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"ON-LINE INTERNAL SURFACE REVERSED-PHASE CLEANING": THE DIRECT HPLC ANALYSIS OF CRUDE BIOLOGICAL SAMPLES

APPLICATION TO THE KINETICS OF DEGRADATION OF OLIGONUCLEOTIDES IN CELL CULTURE MEDIUM

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Abstract—An "on-line" HPLC analysis of crude biological samples is described. A precolumn of internal surface reversed-phase material allows the passage of proteins and other unwanted products while retaining analytes which are transferred, concentrated and chromatographed on a conventional reverse-phase or ion-exchange HPLC column. This protocol allows precise kinetics of the degradation of an oligonucleotide in cell culture to be obtained without radiolabeling or sample preparation.

Selective inhibition of gene expression by oligodeoxynucleotides complementary to mRNA has been widely demonstrated in cell culture [1, 2]. Further development of this exciting approach requires an enhancement of the antisense oligonucleotide's (ON†) enzymatic stability and uptake into cells. In this regard, the basic structure of DNA has been modified in several ways to enhance its pharmacokinetic properties [3, 4].

Unfortunately, practical methodologies to precisely analyse the stability of ONs in biological systems are not available. The method of choice, electrophoretic separation and autoradiography of the degradation of ^{32}P-labeled ONs [3–6], has several disadvantages. An attractive method for determining the kinetics of degradation of ONs in biological systems appears to be that based on an HPLC procedure since synthetic ONs are most often purified and analysed by HPLC. However, this approach has had limited use due to the extensive sample preparation required before analysis and the use of radioactive materials [7].

Due to a need to compare the enzymatic stabilities of a series of modified ONs in various biological media, we re-examined a previously described HPLC method [8] which did not require radiolabelling, extensive sample preparation or an internal standard. We now report our development of this procedure such that rate and mechanism of ON degradation in cell culture media can be readily determined by direct analysis using reversed-phase or ion-exchange HPLC.

MATERIALS AND METHODS

Instrumentation. HPLC was performed on a Waters-Millipore instrument, equipped with two Model 510 and one Model M 45 solvent delivery systems, a Model 680 solvent programmer, a Model 712 autosampler and a Model 990 diode-array UV detector. A six-port 4010 Rheodyne valve allowed switching between the precolumn and either the column or the detector. Precolumn (Ultrabiosep C_8, 10 μm, 4.6 × 10 mm), RP column (Nucleosil C_{18}, 3 μm, 4.6 × 100 mm) and ion-exchanger column (Ultranucleotide PVDI 4000-05, 10 μm, 4.6 × 100 mm) were purchased from SFCC/Shandon. An internal surface reversed-phase (ISRP) column (Ultrabiosep C_8, 10 μm, 4.6 × 150 mm) was kindly furnished by SFCC for the initial experiments. Columns were thermostated at 30° and protected by filters which were changed daily.

Chemicals. Distilled water was purified on a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Acetonitrile (ACN) was of far-UV HPLC grade (Fisons, Loughborough, U.K.). Ammonium acetate, potassium dihydrogen phosphate and potassium chloride were *pro analysi* grade (Merck, Darmstadt, Germany). Cell culture medium RPMI 1640 (ref. 041-02401) and fetal calf serum were purchased from Gibco BRL (Uxbridge, U.K.). Antibiotics were purchased from Bohringer (Mannheim, Germany).

Stock solutions [(a) 1 M ammonium acetate, pH 5.9; (b) 0.2 M potassium phosphate, pH 6.0; (c) 1 M potassium chloride] were filtered (0.22 μm, Millex-GS, Millipore) and stored at +5°. Solvents were prepared by mixing appropriate amounts of stock solutions, ACN and water (v/v) and were degassed under nitrogen bubbling for 2 min [A: (a) 10%, ACN 2%; B: (a) 10%, ACN 9.2%; C: (b) 10%, (c) 20%; D: (b) 10%, (c) 20%, ACN 20%; E: (b) 10%, (c) 40%, ACN 20%]. Phosphate-containing solvents were prepared daily.

ONs were synthesized in our laboratory on a DNA synthesizer (Applied biosystems 381A). A crude 19-mer ON1: αd(GAGTCATGCTCTCCTTGGC), chosen for its enhanced enzymatic stability due to its alpha configuration [1], was initially examined in

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† Abbreviations: ON, oligonucleotide; ISRP, internal surface reversed-phase; ACN, acetonitrile.

order to evaluate the analytical approach. The stability in cell culture medium of a purified 12-mer in beta configuration ON2: βd(ACACCCAATTCT) was then studied. This sequence is complementary to the splice acceptor junction of the HIV/TAT gene [9].

Samples. A stock solution of cell culture medium containing 10% (v/v) heat-inactivated serum (30 min at 56°), penicillin (20 IU/mL) and streptomycin (20 μg/mL) in RPMI was filtered (0.22 μm, Millex-GS) and then divided into 1.5-mL sterile containers which were stored at −20°.

For each kinetic study, one A_{260} unit on ON (about 40 μg in 20–40 μL of water) was diluted to 1 mL with one thawed aliquot of culture medium in an ice bath, vortex-mixed and divided up into 10 conic inserts used in the autosampler. Inserts were tightly stopped and stored immediately at −20°. Sterile procedures were used for all handlings. For each measure, one insert was quickly thawed, vortex-mixed, incubated at 37° for the required duration and frozen again. Immediately before HPLC analysis, an insert was quickly thawed, vortex-mixed, and 50 μL were injected without further treatment. In this way all samples were studied in the same medium and subjected to the same cycles of cooling–heating, with a view to minimizing possible variations [9].

HPLC of biological samples. HPLC analysis of drugs and metabolites in biological samples requires extensive preparation prior to chromatographic injection: these protein-containing fluids may not be directly injected into conventional HPLC columns because proteins denature on the bonded surface and absorb on the porous matrix, causing the column to become ineffective [7].

Over the past 10 years, trends in sample preparation have moved from: (i) the "liquid–liquid extraction" towards the "liquid–solid extraction" methods; (ii) the "off-line" towards the "on-line" procedures; and (iii) the manual towards the automated techniques [10]. Paradoxically, as sample clean-up becomes less time-consuming, it has become more expensive (robotic systems and/or disposable cartridges). Loss of analytes is generally unavoidable. This requires the addition of an internal standard before clean-up. The choice of an internal standard is tedious because it must: (i) be inert towards the medium, (ii) have the same behaviour as analytes during the sample preparation, and (iii) have chromatographic properties fairly different from those of analytes for HPLC analysis. The "on-line" procedures require precolumns packed with either coarse reversed-phase bonded silica particles or ion-exchange materials. In one part of the analysis, the endogenous compounds are eliminated, then analytes are eluted onto the analytical column. Precolumns must be systematically purged of the retained proteins and replaced frequently. Whatever the method, development of a protocol requires many assays and controls.

In 1985, Hagestam and Pinkerton [8] introduced a new concept for direct HPLC analysis of biological samples, the so-called "internal surface reversed-phase" (ISRP). In the materials used, the inner surface of the silica bonded particle is hydrophobic and the outer surface is hydrophilic. Macromolecules too large to penetrate into the pores are quickly eluted in aqueous solvent. Small molecules can penetrate into the pores and are subjected to partition chromatography. In other words, proteins, salts and others hydrophilic compounds such as amino acids contained in cell culture media are first eliminated; hydrophobic compounds are then eluted according to conventional reversed-phase procedures.

These authors connected a low-performance ISRP precolumn to a high-performance C_{18} column via a switching technique [8]. With this "on-line" procedure, the sample was cleaned on the precolumn, then transferred and analysed on a RP column. Surprisingly, further development of this procedure was not reported during a period of time when efforts were directed toward the conception of more selective ISRP materials [11–16]. Research was focused on the detection of traces of drugs in serum and plasma [17–20] and on protein binding studies [21–24]. Now, new generations of high-performance ISRP columns and other restricted access stationary phases [25] are commercially available.

The "on-line ISRP cleaning" concept was rediscovered independently by Haginaka *et al.* (anticonvulsant drugs in serum) [26], Matlin *et al.* (anti-estrogen in diluted plasma) [27], and by us (stability of neutral nucleotide derivatives in cell culture media, human serum and other biological fluids)*. In these studies, an ISRP precolumn was connected to a conventional RP-C18 column.

We have extended this elegant procedure to the analysis of ONs in biological fluids without any pretreatment or internal standard, by connecting a C_8-ISRP precolumn with either a C_{18} reversed-phase or a new ion-exchanger column.

Reversed-phase HPLC of ONs. Synthetic ONs are currently analysed or purified by RP-HPLC [28, 29]. Elution order depends on the length of the sequence, the nature of the nucleobases and any structural modifications. Two effects are antagonistic: hydrophobicity increases with the number of bases and decreases with the number of internucleotidic phosphates. Generally, the first effect predominates such that ONs are eluted according to their length, but inversions may occur. ONs are currently chromatographed by means of gradient elution techniques. By these procedures closely related compounds may be co-eluted (sample ON1, Fig. 1a), providing false information about the purity of the product.

Better resolution may be obtained using isocratic conditions but optimization of these is tedious as the retention time of ONs drastically changes depending on the percentage of organic modifier (x%) in the eluent. For example, the components of the same ON1 sample were not retained on the RP-C18 column for $x > 10\%$ (Fig. 1b) whereas they were not eluted within 2 hr for $x < 8\%$. Optimized analysis was obtained for $x = 9.2\%$ and showed that ON1

* Puech F, Gosselin G, Pompon A, Lefebvre I, Paoletti J, Aubertin AM, Kim A and Imbach JL, Derivated anti-HIV nucleosides: 1. Phosphotriester prodrugs of ddC. *Antiviral Res*, to be submitted.

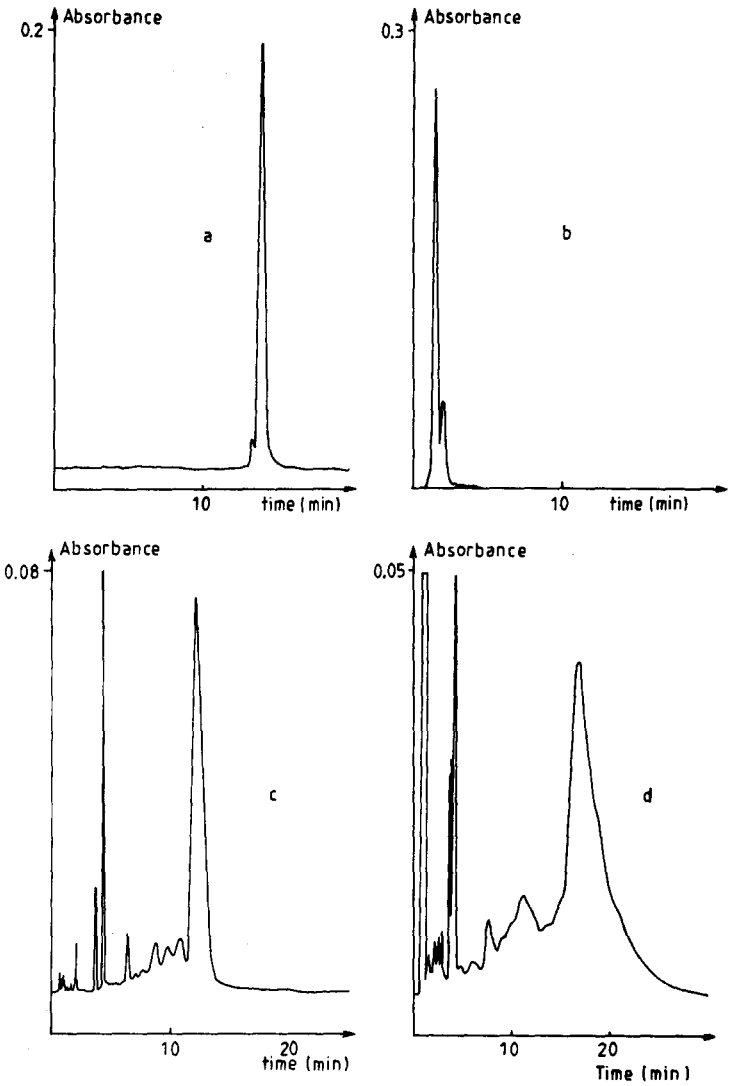


Fig. 1. Analysis of crude ON1. Flow rate 1 mL/min. (a, b, c) C18-RP column, injection 50 μL of 9×10^{-6} M sample in water: (a) linear gradient from 2 to 20% ACN in 20 mn; (b) isocratic 10% ACN; (c) isocratic 9.2% ACN; (d) C8-ISRP column, 50 μL of 9×10^{-6} M sample in cell culture; isocratic 5.4% ACN.

was a mixture (Fig. 1c). This "trapping and abrupt release" behaviour was observed previously in the case of phosphorothioates analogues of ONs [30].

In preliminary experiments for analysing ONs in cell culture, we used the same ON1 and a 15-cm length C8-ISRP column. Whatever the solvent, proteins were eluted with the dead volume while ONs showed the same "all or nothing" behaviour: no elution for $x < 4\%$, no retention for $x > 6\%$. The best analysis was obtained for $x = 5.4\%$ (Fig. 1d).

Taking advantage of these differences resulting from the different hydrophobicities of C8 and C18 materials, we used a short guard-column filled with the same C8-ISRP material as a "reversed protein-filter" before the analytical C18-RP column, according to the following procedure: the system was equilibrated with solvent B ($x = 9.2\%$), the precolumn was then disconnected from the column and connected to the detector by actuating the switching valve (inject position), and was then equilibrated with solvent A ($x = 2\%$). After injection, proteins were quickly eluted. At 4 min, the valve was actuated (load position) and the strength of the solvent was quickly increased (linear gradient from A to B in 1 min). Thus, for an intermediate value of x, analytes were back-flushed and concentrated at the very beginning of the column. When the elution threshold was reached, analytes were chromatographed (Fig. 2). The precolumn was re-equilibrated (inject position, solvent A, 7 min) for a new run.

Ion-exchange HPLC of ONs. Strong anionic exchanger resins are commonly used for HPLC analysis of ONs [31, 32], but their lifetime is generally

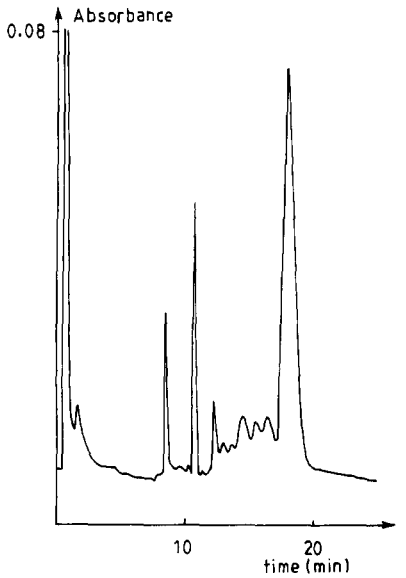


Fig. 2. Direct analysis of crude ON1 in cell culture by "on-line ISRP cleaning" and C18-reversed-phase analysis (see text and compare to Fig. 1c). Flow rate 1 mL/min, injection 50 μl of 9×10^{-6} M sample.

limited. Among the new PVDI columns (silica soaked with polyvinylimidazole), the large-pore PVDI 4000 conceived for the analysis of high weight nucleic acids provided an excellent resolution of medium-size (12–20-mers) ONs and good column stability. Denaturating agent such as urea or guanidine was not required; this results in lower background which improves the UV detection. Capacity factors depend mainly on the number of negative charges, thus elution of sequences of different lengths is performed by increasing the ionic strength of the eluent. Hydrophobicity of the support requires the addition of an organic modifier, which was convenient for our purpose.

We adapted the coupling of a C8-ISRP cleaning precolumn to the ion-exchanger column according to the following procedure: the system was equilibrated with solvent C (20 mM phosphate buffer, 0.2 M KCl) and then the precolumn was connected to the detector. After injection and elution of proteins, the valve was actuated and analytes were back-flushed and concentrated on the column by means of an intermediate solvent D (as C, plus ACN 20%). After 3 min, the ionic strength was increased according to a 27-min linear gradient from D to E (as D, except 0.4 M KCl) and analytes were chromatographed in less than 20 min (Fig. 3a–d). The system was reequilibrated (10 min) for a new run.

RESULTS AND DISCUSSION

Comparison of methods

Use of an ISRP column or coupling of an ISRP precolumn to a RP column gave poor results for the analysis of ONs (signals too large and not well enough resolved); this problem is inherent to the behaviour of ONs on RP materials. Another disadvantage of this technique for the analysis of ONs is that chromatographic conditions must be redefined for each compound of a series, according to its hydrophobicity. Nevertheless, these experiments demonstrated that ONs were not lost by binding to the proteins or on the ISRP precolumn, as shown by comparing chromatograms in Figs 1c and 2: apart from the protein moiety and delay in the retention times of the ON moiety, the shape and areas of the signal were exactly the same as in the control. Moreover, this highly simplified method, which requires only a two-solvents delivery system, gave excellent results when comparing the stability and the degradation mechanisms of a series of less sophisticated compounds (unpublished data).

Association of an ISRP precolumn to an ion-exchanger column gave excellent results for the study of ONs in cell culture without sample preparation. Since the elution order depends almost entirely on the length of the ONs, once the chromatographic conditions have been optimized for one compound, these conditions can be applied without change for the analysis of homologues with quite different hydrophobocities. A disadvantage of this method is that a three-solvents delivery system is needed.

These analyses provided accurate kinetic data which may be used to explore the mechanisms of degradation.

Degradation of a βd-ON in cell culture medium

Chromatograms in Fig. 3a–d show the step-wise evolution of the ON2 sample over time following incubation in cell culture medium at 37°. The signal 12, corresponding to the initial 12-mer, progressively decreases while signal 11 appears, then signal 10 and so on. When the incubation time increases, signal 2, corresponding to the nucleosides and nucleotides, increases like the signals corresponding to short ONs, while signals corresponding to longer ONs regularly decrease. The pattern suggests ON degradation by an exonuclease attack, since an endonuclease activity would not provide a uniform degradation pattern. Furthermore, in accordance with recent data [3, 5] we postulate that this degradation results from a 3′-exonucleasic activity in serum.

The area of each signal (measured at 262 nm) was normalized to the total area. Note that this sum was nearly constant for the different samples (±3%), which indicates the reliability of the method. Postulating that the attack was at the 3′-end, molar extinction coefficients were calculated for the parent compound and the first four fragments by means of the HYPO algorithm which takes into account the sequence and proximity of the bases in the ONs. Concentrations of the different species were calculated by means of these coefficients and the corresponding kinetic curves are represented in Fig. 4. Data were treated by means of the SIMPLEX algorithm according to the simple model:

$$\text{12-mer} \xrightarrow{k_1} \text{11-mer} \xrightarrow{k_2} \text{10-mer} \xrightarrow{k_3} \text{etc.}$$

Treatment of data corresponding to the signal 12

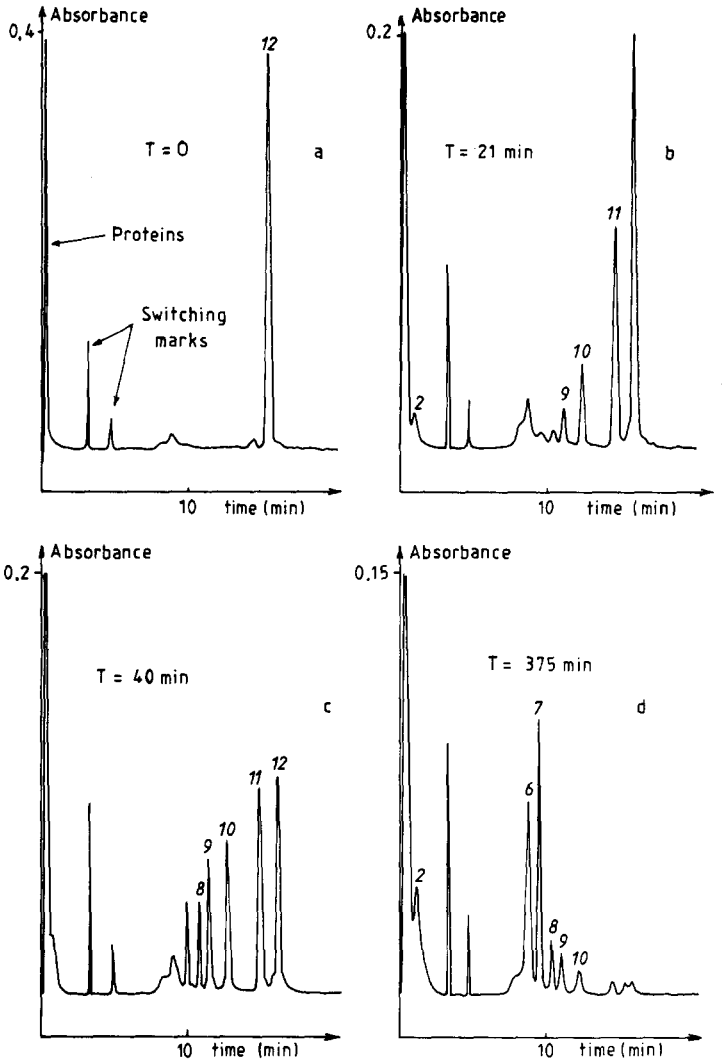


Fig. 3. Direct analysis of ON2 and degradation products after incubation at 37° in cell culture by "on-line ISRP cleaning" and ion-exchange analysis (see text). Flow rate 1 mL/min, injection 50 μL of 9×10^{-6} M sample.

led to the best fit for $k_1 = (2.3 \pm 0.2)\ 10^{-2}\ min^{-1}$ ($R = 0.994$). For the signal 11, results were: $k_1 = (2.1 \pm 0.2)\ 10^{-2}\ min^{-1}$ and $k_2 = (5.2 \pm 0.4)\ 10^{-2}\ min^{-1}$ ($R = 0.991$). Thus, it seems that the rate of enzymatic hydrolysis may depend on the nature of the cleaved link: ($T_{1/2} = 31 \pm 2$ min for the $^{3'}$T/C—cleavage of the parent 12-mer vs $T_{1/2} = 13 \pm 2$ min for the $^{3'}$C/T—cleavage of the first 11-mer fragment). Moreover, until the appearance of short fragments, the sum of concentrations of the parent 12-mer and the postulated first four fragments were quite constant (±1%).

Based on the high values of the extinction coefficients of ONs, the sharpness of the signal and the sensitivity of the UV detector, analysis of ONs with an initial concentration as low as 10^{-5} M may be obtained by this procedure.

In addition, we have shown that even in deactivated culture medium, an extensive degradation of a β-ON arises. The short half-life at 10^{-5} M concentration ($T_{1/2} = 31$ min) observed for the βd-12mer may explain some of the erratic biological evaluations of β-ONs. Decomplementation of the serum may be achieved under various conditions where the 3'-exonuclease activity is never fully destroyed [3], only modulated (unpublished data). The rapid formation of 3'-truncated sequences in culture medium may have an important bearing on the selective action of antisense ONs.

ISRP cleaning

The general method which we propose for the direct HPLC analysis of crude biological samples is summarized in Scheme 1 and needs only the addition of a six-port switching valve to a conventional chromatograph. The pharmacokinetics of 12 compounds at different concentrations in various media (cell cultures, undiluted human serum and others

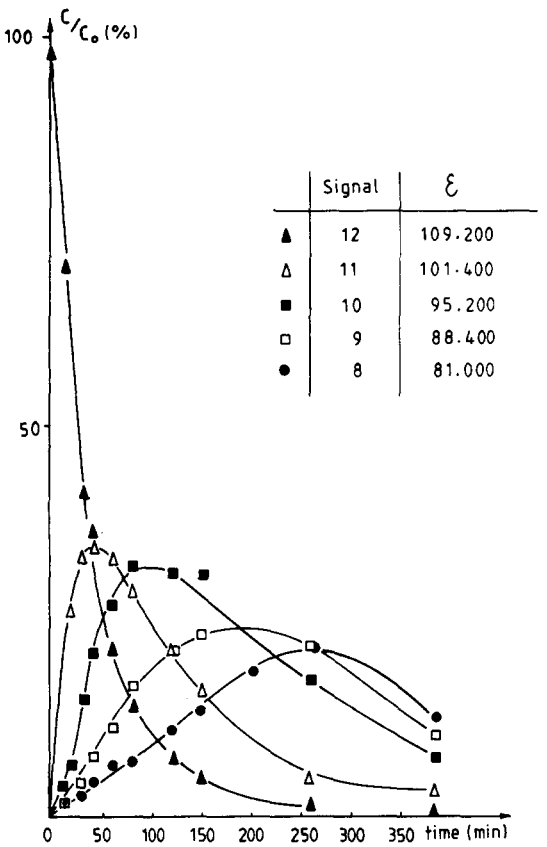
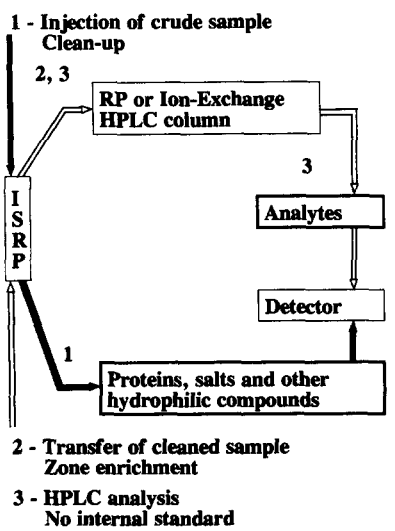


Fig. 4. Kinetic curves: evolution of ON2 at 9×10^{-6} M initial concentration and its degradation products in cell culture medium at 37°.



Scheme 1. The "on-line ISRP cleaning" concept for direct HPLC analysis of biological samples.

biological fluids) have been evaluated recently,* this work was accomplished by several hundred analyses using either the reverse-phase or ion-exchange protocol. Simple kinetic models fit the experimental data ($R > 0.99$ and currently $R > 0.999$ for N = 10) very well, which substantiates the reliability of the method.

Since contaminants are directly eliminated by this new "on-line" procedure, systematic purges and precolumn replacements are not required. As a precaution, the cartridges were replaced when their back-pressure increased to twice the initial measure, corresponding to a few hundred injections.

In no case was loss of analytes observed by binding either on the ISRP material or on the proteins. The only limiting factor for reproducible results was the reliability of the autosampler (less than 2% for 10–100 μL injections). Thus, no internal standard was required. As described previously, back-flushing of the precolumn was preferable to forward-flushing during the transfer of analytes [26].

In summary, the behaviour of a potential drug as sophisticated as antisense ON and its degradation products was analysed at a low concentration (10^{-5} M) in cell culture medium by HPLC without radiolabeling, sample preparation or an internal standard. For this purpose we developed a so far unexploited "on-line switching" technique, with a precolumn containing ISRP material acting as a "reversed-filter" of proteins, and we adapted this technique to ion-exchange HPLC. This is the first report of this procedure in the literature.

We believe that this highly simplified, inexpensive and reliable "ISRP cleaning" method will be of considerable interest for metabolism and pharmacokinetic studies in the future. Further developments of this method are now in progress in our group and will soon be published.

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