowledgments

The contribution of Mrs. Mary Scurati was greatly appreciated.

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