Quantitation of In Vitro Activity of Synthetic Trans-Acting Ribozymes Using HPLC


ABSTRACT

The cleavage activity of synthetic ribozymes needs to be characterized by reliable and rapid methods. A chromatographic method to simultaneously quantitate the amounts of substrate, cleavage fragments and ribozyme is described. The method allows the rapid normalization of analytical data because the sum of the 260-nm peak areas of remaining substrate and obtained fragments is essentially equal to the initial substrate peak area. Moreover, the simultaneous determination of the ribozyme content improves the accuracy of the evaluation of kinetic parameters. Thus, the method is a reliable alternative to the densitometric autoradiographic electrophoretic method.

MATERIALS AND METHODS

Ribozymes and Substrates

Two hammerhead ribozymes specifically targeted against the mRNA encoded by the human gene MGMT were used. The hammerhead catalytic motif was chosen because a short domain (1, 4) of only 32–40 nucleotides (nt) can easily be prepared by automatic chemical synthesis (3, 9). The catalytic complexes ribozyme/substrate Rz-MT1 and Rz-MT2 and the cleavage sites GUA at nt 85 (Rz-MT1) and GUU at nt 741 (Rz-MT2) of the MGMT mRNA (GenBank® Accession No. M29971) are shown in Figure 1.

Synthesis and Deprotection of RNA Sequences

Syntheses of RNA sequences were performed by standard phosphoramidite ribonucleotide chemistry (PerSeptive Biosystems, Framingham, MA, USA) in an Expedite™ 8909 Nucleic Acid Synthesis System (PerSeptive Biosystems). The oligonucleotides obtained were partially deprotected in a 3:1 mixture of concentrated aqueous 33% NH₃ (J.T. Baker, Deventer, The Netherlands) and ethanol (BDH Ltd., Poole, Dorset, England, UK) at 45°C for 12 h, concentrated to dryness and then fully deprotected using a 24-h reaction at room temperature with triethylamine trihydrofluoride (Aldrich Chemicals, Steinheim, Germany) to restore the 2′OH ribose (7). Crude deprotected products, recovered in sterile double-distilled water, were precipitated by n-butanol (J.T. Baker).

Purification of Synthetic Oligoribonucleotides

Pellets dissolved in the running buffer were applied to a preparative column of the strong anion-exchanger Mono Q®-HR 16/10 (Pharmacia Biotech, Uppsala, Sweden) eluted at 6 mL/min (30 min) with a linear 0.25–0.75 M NaCl gradient (Sigma Chemical, St. Louis, MO, USA) in 20 mM Tris-HCl (Merck, Darmstadt, Germany), pH 8.7, plus 10% of HPLC-grade acetonitrile (Labscan, Dublin, Ireland) in a 410-bio Liquid Chromatograph (Perkin-Elmer, Norwalk, CT, USA). The absorbance of the eluted products was monitored at 260 nm.

Figure 1. Schematic representation of ribozyme-substrate catalytic complexes, the cleavage sites and the product of the cleavage. Both cleavage reactions produced asymmetric fragments containing 7 and 5 phosphate residues, which results in different retentions of the components in the ion-exchanger HPLC column.
260 nm in a UV/VIS LC 90-bio Spectrophotometric Detector (Perkin-Elmer). The homogeneous purified oligoribonucleotides were desalted in a sterile Sephadex® G-50 DNA-grade column (Pharmacia Biotech) eluted with sterile double-distilled water, quantitated by UV absorption in a Lambda 11 UV-Vis Spectrophotometer (Perkin-Elmer) and, after concentration to dryness, stored at -20°C until used.

### Ribozyme Reactions In Vitro

The multiple turnover cleavage reactions were performed with 50 nM ribozyme and increasing substrate concentrations ranging from 10–120-fold greater than that of the ribozyme. Briefly, separate solutions of the ribozyme and the substrate in sterile 50 mM Tris-HCl buffer, pH 7.6, were heated at 90°C for 1 min to disrupt any aggregates formed during storage and then allowed to cool at room temperature. Sterile magnesium chloride solution (Merck) was added to a final concentration of 10 mM. The cleavage reactions, carried out at 25°C, were started by mixing equal volumes of the ribozyme and substrate solutions. One hundred-microliter aliquots were collected at the specified times, and reactions were rapidly terminated by heating the samples at 90°C for 1 min after the addition of 10 µL of 500 mM EDTA (Sigma Chemical). Samples, chilled on ice, were stored at -20°C until analyzed.

### Cleavage Evaluation

The samples were applied to a strong anion-exchanger Mono Q-HR 5/5 Analytical Column (Pharmacia Biotech) eluted at 1 mL/min (30 min) with a linear 0.15–0.85 M NaCl gradient in 20 mM Tris-HCl, pH 8.7, plus 10% of HPLC-grade acetonitrile in a 410-bio Liquid Chromatograph at 40°C. The area of eluted products, monitored at 260 nm in a LC 75 Spectrophotometric Detector (Perkin-Elmer) was quantified in an LCI-100 Computing Integrator (Perkin-Elmer) after calibration with authentic samples.

### Table 1. Kinetic Parameters Calculated for the Hammerhead Ribozymes

<table>
<thead>
<tr>
<th>Ribozyme</th>
<th>kcat (min⁻¹)</th>
<th>Km (nM)</th>
<th>kcat/Km (min⁻¹ × nM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rz-MT1</td>
<td>1.13</td>
<td>2150</td>
<td>5.3 × 10⁻⁴</td>
</tr>
<tr>
<td>Rz-MT2</td>
<td>8.04</td>
<td>1200</td>
<td>69.7 × 10⁻⁴</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

The cleavage activity of Rz-MT1 and Rz-MT2 ribozymes targeted against different regions of MGMT mRNA was assayed by using 13-mer minimal synthetic substrates.

Figure 2 depicts a typical example of the chromatographic resolution and progressive catalytic conversion of substrate (S) into products (P1, P2). The sum of the peak areas of the products (P1+P2) tends to equal the reduction of the peak area of the substrate (S). So, at any time (t) the following relation is valid:

\[(P1)_t + (P2)_t + (S)_t = (S)_{t=0}\]

where \((S)_{t=0}\) and \((S)_t\) are the peak areas of the substrate at the starting concentration and at time (t), respectively, while \((P1)_t\) and \((P2)_t\) are the areas of indicated oligonucleotide peaks at time (t). Consequently, it may be sufficient to calibrate the substrate area with standardized sample to derive the molar amounts of the cleavage fragments. The areas of the peaks obtained after multiple injections of replicate samples indicated the high reproducibility of the method. Standard errors ranged within less than 1.0 pmol of the target substrate, representing only 2% of the overall amount used at the 50-pmol absolute concentration. In addition, this method at variance with the densitometric method always allows the determination of the ribozyme content. This represents an undoubted advantage, since the accuracy of evaluating the determination of kinetic parameters was increased. In Figure 3, a typical set of cleavage time courses at various substrate concentrations is reported. Each point represents a single chromatographic run. The \(v_0\) rates of Rz-MT1 and Rz-MT2, at a given substrate concentration, were obtained from the slope of the corresponding plot measured after the polynomial regression fitting of experimental data. The \(v_0\) values normalized for the ribozyme concentrations [Rz] were plotted against the substrate concentration [S] (Figure 4), and the \(k_{cat}\) and \(K_m\) kinetic parameters were calculated from the linear Eadie-Hofstee representation. In this way, Rz-MT2 was roughly 10-fold more efficient than Rz-MT1 (Table 1). Thus, the method is able to differentiate between ribozymes on the basis of the kinetic parameters. The overall ribozyme amount necessary for the determination of kinetic parameters (30 pmol ribozyme, about 0.08 optical density [OD]) represents only a negligible fraction (roughly 0.2%) of the purified product obtained after 1-μmol scale automatic synthesis (on average 68 nmol, about 23 OD). The improved kinetic data largely compensate the disadvantage that the method uses about 1 order of magnitude higher concentrations of ribozyme than those usually used in the radio-densitometric methods.

In conclusion, the described nonradioactive chromatographic method allows a direct, rapid and accurate measurement of all components of the...
catalytic reaction in a highly reproducible way. This feature makes the method appropriate to monitor the cleavage activity of ribozymes that must be chemically stabilized before administration to living cells.

REFERENCES


This work was partially supported by CNR-Progetto Finalizzato “Applicazioni Cliniche della Ricerca Oncologica” and by Ministero della Sanità-Istituto Superiore di Sanità-Progetto “Allestimento di Modelli Animali per l’AIDS”. L.B. is recipient of a fellowship from the Italian Association for Cancer Research. Address correspondence to Lorenzo Citti, Genetica e Biochimica Tossicologica, Istituto di Mutagenesi e Differenziamento, via Svezia 10, 56124 Pisa, Italy. Internet: lcitti@imd.pi.cnr.it

Received 12 February 1997; accepted 12 May 1997.

L. Citti, L. Boldrini, S. Nevischi, L. Mariani and G. Rainaldi
Istituto di Mutagenesi e Differenziamento CNR
University of Pisa
Pisa, Italy