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Short communication

Spectroscopic and chromatographic evidences of NADPH in human placental extract used as wound healer

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Abstract

An aqueous extract of human placenta, which is used as wound healer, has been investigated in terms of fluorescence properties. When excited at 340 nm, it results in fluorescence emission having maxima around 436 nm, which is fairly specific for nicotinamide adenine dinucleotide, reduced form (NADH) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). The excitation spectra, having emission at 440 nm, show patterns comparable to these nucleotides. Thin layer chromatography and reversed-phase (RP) HPLC confirm presence of only NADPH in the extract. The emission and excitation patterns of NADPH purified after HPLC resemble exactly with the reference compound. Its content has been estimated to be 0.018 ± 0.003 mg/ml based on fluorescence emission with respect to a standard calibration curve ($n = 6$). Biological functionality of NADPH in the extract has been confirmed by glutathione reductase assay ($n = 5$).

Keywords: Human placentae; Wound healing; NADPH; Fluorescence; TLC; Reversed-phase HPLC

1. Introduction

Placenta being the only discarded human organ, an extensive research has been done on it. Being a link between mother and fetus, it provides all nutrients and protective agents to the baby. It is a rich source of many biological and therapeutic components [1]. Developed from folk knowledge, fresh amniotic membrane of human placenta is used as a cover to acute burn injuries as a biological dressing [2–6]. An aqueous extract of placenta is used as a licensed drug in post surgical dressings, as a healer in burn injuries and chronic wounds under different trade name in many countries including India [1,6]. Knowledge on active components present in the extract and their mechanisms in wound healing is far from clear. During characterization of such extracts used as drug, a fluorophore has been detected which had excitation and emission properties similar to nicotinamide adenine dinucleotide, reduced form (NADH) and nicotinamide adenine dinucleotide phosphate, reduced form (NADPH). Since NADPH is known to regulate a number of phenomena related to wound healing [7–9], we have

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investigated its presence in different batches of the drug.

2. Experimental

2.1. Reagents

Human placental extract (hereafter called placental extract) was supplied by the drug house M/s Albert David Ltd. (15, Chittaranjan Avenue, Calcutta 700072, India), under the trade name 'Placentrex' which is manufactured under proprietary method. In short, fresh placentae were stored in ice and portions were tested for HIV antibody and Hepatitis B surface antigen. Single hot and cold aqueous extractions were done after incubating dissected and minced placenta at 90 and 6 °C, respectively. This was followed by sterilization of the extract under saturated steam pressure (15 psi at 120 °C for 40 min). Henceforth, heat treatment will be referred to exposure of a sample under such conditions. After filtration and addition of 1.5% (v/v) benzyl alcohol as preservative, ampoules were filled, then they were sterilized once again under the said condition for 20 min. In the first sterilization, the extended duration of heat treatment essentially completed precipitation of a number of macromolecules like proteins. This is apart from adding safety margins to the temperature, time or both to destroy most resistant spore-producing species like *Clostridium tetani*. The terminal sterilization step was to maintain sterility of the products after they were filled and sealed in ampoules. Each milliliter of the drug was derived from 0.1 g of fresh placenta. A single batch was prepared from the pool of several placentae. The preservative does not interfere with visible absorption and fluorescence patterns under the experimental conditions mentioned here.

NADH, NADPH, bovine serum albumin (BSA, fraction V), glutathione reductase (GR) and oxidized glutathione (GSSG) were from Sigma, USA. Aqueous solutions of both the nucleotides (1 mg/ml) were heat treated to generate thermally degraded products, if any; for testing. Ethanol (90%, rectified spirit) was from Bengal Chemicals and Pharmaceuticals Laboratory, Calcutta. Acetic acid (glacial, 99–100%), ethyl acetate (A.R.) and methanol (HPLC grade) were from E-Merck, India. Water used was first passed through the Millipore Elix-10 system followed by the Milli-Q system (cartridge cat #QTUM 001 IX) which yielded water of resistance 30 MΩ. Extinction coefficient of both NADH and NADPH were $6.3 \times 10^3\ M^{-1}\ cm^{-1}$ at 340 nm. Dispensing error was limited to ±3%.

2.2. Optical instruments

Fluorescence was recorded with a Hitachi F-4500 recording spectrofluorimeter with a 3 ml quartz cuvette. Excitation and emission slit widths were 5 nm. Optical absorbance were measured with a digital UV-Vis spectrophotometer (model No. Digispec-200 GL, SICO, India). Absorption spectra were scanned with Specord 200 (Analytica Jena, Germany) recording spectrophotometer.

2.3. Thin layer chromatography

Thin layer chromatography (TLC) were done with four solvent systems: (a) ethanol:ethyl acetate (70:30 v/v); (b) ethanol:water (90:10); (c) ethanol:acetic acid (90:10) and (d) ethanol:acetic acid:water (80:10:10) using 10 cm × 5 cm silica gel 60 F_{254} aluminium-impregnated TLC sheets (Merck, Germany). Aqueous solutions of NADH and NADPH (1 mg/ml) with or without heat treatment served as stocks from which 2.5–3.0 μl were applied on the plates. Placental extract was concentrated 20-fold under vacuum from which 8–10 μl was applied. Chromatograms were developed between 28 and 30 °C and nucleotides were viewed under short wavelength UV irradiation at 254 nm by mineralight lamp (Model No. UVGL-25, UVP, Upland, CA 91786, USA).

2.4. Reversed-phase (RP) HPLC

NADH and NADPH were analyzed using Waters reversed-phase C_{18} μ-Bondapak analytical column (7.8 mm × 300 mm; 125 Å, 10 μm) equilibrated with 10 mM K-phosphate buffer, pH 7.2. It was eluted isocratically with the same buffer at a flow rate of 1 ml/min and was monitored at 340 nm corresponding to the absorption maxima of the reduced nucleotides [10,11]. An aqueous solution of NADH or NADPH in 50 μl containing 5 μg of the nucleotide (with or

without heat treatment) or 20 μl of the placental extract as supplied, was applied to the column.

2.5. Glutathione reductase (GR) assay

A working solution of GR (50 U/ml) was made with the supplied enzyme in 50 mM K-phosphate buffer, pH 7.5 containing 1 mM EDTA and 1% BSA (used as stabilizer of GR). Assay was initiated in a 1.5 ml quartz cuvette at 25 °C by adding 400 μl of GSSG (from an aqueous stock of 2.4 mM), 350–400 μl of 125 mM K-phosphate buffer, pH 7.6 containing 2.4 mM EDTA, 200 μl of placental extract and 1.4 U of GR to a final volume of 1 ml. Alternately, placental extract was replaced by 50 μl of NADPH (from a stock of 1.28 mM) and water to make up the volume to 1 ml. Depletion of NADPH was estimated spectrophotometrically at 340 nm at ambient temperature between 28 and 30 °C [12].

3. Results

3.1. Absorption spectra

The placental extract as supplied was a colorless liquid. Its absorption spectra between 240 and 800 nm was scanned with Millipore water serving as blank. A 50-fold diluted extract with water after base line corrections revealed absorption maxima of 0.7 centered at 260 nm between 240 and 320 nm possibly due to the presence of polynucleotides [1]. Strong non-specific absorption in the far UV zone originated from bio-organic molecules. Weak but significant absorption of the undiluted drug was observed between 320 and 400 nm with a monotonous decrease of intensity. A representative profile has been provided in Fig. 1. Absorption at 340 nm was 0.161 ± 0.011 ($n = 6$).

3.2. Spectrofluorimetric analysis

When excited at 340 nm, there was an emission from the extract between 350 and 600 nm having maxima (em_{max}) at 436.5 ± 1 nm ($n = 6$) (tracing a, Fig. 2A). Gradual change of excitation wavelength between 340 and 360 nm resulted in decrease of emission intensity with red shift of em_{max} by 10 ± 1 nm indicating heterogeneity of the excitation chromophore or presence of interacting molecules affecting excited energy states [13,14]. Emission of standard NADH and NADPH (ex: 340 nm) showed an overlapping spectra between 350 and 600 nm having em_{max} at 450.0 ± 1.0 nm. The em_{max} of these compounds remained unchanged after altering excitation wavelength between 340 and 360 nm. The emission spectra of heat-treated NADH and NADPH, when normalized with the untreated products showed indistinguishable patterns (tracings b and c, Fig. 2A).

While the em_{max} of the extract was fairly constant at 436.5 ± 1.3 nm, spectral amplitudes were found to be variable and thus the symmetry of the spectra were evaluated. The fluorescence intensities (FI) at em_{max}, 400 nm (left to em_{max}) and at 500 nm (right to em_{max}) were variable by ±17.2, ±15.0, and ±12.3%, respectively from an average ($n = 6$). Symmetry of the spectra was indexed from the ratio of intensity at em_{max}

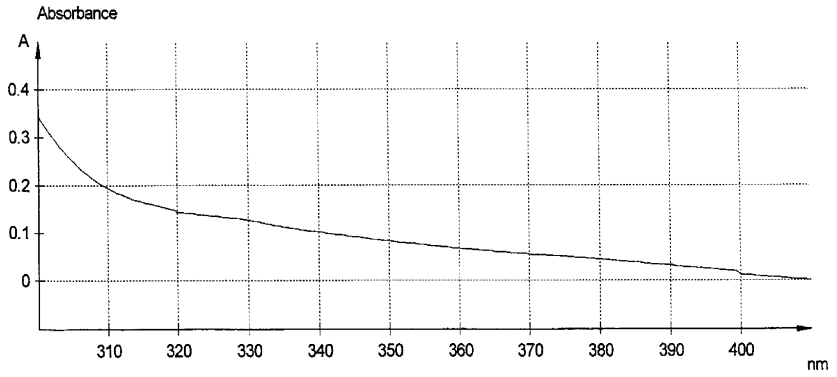

Fig. 1. Absorption spectra of placental extract between 300 and 400 nm.

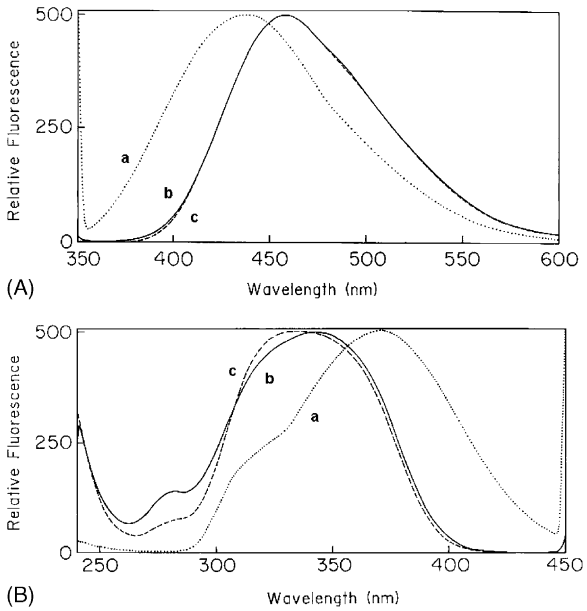

Fig. 2. (A) Emission spectra (ex: 340 nm) of (a) placental extract, (b) NADPH and (c) NADH. (B) Excitation spectra (em: 450 nm) of (a) placental extract, (b) NADPH and (c) NADH.

versus 400 nm or 500 nm and also among the two wavelengths mentioned. The values were 1.15 ± 0.04 (with ±4.2% variation from an average), 2.32 ± 0.13 (±5.56% variation) and 1.97 ± 0.11 (±5.5% variation) (Table 1).

The excitation spectra of placental extract between 250 and 450 nm (em: 450 nm) showed (excitation maxima) ex_{max} at 360.2 ± 1 nm ($n = 6$). Excitation spectra of standard NADH and NADPH (em: 450 nm) showed similar but non-overlapping patterns having ex_{max} at 342.3 and 345.4 nm, respectively (Fig. 2B). Thus, for the extract, a red shift and a blue shift of approximately 15 and 14 nm were observed in the excitation and emission patterns respectively relative to NADH or NADPH.

To exclude the rare possibility of fluorophore contamination as an artifact of manufacturing process, a batch was prepared from water instead of placenta under proprietary method. No fluorescence was observed with the blank batch.

3.3. TLC

In TLC with silica gel, four solvent systems were selected where the R_f of NADH and NADPH varied between 0.4 and 0.9 and their migrations were compared with the components of the extract ($n = 5$). In all sets, heat-treated nucleotides were included. It was agreed that in none of the chromatograms, NADH and NADPH were distinctly separated but the heat-treated samples had differences in R_f with the untreated standards. However, in all chromatograms, prominent spots corresponding to heat-treated NADH/NADPH were detected for the placental extract (Table 2). A representative picture of TLC has been shown in Fig. 3. Placental extract in addition to the nucleotide contained other materials as well which had different R_f ranging from 0 to 1

Table 1
Analysis of fluorescence emission spectra of different batches of the placental extract after excitation at 340 nm

Set[a]	em_{max} (nm)[b]	F.I em_{max}[c] [O]	F.I 400 nm[d] [A]	F.I 500 nm[e] [B]	[O]/[A][f]	[O]/[B][g]	[A]/[B][h]
1	435.2	309	265	141	1.16	2.18	1.87
2	436.0	402	334	164	1.20	2.44	2.03
3	435.6	314	271	143	1.15	2.19	1.89
4	436.4	290	245	128	1.18	2.26	1.91
5	437.1	365	331	158	1.10	2.31	2.09
6	437.5	375	339	162	1.10	2.30	2.08

[a] Sets represent different batches of the placental extract.
[b] em_{max} represents emission maxima.
[c] F.I represents fluorescence intensity in arbitrary units (a.u.), holding excitation and emission slit widths at 5 nm.
[d] F.I 400 nm represents fluorescence intensity at a wavelength left to em_{max}.
[e] F.I 500 nm represents fluorescence intensity at 500 nm, a wavelength right to em_{max}.
[f] Ratio of fluorescence intensity at emission maxima vs. 400 nm.
[g] Ratio of fluorescence intensity at emission maxima vs. 500 nm.
[h] Ratio of fluorescence intensity, at 400 nm vs. 500 nm.

Table 2
Silica gel TLC profiles of NADH, NADPH and the placental extract ($n = 5$)

Solvent system	R_f (nucleotides)	R_f (placental extract)
Ethanol:ethyl acetate (70:30)	NADH = 0.84 NADPH = 0.85 NADH(H)[a] = 0.75 NADPH(H) = 0.73	0.72, (0.80)[b]
Ethanol:water (90:10)	NADH = 0.79 NADPH = 0.79 NADH(H) = 0.65 NADPH(H) = 0.67	0.67
Ethanol:acetic acid (90:10)	NADH = 0.75 NADPH = 0.73 NADH(H) = 0.55 NADPH(H) = 0.54	0.54, (0.65)
Ethanol:acetic acid:water (80:10:10)	NADH = 0.47 NADPH = 0.85 NADH(H) = 0.44 NADPH(H) = 0.67	0.68, (0.79)

[a] (H) indicates aqueous samples after heat treatment.
[b] Value within parenthesis indicates minor spot.

in various solvents but the intensities was very low. Spots from placental extract had trailing and were diffuse possibly due to salts and other accompanying materials.

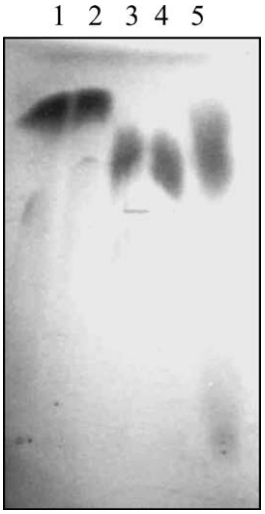

Fig. 3. Thin layer chromatographic profiles of NADH (lane 1); NADPH (lane 2); NADH (heat treated) (lane 3); NADPH (heat treated) (lane 4) and placental extract (lane 5). Solvent was ethanol:water = 90:10.

3.4. RP-HPLC

The RP-HPLC profile of placental extract monitored at 340 nm under defined conditions has been shown in Fig. 4. It shows occurrence of two major (retention time $R_t = 9.53 \pm 0.05$ and 11.42 ± 0.05 min) and two minor ($R_t = 13.5 \pm 0.06$ and 17.1 ± 0.07 min) peaks ($n = 4$). Retention of NADPH and NADH under identical conditions corresponded to 9.51 ± 0.05 and 20.1 ± 0.06 min, respectively (insets A and B, Fig. 4). Downward arrows have marked the positions of standard nucleotides in the original chromatogram. In a control experiment, it was verified that the retention times of heat-treated NADH and NADPH in HPLC were identical with those of standard samples without any significant detection of degraded product [15]. Thus, HPLC analysis strongly suggested presence and absence of NADPH and NADH in the extract respectively.

To confirm that the component of the drug eluting at 9.53 ± 0.05 min was indeed NADPH, its emission spectra between 350 and 550 nm (ex: 340 nm) and excitation spectra between 250 and 450 nm (em: 450 nm) were scanned. When these were normalized with that of standard NADPH, overlapping patterns were obtained (Fig. 5A and B). It was also verified that out

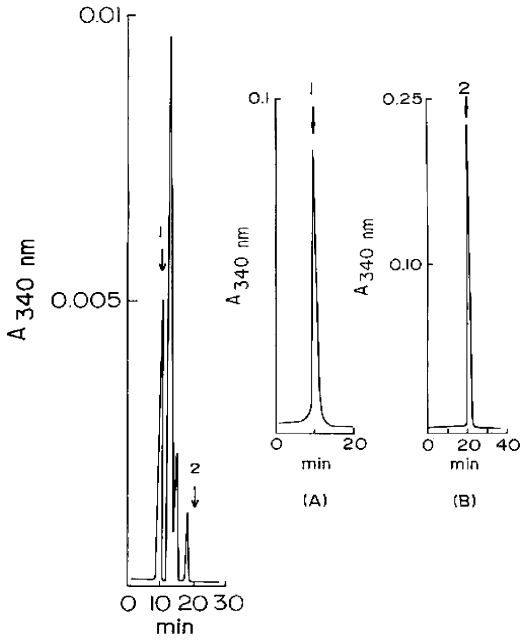

Fig. 4. Reverse phase HPLC profile of placental extract monitored at 340 nm. Retention time (R_t) of the four peaks were 9.5, 11.42, 13.5 and 17.1 min, respectively. Inset: (A) elution profiles of NADPH, $R_t = 9.53$ min and (B) NADH, $R_t = 20.1$ min. Positions of (A) and (B) in the original diagram have been marked as 1 and 2, respectively.

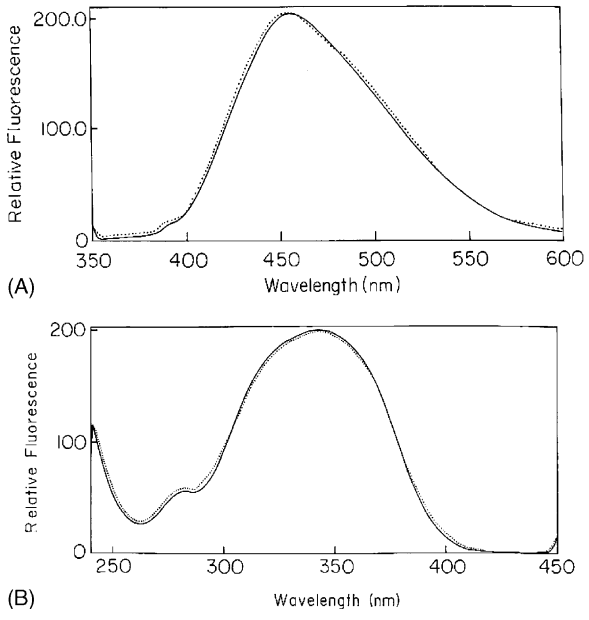

Fig. 5. (A) Emission spectra (ex: 340 nm) and (B) excitation spectra (em: 450 nm) of HPLC fraction of placental extract having the same retention time with standard NADPH. The profiles of NADPH, generated under identical conditions were indistinguishable with the placental extract and have been marked by dotted tracings.

of the four components separated in HPLC, only the peak corresponded to NADPH was fluorescent in the concerned zone.

3.5. Glutathione reductase assay

Under the conditions of assay, time-dependent decrease of absorption was observed at 340 nm; for example, with 200 μl of the extract a decrease of 40 ± 5 B.U. (1 B.U. = 0.001, $n = 4$) was obtained. However, both the rate and amplitude of the reaction were not linearly dependent on the extract concentration possibly because of presence of inhibitor/s in the extract.

3.6. Quantitation of NADPH

Once it was confirmed that NADPH was the only fluorophore in the extract emitting in the concerned spectral zone, attempt was made for its estimation. A calibration curve was constructed with NADPH between 2.5 and 20 nM in water against fluorescence intensity (ex: 340 nm; em: 450 nm; slit width 5 nm). A linear dependence was observed ($R^2 = 0.988$, R = regression coefficient) where variation of fluorescence intensities for each point was ±3%. Similar calibration curve was constructed with heat-treated NADPH using the same concentration zone where linear dependency was also observed ($R^2 = 0.972$). It was further noted that the change of fluorescence intensity with concentration of NADPH in either case was comparable by ±2.85% (ΔF.I./nM NADPH = 10.2 versus 10.8 for heat-treated sample). The placental extract also demonstrated linear dependency of fluorescence intensity with concentration under identical conditions ($R^2 = 0.979$). Assuming that there is no quencher molecule in the extract, a computation within these two linear zones yielded NADPH content of the extract to be 0.018 ± 0.003 mg/ml ($n = 6$).

Quantification of NADPH by glutathione reductase was not successful because when the extract was concentrated in an attempt to purify the nucleotide, a

fraction of its components were precipitated. Thus, while correlating, an underestimation of the nucleotide was observed from fluorescence estimation.

4. Discussion

While characterizing human placental extract used as a wound healer, it was observed that it contained an excitation and emission fluorophore, which was very similar to but not identical with NADH or NADPH (Fig. 2). An obligatory requirement for such fluorescence was absorption at 340 nm that had been verified from its absorption spectra (Fig. 1). Existence of either or both of the nucleotides have been indicated by silica gel TLC though the nucleotides could not be properly resolved (Fig. 3). In an attempt to distinguish between them, 5 cm × 10 cm PR-18 F_{254s} TLC plates (E. Merck) were also employed using 50% ethanol and 50% methanol as solvent systems under chromatographic conditions as stated earlier. In summary, the results were similar to silica gel TLC; heat-treated samples had minor difference in migration compared to the controls but NADH and NADPH remained indistinguishable. The placental extract in all TLC showed prominent spots corresponding to the heat-treated NADH/NADPH. RP-HPLC analysis of the extract clearly demonstrated presence and absence of compounds of identical retention time with that of NADPH and NADH, respectively (Fig. 4). When the fluorescence properties of the fraction corresponding to NADPH were characterized, they matched exactly with standard NADPH thus confirming its presence in the extract (Fig. 5). In retrospect, the deviation of the spectral properties of the crude extract from standard NADPH (Fig. 2), could safely be predicted due to the presence of other compounds. Emission spectra of the extract were analyzed in terms of its emission intensity and maxima, as well as symmetry pattern both at the left (400 nm) and right (500 nm) of em_{max}. While the fluorophore concentration was variable by ±17.2%, variation of the symmetry parameters was limited to 5.5% (Table 1).

TLC on silica gel or RP-plates suggested that both NADH and NADPH were resistance against decomposition or fragmentation under heat treatment but their migrations were different compared to controls. Though origin of the difference was not sought for, minor structural modifications leading to alteration of the degree of solvation or unstable intramolecular association might be the reasons. It has been verified that NADPH once exposed to heat treatment, was not biologically inactivated. Retention of biological functionality and fluorescence properties of NADPH or placental extract after heat treatment reinforced that the functional site of NADPH, i.e. the nicotinamide moiety is thermally stable as reported earlier [16]. Taking the advantage of fluorophoric homogeneity of the extract at the NADPH emission zone, the nucleotide content had been estimated from emission intensity with respect to NADPH calibration curve. It was found to be 0.018 ± 0.003 mg/ml. Reaction with glutathione reductase indicated biological potency of NADPH in the extract. In summary, consistent presence of NADPH in the extract has been demonstrated and its biological functionality has been confirmed. Since the role of NADPH in wound healing through varied mechanisms are well known [17–22], it is likely to act as an active component in the extract analyzed.

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