Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 847 (2007) 153–161
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Identification and quantification of GC-rich oligodeoxynucleotides in tissue extracts by capillary gel electrophoresis

Bernhard O. Noll *, Harald Debelak, Eugen Uhlmann

Coley Pharmaceutical GmbH, Elisabeth-Selbert-Strasse 9, D-40764 Langenfeld, Germany

Received 23 May 2006; accepted 25 September 2006
Available online 17 October 2006

Abstract

Capillary gel electrophoresis (CGE) is a widely used method for quantification of oligonucleotide-based drugs, such as CpG oligodeoxynucleotides (CpG ODN), aptamers and small interfering ribonucleic acids (siRNAs) that allows accurate quantification of parent compound as well as metabolites. Stable secondary structure formation of these molecules frequently prevents analysis by conventional CGE methods and impedes pharmacokinetic assessment. Herein, we describe development of a CGE method for identification and quantification of complex mixtures of secondary structure forming GC-rich ODN in biological samples at dose levels of 0.5 mg/kg and above. Samples containing GC-rich CpG ODN and metabolite markers were treated by solid-phase-extraction (SPE) and subsequently analyzed by CGE using a 50 cm neutrally coated capillary at 60 °C together with a 7 M urea buffer system containing 30% dimethylsulfoxide (DMSO). Peak resolutions ≥1 were typically achieved, enabling pharmacokinetic assessment of secondary structure forming oligonucleotides in biological samples that hitherto were unsusceptible to quantitative analysis.

Keywords: CpG; Oligonucleotide; Capillary gel electrophoresis; GC-rich; Secondary-structure

1. Introduction

The field of nucleic acid based drugs has been growing steadily in recent years. Members of the class of ODN and ORN drugs include CpG oligodeoxynucleotides (CpG ODN), aptamers, antisense-ODN and siRNAs, several of which are in clinical development for various disease indications including cancer, infectious diseases and allergy and asthma [1–6]. Naturally, development of these drugs requires analytical methods to investigate pharmacokinetic properties and to characterize metabolites that form *in vivo*.

For many years capillary gel electrophoresis (CGE) has been an established tool in ODN analysis and continues to develop rapidly [7–9]. The most common CGE methods for analysing biological samples are based on solid-phase-extraction (SPE) and subsequent CGE separation [10]. To date, CGE is the most widely used method for separation and quantification of parent compound as well as metabolites of ODN in biological samples, showing a LLOQ of 0.07 μg/ml in urine, plasma or serum and 0.35–1.2 μg/g in tissue [11]. CGE in combination with mass spectrometric methods, either online or offline, can be used to identify and quantify ODN and metabolites [12–15].

In CGE analysis, the separation of parent compound from metabolites is based on mass and charge selectivity and single stranded nucleic acids usually require denaturation [16–18]. CGE is usually carried out using a neutrally coated fused silica capillary filled with replaceable linear polymer in combination with a Tris/borate/EDTA buffer-system including 7 M urea as a denaturing agent. However, many single stranded nucleic acids, especially when G- or GC-rich, are able to form stable secondary structures even in the presence of denaturing agents, causing decreased peak resolution between parent compound and chain shortened fragments and leading to failure of CGE separations

Abbreviations: ODN, oligodeoxynucleotide; ORN, oligoribonucleotide; siRNA, small interfering ribonucleic acid; CpG ODN, immunomodulatory oligodeoxynucleotide containing a CpG motif; C-Mix, ODN solution of CpG C-Class ODN C1 and its first five metabolites from the 5′-end; B-Mix, ODN solution of CpG B-Class ODN B 1 and its first five metabolites; CGE, capillary gel electrophoresis with UV detection; FA, formamide; NMF, N-methylformamide; EC, ethylene-carbonate; DMSO, dimethylsulfoxide; SPE, solid-phase-extraction

* Corresponding author. Tel.: +49 2173 3997 2231; fax: +49 2173 3997 2399.
E-mail address: bnoll@coleypharma.com (B.O. Noll).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.046

[19,20]. This is especially true for a recently described class of immunomodulatory CpG ODN, namely C-Class CpG ODN [21–25], one of which is currently being tested in clinical studies for treatment of chronic viral infections. The distinct structural feature of this class of ODN is a palindromic base sequence, which usually is GC-rich and very likely the site of secondary structure formation in solution, namely dimer and/or hairpin-formation, caused by inter- or intra-molecular Watson–Crick base pairing. In fact, the unique cytokine pattern elicited by C-class ODN *in vitro* and *in vivo* is most likely linked to the ability to form secondary structures and may therefore be inherent in the structural characteristic of this ODN class. However beneficial in the biological setting, unresolved secondary structures cause decreased peak resolutions between parent compound and its chain-shortened metabolites, making it impossible to accurately quantify parent compound and metabolites using standard denaturing CGE conditions (unpublished results). Various strategies have been tested in the past in an effort to improve the denaturing power of gel electrophoresis. Elevated temperatures were found helpful in CGE of long DNA fragments by reducing separation time and by resolving compressions [20,26,27] as well as by improving selectivity [28,29]. Furthermore, addition of different organic solvents was reported to improve peak resolution of the CGE. The described solvents include ethylene glycol, *N*-methyl-formamide (NMF) [30] formamide (FA) [31–33] and dimethylsulfoxide (DMSO) [34], for example by using 5% DMSO together with 2 M urea at 70 °C to remove compressions in DNA sequencing [35,36].

For our studies, we used a mixture of a 22mer C-class CpG ODN (ODN C1) and five metabolite markers (C-Mix) derived from the parent sequence by consecutive single base deletions at the 5′ end. All oligonucleotides were fully modified phosphorothioates. Each ODN of the mixture contained the same 12-base palindromic GC-rich sequence at the 3′-end, and therefore, was able to form duplexes with all other ODN in the sample. C-Mix was used as a model for a complex mixture of secondary structure forming GC-rich ODN. Our goal was to enhance CGE peak resolution (USB) ≥1 and to enable identification of metabolite length by CGE migration time. We evaluated several modifications to the established CGE method including: (i) elevation of capillary temperature; (ii) increase in capillary length; (iii) addition of varying concentrations of different organic solvents to the buffer system such as FA, NMF EC, and DMSO; and finally, (iv) sample preparation (pre-heating of the samples and addition of up to 80% organic solvents) and injection conditions (voltage and pressure). A second goal of this study was to demonstrate suitability of the modified separation method for analysis of biological samples containing GC-rich ODN in combination with SPE. We found that a CGE method using 30% DMSO together with a borate buffer containing 7 M urea and a capillary temperature of 60 °C gave the best results in resolving secondary structures of complex mixtures of oligonucleotide-based drugs in solution or in biological samples. This method solves the problem of quantitative pharmacokinetic and pharmacodynamic assessment of oligonucleotide-based drugs, which are able to form stable secondary structures.

2. Experimental

2.1. Chemicals and test articles

All ODN used were fully phosphorothioate oligodeoxynucleotides that were manufactured at Coley Pharmaceutical GmbH (Langenfeld). C-Mix contained ODN C1 and C2–C 6 (approx., 250 μg/ml of each ODN in water). B-Mix contained ODN, B1 and B2–B6 (approx., 250 μg/ml of each ODN in water). For sequences refer to Table 1. FA, DMSO, EC, NMF and urea were purchased from Fluka (Buchs, Switzerland), proteinase K was purchased from Qiagen (Hilden), EDTA; Tween 20; Triton X100; Guanidinium HCl; DTT; Tris; NaBr; KCl were purchased from Merck (Darmstadt). Phenol/chloroform/isoamylalcohol (25:24:1) was purchased from Sigma (St. Louis, USA). Human serum was obtained from healthy volunteer blood donors (Blood Bank, University of Düsseldorf). Livers from untreated mice were provided by Coley Pharmaceutical Canada, Kanata, ON, Canada.

2.2. Size-exclusion-chromatography

HPLC analysis was carried out using a System Gold 126 Solvent Module, System Gold 508 Autosampler, and System Gold 168 Detector (Beckman-Coulter, Fullerton, USA). Chromatograms were analysed using the 32 Karat 5.0 software (Beckman-Coulter, Fullerton, USA). The corrected peak area of the main peak of each sample was recorded in percentage of total corrected peak area.

Chromatographic conditions: (i) column: Superdex HR 75 10 × 300 mm (GE Healthcare, formely Amersham Pharmacia #17-1047-01); (ii) column temperature: 25 °C; (iii) autosampler temperature: 25 °C; (iv) mobile phase: Dulbecco's phosphate buffered saline (D-PBS) (136.89 mM NaCl; 8.06 mM Na_2HPO_4; 1.48 mM; KH_2PO_4; 2.68 mM KCl; pH 7.3); (v) detection wavelength: 260 nm; (vi) flow rate: 0.5 ml min^{-1}; (vii) injection volume: 10 μl; (viii) data acquisition rate: 2 pt. s^{-1}.

2.3. Melting-curve-measurement

Temperature dependent UV absorption was measured using a Cary 100 Bio Photospectrometer (Varian, Palo Alto, USA) equipped with a Cary temperature controller. Melting curves were measured at 260 nm for five ODN concentrations in 1 × Dulbecco's PBS buffer without preheating. The samples were placed in the spectrophotometer, cooled to 10 °C, and gradually heated to 95 °C at rate of 0.5 °C min^{-1}. Two heating and two cooling cycles were performed sequentially. The change in absorbance versus temperature melting curves were fitted using the Marquardt-Levenberg Fitting Routine to the following model: $\varepsilon\ (T) = (M_{ss} \times T + B_{ss}) \times \alpha + (M_{ds} \times T + B_{ds}) \times (1 - \alpha)$, where the left term of the right expression represents the mole-fraction weighted, linear component of the melt curve due to single-stranded molecules and the right term represents the mole-fraction weighted, linear component due to double stranded complexes. The mole fraction α, is a function of temperature (T), equilibrium constant and total strand concentration

Table 1
Parent compounds, metabolites and internal standard ODN

ODN #	Sequence	Description	Bases
B 1	CG TCG TTT TCT CGT TTT GTC GTT	B-Class CpG ODN	23
B 2	G TCG TTT TCT CGT TTT GTC GTT	B-Class CpG ODN	22
B 3	TCG TTT TCT CGT TTT GTC GTT	B-Class CpG ODN	21
B 4	G TCG TTT TGT CGT TTT GTC G	B-Class CpG ODN	20
B 5	G TCG TTT TGT CGT TTT GTC	B-Class CpG ODN	19
B 6	G TCG TTT TGT CGT TTT GT	B-Class CpG ODN	18
C 1	T CGT CGT TTT CGG CGC GCG CCG	C-Class CpG ODN	22
C 2	CGT CGT TTT CGG CGC GCG CCG	C-Class CpG ODN	21
C 3	GT CGT TTT CGG CGC GCG CCG	C-Class CpG ODN	20
C 4	T CGT TTT CGG CGC GCG CCG	C-Class CpG ODN	19
C 5	CGT TTT CGG CGC GCG CCG	C-Class CpG ODN	18
C 6	GT TTT CGG CGC GCG CCG	C-Class CpG ODN	17
IS	T TTT TTT CGT CGT TTT CGG CGC GCG CCG	Internal standard	28

in the case of bi-molecular equilibriums, and of temperature and equilibrium constant in the uni-molecular case. The $\varepsilon(T)$ function assumes the reaction duplex → single stranded has no intermediates, i.e. it is a two-state reaction.

2.4. SPE

For recording the standard curve, different amounts of ODN C1 were spiked into 100 μl of human serum. 1.25 μg of internal standard (IS) and different amounts of ODN C1 (150, 50, 16.6, 5.5, 1.9 or 0 μg/ml) were added to the serum samples. Duplicates were prepared for each concentration of analyte. Liver lysate was prepared by digesting 100 mg mouse liver in 500 μl extraction buffer (50 mM Tris pH 8; 0.5% Triton × 100). ODN C1 was added and incubated for the indicated time at 37 °C. The SPE method used at Coley is a modified procedure to a previously published method [10]. Samples were incubated for 2 h at 55 °C in proteinase K buffer (1 mg/ml proteinase K in 30 mM Tris pH 8; 30 mM EDTA; 5% Tween 20; 0,5% Triton × 100; 800 mM Guanidinium HCl) and subsequently applied to phenol extraction. The resulting supernatant was applied on a SAX-SPE column (Agilent-Accubond II SAX cartridge; 100 mg, 1 ml; Part no. 188-1610) using SAX buffer (25 mM Tris/HCL, pH 9.0; 0.25 M KCl; 20% acetonitrile) eluted with the same buffer containing 1 M NaBr and subsequently applied to a RP-SPE column (Glen Research Poly Pak Barrel, 125 mg; Part no. 60-2100-30) using RP-dilution buffer (25 mM Tris/HCl, pH 9.0; 0.5 M KCl; 1 M NaBr). The eluate of the RP-column was dialyzed against deionized water and applied to CGE.

2.5. CGE

For CGE, a PACE MDQ system (Beckman-Coulter, Fullerton, USA) equipped with the 32 Karat 5.0 software (Beckman-Coulter, Fullerton, USA) was used. The desalted sample solutions were injected either electrokinetically using water preinjection or by pressure injection into a gel-filled capillary from the sample side. The electrophoretic conditions were: (i) capillary: eCAP DNA, neutral, 21 cm or 50 cm effective length, 100 μm I.D. (Beckman-Coulter Part no. 477477); (ii) capillary temperature: 25 °C, 30 °C, 40 °C, 50 °C or 60 °C; (iii) sample storage temperature: 10 °C; (iv) gel: ssDNA 100 R (Beckman-Coulter Part no. 477621) dissolved in either 4 ml or 5 ml of buffer and 0–50% of organic solvents (exact amounts and type of solvent indicated in the Section 3); (v) buffer: Tris/boric acid/EDTA buffer containing 7 M urea (Beckman-Coulter Part no. 338481) and 0–50% of organic solvent (indicated in the Section 3); (vi) detection wavelength: 254 nm; (vii) separation voltage: 300–600 V/cm; (viii) injection time: 2–45 s; (ix) injection voltage: 10 kV; (x) injection pressure: 9–25 psi; (xi) run time: 45–60 min; (xii) data acquisition rate: 4 pt. s^{-1}.

2.6. Calculations for CGE evaluation

Electropherograms were analyzed by integration using the 32 Karat Software Version 5.0 (Beckman-Coulter, Fullerton, USA), migration times and the corrected peak areas of the main peaks for each sample were recorded using the formula: $A_{corr} = v \times A = L_d \times A/t$ where v = velocity; L_d = capillary length to detector; t = migration time; A = uncorrected peak area; A_{corr} = corrected peak area. For the evaluation of different buffer systems, typically duplicates or triplicates were recorded for the different separation conditions. All described ODN concentrations were calculated using area under the curve integrations of the CGE electropherograms comparing a defined amount of internal standard ODN (IS) to the analyte ODN C1. Concentrations of ODN C1 were then calculated using the following formula: ODN concentration (μg ODN/ml plasma or lysate) = A/B, where A = extinction coefficient of analyte × corrected area of analyte × amount of standard (μg) × 1000, and B = extinction coefficient of standard × corrected area of standard × sample volume (ml). Data was expressed as means of values obtained with three individual samples for each given time and dose. Typical integration parameters were: (i) threshold: 300; (ii) peak width: 1.0; and (iii) peak sensitivity: 300.

2.7. Sample pretreatment

C-Mix was diluted 1:5 with water/FA or water/DMSO to obtain a solution (50 μg/ml of each ODN) containing either

0, 30, 50, or 80% FA or 10, 20, 30, or 50% DMSO in water. Before injection into the capillary, samples were heated to 95 °C for 5 min, then chilled on ice and subsequently applied to CGE analysis.

3. Results & discussion

3.1. Characterization of ODN C1 under native conditions

To characterize the prevalent secondary structures of ODN C1 in solution, we measured temperature dependent UV-absorption and performed SEC in physiological buffers. Native SEC analysis was performed at concentrations of 125, 12.5, 6.25 or 3.125 μM ODN C1 in PBS. Chromatograms showed two distinct peaks for ODN C1 at retention times (t_r) correlating to dimer (t_r = 21.5 min) and monomer (t_r = 25.4 min), indicating the presence of both forms and confirming the prevalence of dimers at the tested concentrations. In contrast, ODN B1 showed a single monomer peak (t_r = 25.7 min) at all concentrations (Fig. 1). Peak areas of the dimeric complex of ODN C1 was determined (96.5% at 125 μM, 95% at 12.5 μM in PBS; 94.0% at 6.25 μM; 93.8% at 3.125 μM). The suggested model for the secondary structures formed by ODN C1 is displayed in Fig. 2, where the molecule is able to interchange between a monomeric hairpin

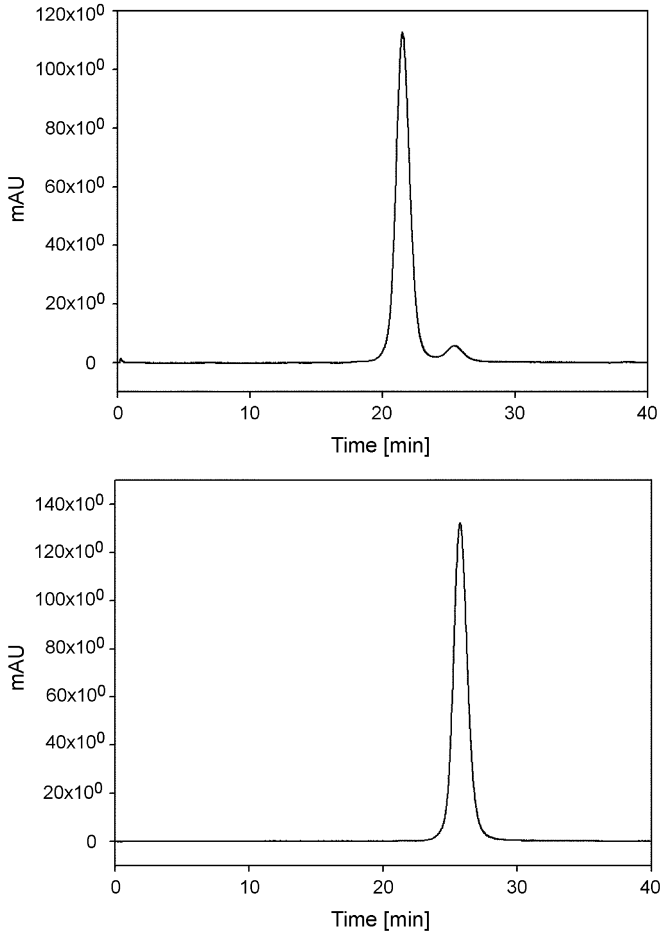

Fig. 1. SEC of ODN C1 (upper panel) and ODN B1 (lower panel). D-PBS was used as sample and running buffer. ODN concentrations were 6.25 μM.

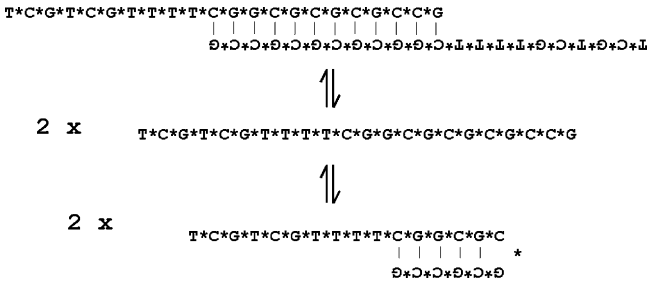

Fig. 2. Model for secondary structure formation of ODN C1 in solution. An equilibrium exists in solution between a dimer of ODN C1 associated at the 3′ GC-rich region (upper structure) or two monomeric structures, either random coiled (middle structure) or displaying a 3′-hairpin (lower structure). Watson–Crick base pairs are symbolized by dashes, asterisks indicate phosphorothioate bonds.

form and dimeric duplex. In both cases Watson–Crick base pairing between the bases of the 3′ palindromic region is responsible for the proposed structures. Melting curves were recorded using solutions of 1.25–8.75 μM ODN C1 in physiological buffer (D-PBS). The calculated melting points in the heating cycle varied between 62 and 65 °C depending on concentration (Table 2). The low hyperchromicity of these solutions (<5%) reflects the fact that only 12 out of the 22 bases of ODN C1 participate in duplex formation. The measured melting curves were fitted using the Marquardt-Levenberg algorithm. When T_m was plotted over ln (C_t), where C_t is the total strand concentration, a linear relation was detected, displaying a correlation factor r^2 of 0.9941 (data not shown). According to the equilibrium equation for bi-molecular reactions of self-complementary strands: $1/T_m = (R/\Delta H) \times \ln(C_t) + \Delta S/\Delta H$ (where R = gas constant; T_m = melting temperature; H = enthalpy; S = entropy and C_t = total strand concentration) this indicated a bimolecular mechanism of the melting process, suggesting the presence of dimeric duplexes rather than hairpins in the analysed concentration range.

3.2. Initial CGE separations of B-Mix and C-Mix

For a mixture of non-secondary structure forming ODN (B-Mix), resolutions >1 between peaks were easily achieved using CGE conditions as suggested by the 100ssDNA Kit (Beckman-Coulter, Fullerton, USA) (Fig. 3, upper left panel). However, when the same conditions were applied to C-Mix, no separation between single peaks could be observed (Fig. 3, upper right panel). This phenomenon was attributed to the presence of unresolved secondary structures at the 3′ends of the ODN strands, since each ODN of C-Mix contained the same 12 bases long, palindromic GC-rich sequence at the 3′-end and therefore, was able to form duplexes with any other ODN in the sample. Accordingly, native CGE-analysis of C-Mix resulted in a largely unresolved pattern of at least 10 overlapping peaks, most likely due to the formation of dimers and heterodimers in addition to, the respective monomer peaks (data not shown). Although, native conditions allowed separation of different species of ODN C1 in solution, neither native CGE nor standard CGE using the 7 M urea buffer system was considered useful for identification

Table 2
Calculated melting temperatures (T_m) displayed in (°C) and thermodynamic parameters from thermal melting experiments of ODN C1

Concentration (M)	dH (kcal/mol)	dS (cal/K × mol)	dG (kcal/mol)	Tm (°C)
8.75×10^{-06}	−78.85	−210.24	−13.65	64.72
7.00×10^{-06}	−73.79	−195.33	−13.20	63.90
5.25×10^{-06}	−67.41	−176.23	−12.75	63.26
3.50×10^{-06}	−63.40	−164.16	−12.49	62.10

ODN concentrations are given in the left column. The other columns depict: Enthalpy = dH; Entropy = dS; free enthalpy = dG.

and quantification of single metabolites in complex mixtures of secondary structure forming C-Class ODN, as would be present in extracts of biological samples.

3.3. Capillary temperature

Temperature is a critical parameter in the separation of ODN, since many secondary structures can be resolved at elevated temperatures. The reported temperature optimum for separations of DNA fragments or GC-rich ODN lies between 60 and 70 °C [35–37]. In our experiments, capillary temperatures between 30 and 50 °C did not result in sufficient peak separation for the C-Mix for any of the tested buffer systems (data not shown). Best separations for the C-Mix were achieved at the highest tested capillary temperature (60 °C). Separation time decreased when capillary temperature increased, owing to the reduced electrical resistance and, on this account, higher current. However, separation was not sufficient using the 7 M urea buffer system without additives (Fig. 3, lower left panel). We did not increase temperature above 60 °C, since urea is thermally unstable [38] and at temperatures over 70 °C the products of thermal decomposition of urea have a deleterious effect on separation performance [36]. In addition, capillary temperatures above 30 °C led to increased fluctuations in the baseline as well as the current. Baseline noise levels increased approximately three-fold when comparing separations at 25 °C and 60 °C. This appeared to be largely independent from buffer or gel composition. The high temperatures required for resolving ODN secondary structures may be in part caused by the high local concentration of ODN in the capillary. Since, ODN C1 strongly favours the formation of a dimeric complex, the melting temperature is expected to increase with concentration and thus, require stronger denaturing condition in the capillary at higher ODN concentrations.

3.4. Capillary length

To evaluate separation efficiency we focused on two parameters of the CGE-analysis. Firstly, since we aim for a method

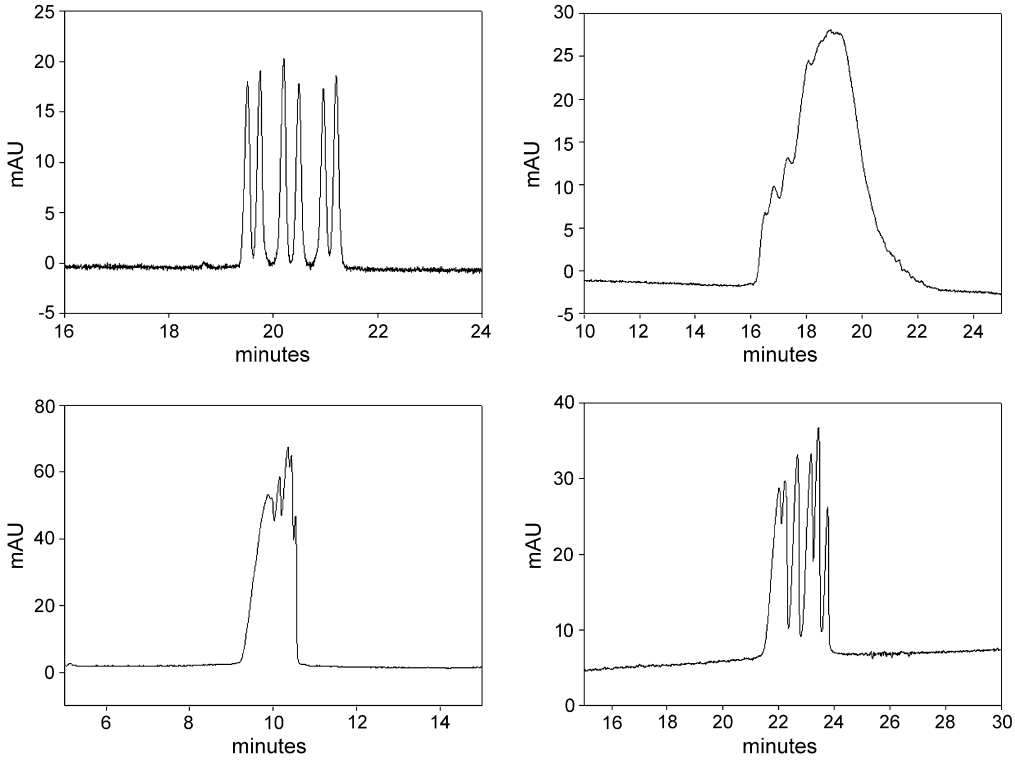

Fig. 3. Electropherogram of B-Mix (upper left panel; 21 cm neutral capillary, 25 °C) or C-Mix applied by voltage injection using CGE with 7 M urea/TBE buffer system at different temperatures. Upper right panel: 21 cm neutral capillary, 25 °C; lower left panel: 21 cm neutral capillary, 60 °C; lower right panel: 50 cm neutral capillary, 60 °C.

Table 3
Peak resolutions (USP) of parent compound and metabolite markers of different CGE runs

	Resolution between peaks	7 M urea/21 cm Cap B-Mix	7 M urea/50 cm Cap C-Mix	10% DMSO/50 cm Cap C-Mix	30% DMSO/21 cm Cap C-Mix	30% DMSO/50 cm Cap C-Mix
1	1/2	1.3235	1.0492	1.2131	0.5952	1.5172
2	2/3	2.5874	0.6923	0.8421	0.3697	1.0874
3	3/4	1.5863	1.1807	1.3580	0.6957	1.7778
4	4/5	2.4941	1.0112	1.1951	0.4459	1.5701
5	5/6	1.2477	0.3387	0.5185	0.2660	0.9344

Gel type, capillary length and sample type are listed in table. All CGE separations for C-Mix were carried out at 60 °C, CGE separation of B-Mix was carried out at 25 °C. Peak resolution was calculated using the formula: $R_s = 2(t_{M2} - t_{M1})/(w_{b1} + w_{b2})$, where R_s is peak resolution, t_{M1} and t_{M2} are the migration times of peak 1 and 2, respectively and w_{b1} and w_{b2} are the mean peak widths at the bases of peak 1 and 2.

enabling us to assign the detected peaks in an unknown ODN mixture to certain ODN lengths, we plotted ODN length over migration time and determined "goodness of fit" (r^2) to a linear curve. Secondly, we calculated the resolution between the single ODN peaks (R_s), using peak spacing and peak width for calculation of peak resolution. With or without organic additives to the 7 M urea buffer system, the standard capillary length (21 cm effective capillary length) was not sufficient to achieve complete peak separation for the C-Mix (Fig. 3, upper right panel & lower left panel). CGE analysis was performed using longer capillaries since, according to the principles of CGE separation, peak spacing per base is a linear function of capillary length when all other variables are held constant [39]. Sufficient resolutions were obtained using a capillary of 50 cm effective length in combination with capillary temperatures of 60 °C. Compared to the 21 cm capillary, an increase of 0.7–1.0 in resolution between peaks was observed for the 30% DMSO gel using the 50 cm capillary (Table 3). However, the gain in resolution was achieved at the cost of additional separation time, which increased proportionally.

3.5. Addition of organic solvents

When using the established 7 M urea/TBE buffer system without additives at 60 °C, resolution of the single peaks of the C-Mix varied between 0.3 and 1.2 (Table 3). Including 30% EC in the buffer system decreased peak resolution, indicating that the denaturing properties of EC in combination with urea were lower for GC-rich ODN than for the common 7 M urea/TBE buffer system (Fig. 4, upper left panel). NMF has been described to show higher stability in CGE than FA, and high electrophoretic mobility of analytes due to its high dielectric constant [30]. Since, addition of 30% NMF to the 7 M urea buffer system resulted in insolubility of urea, urea concentration

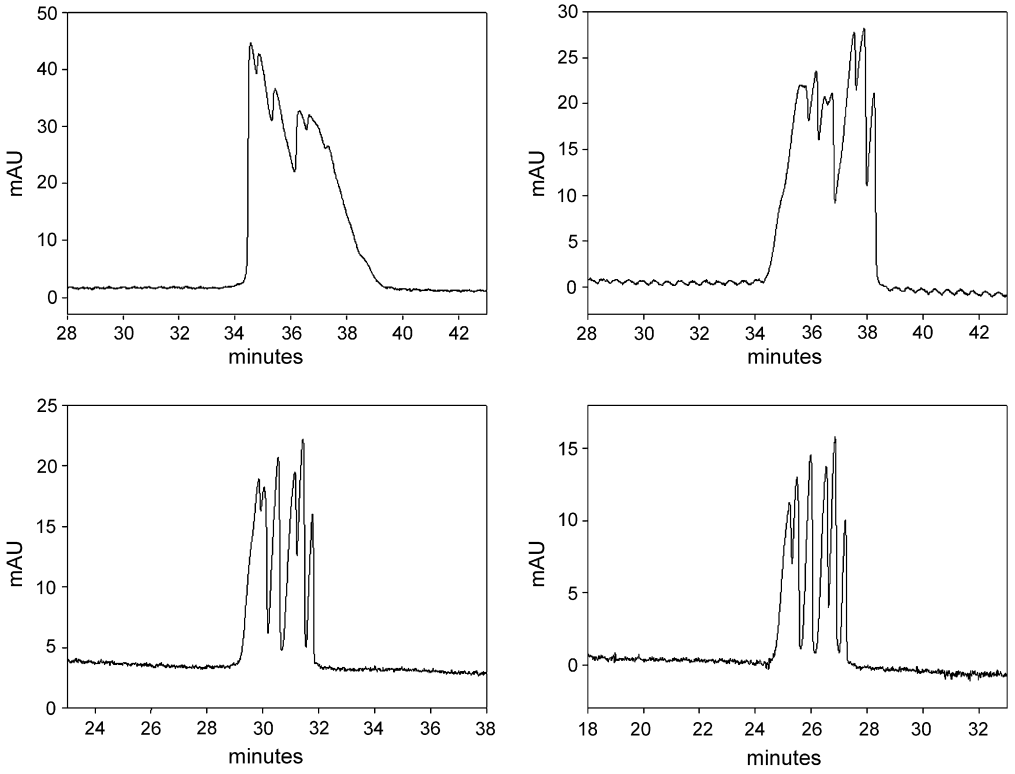

Fig. 4. Electropherogram of C-Mix applied by voltage injection using CGE with a 50 cm neutral capillary at 60 °C and different organic additives to a TBE/urea buffer system. Upper left panel: 30% EC; upper right panel: 30% NMF; lower left panel: 30% FA; lower right panel: 10% DMSO.

in the buffer was reduced from 7 to 3 M before addition of NMF. For the resulting buffer system, resolution was lower compared to the 7 M urea/TBE buffer system, indicating that the loss in denaturing power caused by reduction of the urea concentration could not be overcome by the possibly stronger denaturing properties of NMF (Fig. 4, upper right panel). Resolution of the CGE separation did not change by including up to 50% FA to the 7 M urea/TBE buffer system (Fig. 4, lower left panel), however, when performing CGE including 10% DMSO resolution was significantly improved (Fig. 4, lower right panel, Table 2). In addition to DMSO being a stronger denaturing agent than FA [40,41] its thermal stability is much higher [42] whereas FA is prone to thermal decomposition at elevated temperatures [20]. Best separations were achieved using a buffer system including 7 M urea and 30% DMSO using a capillary with an effective length of 50 cm (Fig. 5, Table 2). A linear relation between ODN length and migration time was demonstrated, enabling identification of metabolite lengths in electropherograms of samples including unknown components (Fig. 6). Although, migration times displayed fluctuations of up to 10%, mostly depending on the age of gel and capillary, variations of the relative migration time appeared to be minor. Small deviations from the ideal linear relation of ODN length and relative migration time were highly reproducible and most likely due to the specific base sequences of the respective ODN (Fig. 6) [43]. A significant increase in separation time was observed when DMSO was used as buffer additive, approximately doubling separation time for the 30% DMSO system compared to the 7 M urea/TBE buffer system (45 min compared to 21 min). This was attributed to the lower electrical conductivity of DMSO compared to water as well as increasing solvent viscosity and dielectric friction caused by interaction of charged ODN molecules and the organic solvent [44]. We tried to limit the amount of DMSO to a minimal concentration, which still gave us resolutions (USB) >1. Increasing DMSO concentrations to 40% did not significantly increase peak separation relative to the minimal concentration, but further increased separation time. Therefore, we suggest using concentrations of 20–30% DMSO for optimal separation of C-Mix. Further improvement on peak separation was observed, when gel density was increased by adding only 4 ml of buffer to the gelmatrix instead of 5 ml.

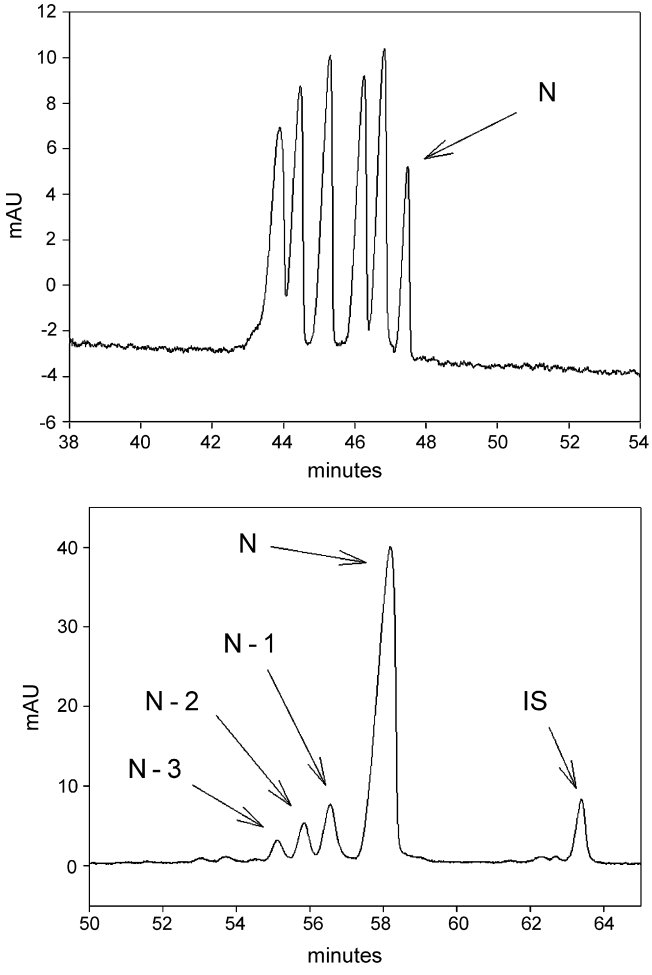

Fig. 5. Electropherogram of C-Mix applied by voltage injection using CGE with 7 M urea/TBE buffer system including 30% DMSO in a 50 cm capillary at a capillary temperature of 60 °C (upper panel). Electropherogram of an SPE extract from mouse liver lysate spiked with CPG C 1 after 24 h incubation at 37 °C (lower panel). IS = internal standard; N = ODN C1; $N-1$, $N-2$ and $N-3$ = metabolites Resolutions (USB): N to $N-1 = 2.09$; $N-1$ to $N-2 = 1.19$; $N-2$ to $N-3 = 1.30$. Run time differences are due to different gel densities. The upper gel was dissolved in 5 ml buffer, the lower gel in 4 ml buffer.

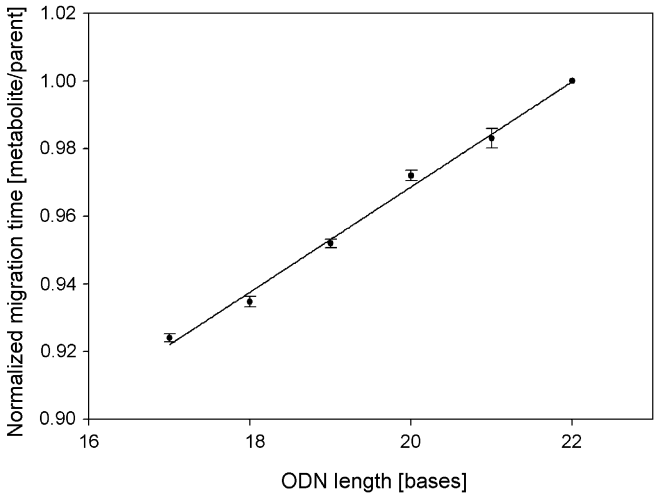

Fig. 6. Standard curve for determination of metabolite length. CGE was performed using 7 M urea containing 30% DMSO at 60 °C and C-Mix. Normalized migration time was plotted over metabolite length and a linear fit was performed using the linear equation $y = a \times x + b$. Error bars were calculated from nine independent injections. Goodness of fit (r) was calculated using Pearson's formula: $r = (N \times \sum X \times Y - \sum X \times \sum Y)/\sqrt{(N \times \sum X^2 - (\sum X)^2)} \times \sqrt{(N \times \sum Y^2 - (\sum Y)^2)}$ and was expressed as r^2. Correlation coefficient $r^2 = 0.9940$.

3.6. Sample pretreatment & injection conditions

Addition of up to 80% FA or up to 50% DMSO to the samples before injection (with or without denaturing by heating to 95 °C) did not result in significant differences in peak resolution compared to untreated samples in water. This was true for all

Table 4
Concentrations of ODN C1 (μg/ml) calculated using the standard curve for human serum samples spiked with ODN C1 and internal standard IS

Conc. (μg/ml)	Day 1		Day 2		Day 3		Mean	SD	CV (%)
	1	2	1	2	1	2			
50	56.41	59.11	45.88	45.10	46.63	47.27	50.07	±6.06	12.1
10	9.86	7.79	9.89	10.63	8.54	8.85	9.26	±1.05	11.3
2	1.53	1.71	2.04	2.05	1.86	nd	1.84	±0.22	12.0
0.4	0.36	nd	1.09	1.28	0.92	0.85	0.90	±0.35	38.2
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0

For each concentration duplicate samples were prepared in three independent preparations. nd = not detected (standard and analyte); SD = standard deviation; CV = coefficient of variation.

tested buffer systems (data not shown). This finding indicates the ability of GC-rich ODN to re-form secondary structures already resolved in the sample over the course of a CGE separation when using insufficient denaturing conditions in the capillary. Pressure and voltage injection were evaluated using buffer systems with or without organic additives. For voltage injection, injection times between 2 and 45 s were used with voltages up to 10 kV. For pressure injections, injection times up to 60 s at pressures of up to 25 psi were used. In our assays, similar resolutions were achieved using both techniques. However, several disadvantages were associated with pressure injection. Compared to voltage injection, a smaller amount of ODN could be loaded onto the capillary, resulting in reduced sensitivity (approximately, 1/3 of the UV-absorption at similar injection times). Longer injection times (>20 s) or higher pressures (up to 25 psi) could not completely compensate for this loss in sensitivity. In addition, reproducibility appeared to be lower using pressure injection. Furthermore, when applying samples extracted from serum using pressure injection, we often observed shifts in the baseline and we generally encountered difficulties in detecting the ODN (data not shown). Therefore, a method using voltage injection was preferred for the analysis of extracted serum samples. Best results for CGE separations were achieved using the highest injection voltage setting (10 kV) in combination with water pre-injection (2 s). Injection times were increased up to 45 s without loss of resolution. In addition, peak intensity had an influence on CGE separation. Best separations for solutions containing mixtures of ODN of similar length were achieved when single peak intensities did not exceed 0.04 AU. When peak intensities were below 0.01 AU the relative increase in baseline caused loss of sensitivity. Significantly decreased resolution and peak broadening was observed for C-Mix when the total signal intensity exceeded 0.05 AU per peak. We attributed this effect to overloading the capillary at the sample side during injection. This effect can be explained by higher local concentrations of ODN, resulting in a shift of the equilibrium to the dimeric forms. Consequently, we adjusted injection times for peak intensities between 0.01 and 0.04 AU.

3.7. Quantification of ODN C1 using a standard curve and separation of a liver lysate sample

To demonstrate linearity of the CGE analysis following SPE, a standard curve was recorded. Human serum samples were spiked with 150, 50, 16.6, 5.5 and 1.9 μg/ml ODN C1. Since overall recovery of extraction and analysis was described to be approximately 50% [10] a defined amount of IS was added and used for quantification. After SPE, CGE analysis was performed using the 30% DMSO buffer system. The correlation coefficient (r^2) of the resulting standard curve was calculated from the means of the duplicates for all data points. R^2 was 0.99994 (Fig. 7), demonstrating linearity in the tested concentration range (2–150 μg/ml) and proving the method applicable for accurate quantification of ODN C1 and its metabolites in biological samples. By performing three independent experiments the LLOQ for ODN C1 was estimated to be below 1.9 μg/g (Table 4). Since baseline noise was approximately 0.0003 mAU, the LLOQ was estimated to be 1 μg/g, equalling 0.003 mAU, when injections were optimized to give absorptions of 0.04 mAU for an IS concentration of 12.5 μg/ml. These values were closely matching the sensitivity described for conventional CGE (0.35–1.2 μg/g) [11]. At analyte concentrations five or more times higher than the IS concentration, variability increased, mostly due to larger errors in peak integration when comparing peaks of great differences in size (Fig. 7).

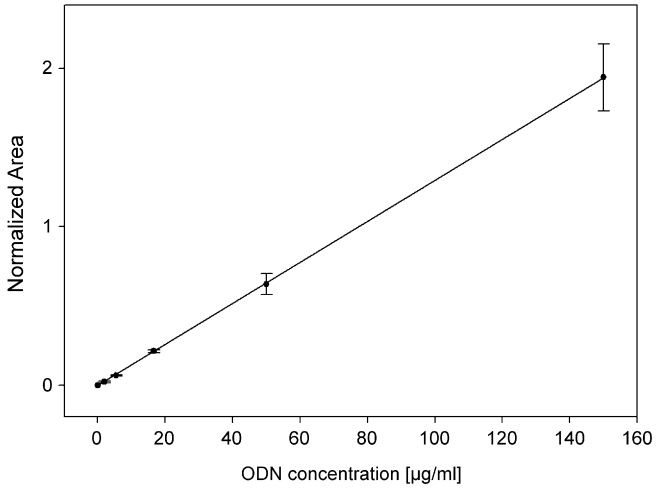

Fig. 7. Standard curve for human serum samples spiked with ODN C1 using ODN IS as an internal standard. For each concentration duplicate samples were prepared and extracted using SPE. CGE was performed using 7 M urea containing 30% DMSO at 60 °C. The standard curve was obtained by plotting the ODN concentrations calculated from the corrected areas of the electropherograms over the amount of ODN added to the samples before SPE. Means from the duplicates were plotted. Correlation coefficient $r^2 = 0.99994$.

Subsequently, liver lysate was spiked with 50 μg/ml ODN C1 and incubated at 37 °C for 24 h. IS was added and ODN were isolated by SPE. Extracts were analysed by CGE. Fig. 5 (lower panel) shows the generated electropherogram of ODN C1 with *in vitro* metabolites. Using the described CGE method peak separations to almost baseline (>1) were reached. Resolutions (USB) were: N to $N-1 = 2.09$; $N-1$ to $N-2 = 1.19$; $N-2$ to $N-3 = 1.30$, demonstrating applicability of the method for separation of shorter ODN fragments. We expect this method to be applicable for studies using dose levels of 0.5 mg/kg and above. However, for lower dose levels, as were reported in clinical studies of immunostimulatory ODN [3,4] sensitivity of CGE using UV detection may generally be to low.

4. Conclusion

We have successfully developed a modified CGE method that allows identification and quantification of complex mixtures of GC-rich ODN. The stable secondary structures formed by these ODN render classical CGE unsuitable for analysis. Increase of separation temperature and capillary length in combination with the addition of up to 30% DMSO to the established 7 M urea/TBE buffer system was required to achieve sufficient peak resolutions. Following extraction from liver lysate, GC-rich ODN were successfully analysed by CGE and peaks separated to baseline. This method solves the problem of quantitative pharmacokinetic and pharmacodynamic assessment of secondary structure forming GC-rich ODN in tissues and biological samples that heretofore were unsusceptible to quantitative analysis.

Acknowledgements

The authors wish to thank Tanja Sniatala and Angela Lohner for their excellent experimental work in CGE and HPLC, Matthias Görlach and Dirk Razey for oligonucleotide synthesis, Mike McCluskie for providing mouse tissues and Heather Davis for her help with the manuscript.

References

[1] A.M. Krieg, Curr. Oncol. Rep. 6 (2004) 88.
[2] D.M. Klinman, Nat. Rev. 4 (2004) 1.
[3] C.L. Cooper, H.L. Davis, M.L. Morris, S.M. Efler, M.A. Adhami, A.M. Krieg, D.W. Cameron, J. Heathcote, J. Clin. Immunol. 24 (2004) 693.
[4] A.M. Krieg, S.M. Efler, M. Wittpoth, M.J. Al Adhami, H.L. Davis, J. Immunother. 27 (2004) 460.
[5] A. Dove, Nat. Biotechnol. 20 (2002) 121.
[6] S. Agrawal, E.R. Kandimalla, Nat. Biotechnol. 22 (2004) 1533.
[7] A.S. Cohen, S. Terabe, J.A. Smith, B.L. Karger, Anal. Chem. 59 (1987) 1021.
[8] W.C. Quigley Wes, N.J. Dovichi, Anal. Chem. 76 (2004) 4645.
[9] L.A. DeDionisio, Methods Mol. Biol. 162 (2001) 353.
[10] J.M. Leeds, M.J. Graham, L. Truong, L.L. Cummins, Anal. Biochem. 235 (1996) 36.
[11] R.Z. Yu, R.S. Geary, A.A. Levin, Curr. Opin. Drug Discov. Devel. 7 (2004) 195.
[12] J. Hernandez-Borges, C. Neususs, A. Cifuentes, M. Pelzing, Electrophoresis 25 (2004) 2257.
[13] A. von Brocke, T. Freudemann, E. Bayer, J. Chromatogr. A 991 (2003) 129.
[14] A.S. Cohen, A.J. Bourque, B.H. Wang, D.L. Smisek, A. Belenky, Antisense Nucleic Acid Drug Dev. 7 (1997) 13.
[15] B.O. Noll, M.J. McCluskie, T. Sniatala, A. Lohner, S. Yuill, A.M. Krieg, C. Schetter, H.L. Davis, E. Uhlmann, Biochem. Pharmacol. 69 (2005) 981.
[16] T.I. Todorov, M.D. Morris, Electrophoresis 23 (2002) 1033.
[17] C. Heller, Electrophoresis 20 (1999) 1962.
[18] C. Heller, Electrophoresis 20 (1999) 1978.
[19] J.M. Bowling, K.L. Bruner, J.L. Cmarik, C. Tibbetts, Nucleic Acids Res. 19 (1991) 3089.
[20] K.D. Konrad, S.L. Pentoney Jr., Electrophoresis 14 (1993) 502.
[21] G. Hartmann, J. Battiany, H. Poeck, M. Wagner, M. Kerkmann, N. Lubenow, S. Rothenfusser, S. Endres, Eur. J. Immunol. 33 (2003).
[22] J.D. Marshall, K. Fearon, C. Abbate, S. Subramanian, P. Yee, J. Gregorio, R.L. Coffman, G. Van Nest, J. Leukoc. Biol. 73 (2003) 781.
[23] M. Jurk, B. Schulte, A. Kritzler, B. Noll, E. Uhlmann, T. Wader, C. Schetter, A.M. Krieg, J. Vollmer, Immunobiology 209 (2004) 141.
[24] E. Uhlmann, J. Vollmer, Curr. Opin. Drug Discov. Devel. 6 (2003) 204.
[25] J. Vollmer, R. Weeratna, P. Payette, M. Jurk, C. Schetter, M. Laucht, T. Wader, S. Tluk, M. Liu, H.L. Davis, A.M. Krieg, Eur. J. Immunol. 34 (2004) 251.
[26] J. Zhang, Y. Fang, J.Y. Hou, H.J. Ren, R. Jiang, P. Roos, N.J. Dovichi, Anal. Chem. 67 (1995) 4589.
[27] Z. Kabatek, K. Kleparnik, B. Gas, J. Chromatogr. A 916 (2001) 305.
[28] H. Lu, E. Arriaga, D.Y. Chen, D. Figeys, N.J. Dovichi, J. Chromatogr. A 680 (1994) 503.
[29] K. Kleparnik, F. Foret, J. Berka, W. Goetzinger, A.W. Miller, B.L. Karger, Electrophoresis 17 (1996) 1860.
[30] P. Lindberg, J. Roeraade, J. Liq. Chromatogr. 22 (1999) 307.
[31] M.J. Rocheleau, R.J. Grey, D.Y. Chen, H.R. Harke, N.J. Dovichi, Electrophoresis 13 (1992) 484.
[32] D.Y. Chen, H.P. Swerdlow, H.R. Harke, J.Z. Zhang, N.J. Dovichi, J. Chromatogr. 559 (1991) 237.
[33] S.H. Chen, R.T. Tzeng, Electrophoresis 20 (1999) 547.
[34] C. Zimmer, G. Luck, Biochim. Biophys. Acta 287 (1972) 376.
[35] O. Salas-Solano, E. Carrilho, L. Kotler, A.W. Miller, W. Goetzinger, Z. Sosic, B.L. Karger, Anal. Chem. 70 (1998) 3996.
[36] L. Kotler, H. He, A.W. Miller, B.L. Karger, Electrophoresis 23 (2002) 3062.
[37] H. Zhou, Y. Zhang, Z. Ou-Yang, Phys. Rev. E. Stat. Phys. Plasmas. Fluids Relat. Interdiscip. Topics 62 (2000) 1045.
[38] E. Nachbaur, E. Baumgartner, J. Schober, Proc. Eur. Symp. Therm. Anal. 2 (1981) 417.
[39] C. Heller, Electrophoresis 22 (2001) 629.
[40] J.F. Escara, J.R. Hutton, Biopolymers 19 (1980) 1315.
[41] J.R. Hutton, Nucleic Acids Res. 4 (1977) 3537.
[42] D.L. Head, C.G. McCarty, Tetrahedron Lett. 16 (1973) 1405.
[43] R. Frank, H. Koster, Nucleic Acids Res. 6 (1979) 2069.
[44] K.I. Roy, C.A. Lucy, Electrophoresis 23 (2002) 383.