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Characterization and quantitation of the active polynucleotide fraction (PDRN) from human placenta, a tissue repair stimulating agent

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Abstract

The polydeoxyribonucleotide (PDRN) fraction is an extract which forms the active component in a new formulation of the drug Placentex (a tissue repair stimulating agent), obtained from human placenta through an original proprietory extraction method. From a comparison of the UV, NMR and IR spectra of this fraction (before and after nuclease treatment) with that of a similar standard (Sigma D1501), it was shown that the active substances in the PDRN fraction mainly consist of a mixture of DNA fragments. By gel electrophoresis, the molecular weights of the DNA fragments were shown to range from 50 to 2000 base pairs. Finally, an HPLC method is described, based on an anion-exchange material capable of determining the amount of PDRN in different batches of the extract, which varied from 80 to 90%.

Keywords: Electrophoresis; High-performance liquid chromatography; Human placenta; Polydeoxyribonucleotide

1. Introduction

A great variety of substances with biological and/or therapeutic activity, which are present in human placenta, have been isolated and identified as hormones, proteins, glycosaminoglycans (GAG), nucleic acids, etc. Although some of them can be chemically or biologically synthesized, many (nucleic acids and GAG, for example) can be obtained only by isolating them from a natural source. These extracts, with complex compositions, containing many components, depend on the extraction methods and consequently show different pharmacological activity.

The biological study of the fractions extracted from human placenta led to the choice of one of these fractions with specific pharmacological activity in stimulating the tissue repair process [1–6]. Since this specific activity is primarily due to a stimulating effect of the DNA fragments, which are known to allow the release of growth factors by the platelets [7,8], the amount of polydeoxyri-

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bonucleotide (PDRN) in the extract has been improved through a new extraction method (data not shown) which allowed us to obtain the final compound with specific therapeutic activity.

Up to now, the quality control of the placenta extracts involved classical analytical methods which were not satisfactory, being both time consuming and/or lacking in specificy. UV analysis, even though widely used to obtain quantitative responses, is non-specific, and the diphenylamine technique does not give information about the molecular weight distribution. Gel electrophoresis seems to have advantages over chromatographic methods, but the lengthy analysis time is not suitable for routine testing of industrial batches.

Hence a regulatory analytical procedure which would validate a specific HPLC method has been developed. This should be able to account for the polydeoxyribonucleotidic nature of the extracts and quantitate this PDRN fraction in the large amounts of samples that usually occur in industrial production.

2. Experimental

2.1. Reagents and standards

Calf thymus DNA, deoxyribonuclease I type II (DNase), ribonucleic acid type IV (RNA), ribonuclease A type I–AS (RNase), chondroitin sulphate A, B and C, hyaluronic acid and albumin were obtained from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl), sodium chloride, magnesium chloride, EDTA, boric acid and all other chemicals were purchased from Merck (Darmstadt, Germany).

The standards 1 kb DNA ladder, 123 bp DNA ladder and DNA Hae III fragments were obtained from Gibco and agarose from BRL.

2.2. Instrumentation

The HPLC equipment (Perkin-Elmer) consisted for a Model 250 Biocompatible Binary pump with a Rheodyne Model 7125 injection valve and a 20 μl loop and a Model 290 Bio UV–visible spectrophotometric detector. The data were processed with a PE Nelson Model 1020 integrator. A 100 × 4.6 mm i.d. Gen Pack Fax column (Waters) was thermostated at 60°C.

Electrophoresis was carried out on a EC Maxicell Submarine gel system equipped with a PS 500 XTDC power supply (Hoefer Scientific Instruments), a UVP transilluminator, a UVP camera, a Mitsubishi video-copy processor and UVP Gel Base GDS 5000 software.

^{1}H NMR spectra were obtained on a Bruker AMX 500 (500 MHz) spectrometer with tetramethylsilane (TMS) as internal standard.

IR spectra were recorded in potassium bromide disks on a Bruker IFS 25 spectrophotometer.

UV spectra were recorded in 95% ethanol on a Beckman DU 65 spectrometer in the range 400–200 nm.

2.3. Chromatographic conditions

To quantitate PDRN and to determine its molecular weight distribution, two separate methods were applied, both using buffer A (0.025 mM Tris–HCl adjusted to pH 8 with 1 M NaOH) and buffer B (0.025 mM Tris–HCl + 1M NaCl adjusted to pH 8 with 1 M NaOH) at 60°C with a flow rate of 0.7 ml min^{-1}. Method 1 was used to determine PDRN: 1 min with 60% buffer B–40% buffer A, linear gradient up to 100% buffer B in 10 min, 4 min with 100% buffer B. Method 2 was used to determine the molecular weight distribution: 1 min with 40% buffer B–60% buffer A, linear gradient up to 80% buffer B–20% buffer A in 30 min, 5 min with 80% buffer B.

A stock solution of placenta extract, concentration 500 μg ml^{-1}, was prepared to obtain the calibration curve. Aliquots of this solution were further diluted to obtain a series of solutions with concentrations of 100–500 μg ml^{-1}. The precision and accuracy were calculated from this curve. Recoveries are given in Table 1. Using the above data, the method response was shown to be proportional to concentration with a correlation coefficient $r^2 = 0.999$. The SD was 4.81 μg ml^{-1} and the RSD was 1.53%.

The reproducibility and stability of the method and samples were measured with three of these

Table 1
PDRN recovery

PDRN added (μg ml^{-1})	Mean recovery (μg ml^{-1})	Difference (%)
100	103.63	+0.95
200	199.32	−1.71
300	308.05	+0.87
400	397.66	−1.43
500	510.50	+2.36

solutions (Table 2). The results were obtained on different days by two different analysts, sometimes letting the samples stand for one day: the reproducibility (RSD) was $< 2\%$ and the assay stability was $> 98\%$.

A series of placenta extracts from industrial production were then analysed following chromatographic method I. The amount of PDRN was determined versus a standard sample according to the following equation

$$\text{Concentration (\%)} = \frac{A_i P_s}{A_s P_0} \cdot 100$$

where A_i = peak area given by an unknown placenta sample, P_s = weight of the placenta sample, A_s = peak area given by the standard and P_0 = weight of the standard.

Table 2
Reproducibility and stability of PDRN

	Concentration (μg ml^{-1})			SD[b]
	500	300	100	
RT (min)	8.497	8.398	8.457	0.050
	8.503	8.417	8.430	0.048
	8.497	8.430	8.437	0.037
	8.517	8.443	8.457	0.039
	8.530	8.423	8.437	0.058
	8.523	8.417	8.476	0.053
Average	8.51	8.42	8.45	
SD[a] (min)	0.01	0.02	0.02	
RSD (%)[a]	0.17	0.19	0.19	
Overall SD	0.041			

[a] Intra-batch.
[b] Inter-batch.

2.4. Electrophoresis conditions

Electrophoresis of DNA samples was carried out on a 0.8% agarose plate in Tris–borate buffer adjusted to pH 8.3. The gel was stained with ethidium bromide. Volumes of 15 μl of 0.5 mg ml^{-1} samples and of standards were loaded into the wells. Electrophoresis was performed towards the anode at 50 V for 9 h at 20°C and the results were photographed with a UV transilluminator.

3. Results and discussion

3.1. Identification and characterization of PDRN

To show the PDRN composition of the fraction obtained from the placenta extracts, a spectrometric and electrophoretic analysis was carried out using calf thymus DNA (Sigma D1501) and human placenta (Sigma D7011) as a sample control. All the above experiments were repeated after biochemical treatment with DNase and RNase.

A first identification of the PDRN nature of the extracts analysed came from the UV absorbance ratio $A_{260/280} = 1.83 \pm 0.05$, which is very close to that of pure DNA ($A_{260/280} = 1.8$), compared with that of RNA ($A_{260/280} = 2.02 \pm 0.05$).

^{1}H NMR spectrum, in which the characteristic signals of the aromatic protons, the deoxyribose moiety and the alkyl protons can be distinguished, is similar to the standard spectrum (Sigma D1501). Minor differences were observed at $\delta 4.5$ and in the range $\delta 3.8$–3.2, where there are signals of impurities in the extract, such as glycosaminoglycans and amino acids. For example, the signals at $\delta 3.3$–3.6 are probably due to the 2 and 3 protons of the uronic acid present in the gylcosaminoglycan structures. The typical sharp signals close to $\delta 2$ seem to confirm this explanation. The small amount of these impurities is not detected in the IR spectrum, which is substantially superimposable on that of the standard (Fig. 1). No modifications of the IR and NMR spectra were observed after the biochemical treatment with RNase.

The molecular weights of the DNA fractions from human placenta (PDRN) were determined

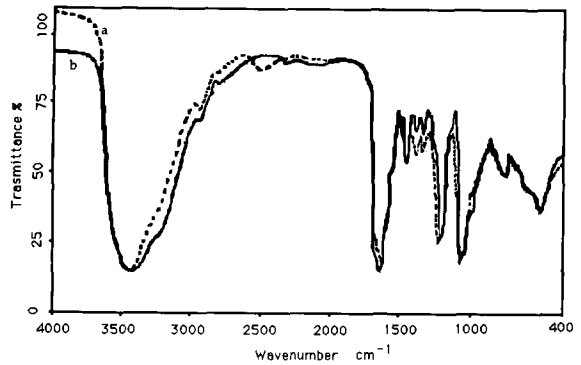

Fig. 1. IR spectra of (a) PDRN and (b) calf thymus DNA standard.

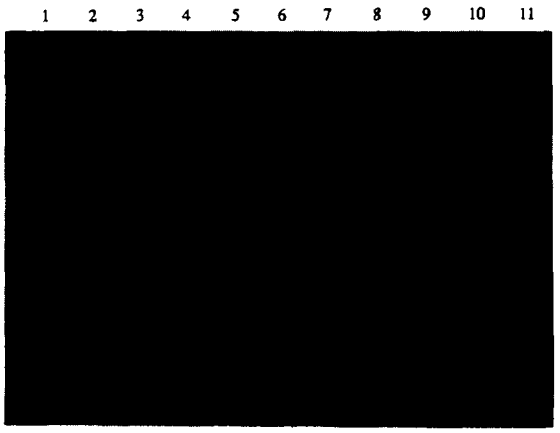

Fig. 2. Electrophoresis of the 1 kb DNA ladder (line 1), 123 bp DNA ladder (line 2) and 174 RF DNA/Hae III fragments (line 3), of the placenta extract samples (lines 4–9) and of DNA calf thymus and DNA human placenta (lines 10 and 11).

by electrophoresis. As can be seen in Fig. 2, the known standard molecular weights (as 1 Kb 1, 2, 3, etc.) are identified in neat spots, each coinciding with the known base pairs. The PDRN samples give a long uninterrupted line (a similar behaviour occurs with the standard DNA thymus and placenta samples) from which the minimum, maximum and median molecular weights can be determined. Then, using a UV transilluminator and densitometer, the above PDRN line is seen as a Gaussian curve (see Experimental for details and Fig. 3 for densitometric analysis). The samples and standard spots disappeared after treatment with DNAse, while remaining the same after treatment with RNAse, thus confirming the deoxyribonucleotide nature of the samples.

3.2. Chromatographic analysis of PDRN

As indicated in Fig. 4, which is a representative chromatogram of an extract, the elution of the PDRN yielded a main peak with favourable symmetry and acceptable retention time. It is worth noting that all the potential impurities of PDRN are either eluted at different times or are not detected at all; hyaluronic acid and chondroitin sulphate A, B and C do not absorb UV radiation and albumin, amino acids and RNA are not retained in the anionic column under these conditions. On the other hand, the DNAse treatment resulted in the disappearance

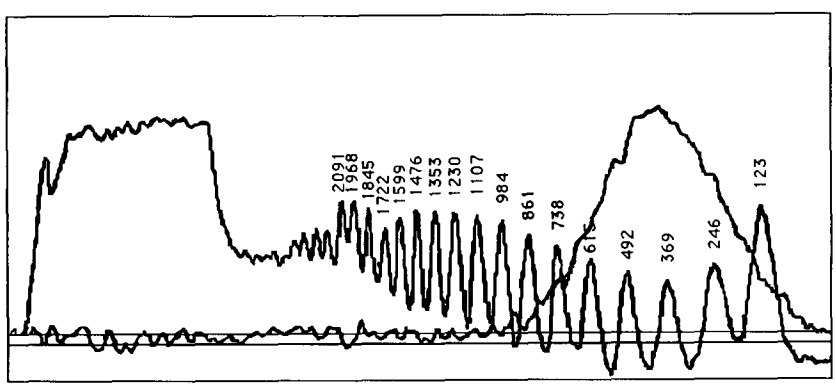

Fig. 3. Densitometric analysis of (a) 123 bp DNA ladder standard and (b) a DNA from an average placenta extract.

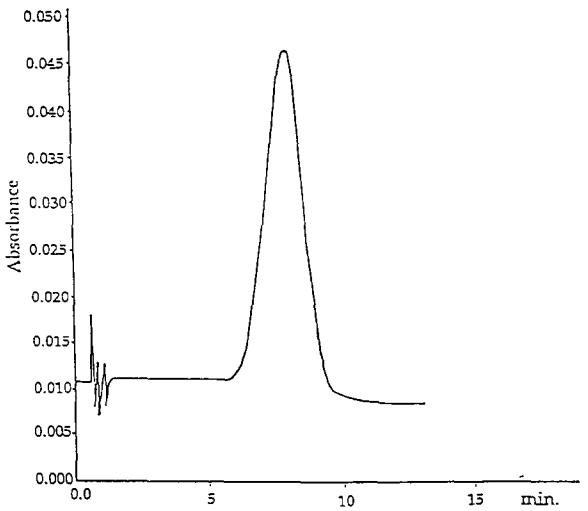

Fig. 4. Chromatogram of the placenta extract obtained using method 1.

of the peak, indicating that there are no detectable amounts of RNA.

The quantitation method, was verified with regard to specificity, reproducibility, stability, precision and accuracy (see Experimental), was applied to many industrial batches, to estimate both the range of the PDRN concentration and the reproducibility of the extraction method. The amounts of PDRN in these industrial batches ranged from 81.3 to 90.5% (first column in Table 3), and were obtained by comparing the area of this peak with that of the standard (Sigma D1501) (see Experimental). They account for the stability of the extraction method and its improvement compared with the earlier extraction method which had led to a concentration of 50%.

Using the same chromatographic method, with only minor modifications (see method 2 in Experimental), it was possible to verify the molecular weight distribution of the PDRN which we achieved through the electrophoresis. Comparing the start, apex and end of the peak for the sample with those for a standard obtained with appropriate restriction enzymes, the PDRN peak start coincided with 100 bp and the peak end corresponded to 2000 bp (columns 2–4 in Table 3), either by superimposing the two chromatograms obtained under the same conditions or by inspection of the chromatogram of the sample added to the standard.

Table 3
Analytical results for industrial batches

Batch no.	HPLC assay	Molecular weight (bp)		
	SD (%)	Peak start	Peak apex	Peak end
1	90.3	100	370	980
2	84.0	100	400	1080
3	82.0	120	520	1600
4	86.3	150	460	1100
5	84.5	180	520	1180
6	90.5	180	410	1050
7	84.2	150	360	900
8	81.3	160	400	1020
9	81.8	160	620	2000
Average	84.99			
SD (%)	3.44			
RSD (%)	4.05			

In conclusion, this multivariate analysis (UV, IR, NMR, electrophoresis) has proved that this HPLC method gives reliable quantitative and/or qualitative responses for placenta extracts and its active ingredient (PDRN). The large number of analyses performed testify not only to the ability of the DEAE anion-exchange column to give a complete resolution of all DNA fragments, as reported [9], but also to its advantageous feature of not being affected by the impurities (10–20%) in industrial preparations.

References

[1] L. Clejan and H. Menahe, Acta Haematol., 58 (1977) 84–88.
[2] H. Pande, J. Calaycay, D. Hawke, C.M. Ben-Avram and J.E. Schively, J. Biol. Chem., 260 (1985) 2301–2306.
[3] M. Hayashil and K.M. Yamada, J. Biol. Chem., 257 (1982) 5263–5267.
[4] M.P. Rathbone, S. Deforge, B. Deluca, B. Gabel, C. Laurenssen, P. Middlemiss and S. Parkinson, Med. Hypoth., 37 (1992) 213–219.
[5] M.P. Rathbone, L. Christianson, S. Deforge, B. Deluca and J.W. Gysbers, S. Hindley, M. Jovetich, P. Middlemiss and S. Takhal, Med. Hypoth., 37 (1992) 232–240.
[6] D. Wang, N. Huang and L.A. Heppel, Biochem. Biophys. Res. Commun., 166 (1990) 251–258.

[7] C.A. Dorsch and J. Killmayer, Arthritis Rheum., 23 (1983) 179–183.
[8] B.A. Fiedel, S. Schoenberger and H. Gewurz, J. Immunol., 123 (1979) 2479–2484.
[9] D.B. Stowers, J.M.B. Kleim, P.M. Paul, Y.S. Lyoo, M. Merion and R.M. Benbow, J. Chromatogr., 444 (1988) 47–65.