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Journal of Chromatography B, 820 (2005) 197–203
JOURNAL OF CHROMATOGRAPHY B
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The synthesis and initial characterization of an immobilized DNA unwinding element binding (DUE-B) protein chromatographic stationary phase

Ruin Moaddel [a,b,*], Gerry B. Price [c,✠], Jean-Marc Juteau [d], Michael Leffak [e], Irving W. Wainer [a,2]

[a] *Department of Pharmacology, School of Medicine, Georgetown University, Washington, DC, USA*
[b] *Gerontology Research Center, National Institute on Aging/NIH, Baltimore, MD, USA*
[c] *Department of Oncology, McGill University, Montreal, Canada*
[d] *Replicor Inc., Laval, Que., Canada*
[e] *Wright State University School of Medicine, Dayton, OH, USA*

Received 24 November 2004; accepted 13 March 2005
Available online 25 April 2005

Abstract

The DNA unwinding element binding protein (DUE-B) plays a key role in DNA replication. The DUE-B protein has been immobilized on a liquid chromatography support and the resulting immobilized protein column was used for the on-line screening of a series of steroids. The DUE-B protein was expressed with an added C-terminal sequence of six adjacent histidine residues, a His_6-tag and immobilized on a chiral ligand exchange support, the CLC-L column, using Ni^{2+} as the coordinating metal ion. The chromatographic retentions of 12 steroids were determined on the DUE-B/CLC-L column. The magnitudes of the steroid-immobilized DUE-B interactions, reflected by the observed retention times, correlated to the effect of the steroids in the cell-free replication system, i.e. the longer the retention, the greater the increase in DNA replication. The coefficient of determination for the %DNA activities linear relation to retention time was 0.9694. The data suggest that the DUE-B/CLC-L phase can be used for on-line pharmacological studies. The results also indicated that His-tagged proteins can be directly immobilized on the CLC-L stationary phase and the resulting columns used as rapid screens for the isolation and identification of small molecule or protein ligands from complex biological or chemical mixtures.
Published by Elsevier B.V.

Keywords: Affinity chromatography; Immobilized protein; DNA replication; Metal complexation chromatography; On-line screening

1. Introduction

While estrogens have been extensively used in hormone-replacement therapy and oral contraception, they have also been associated with an increased risk of breast cancer, as several in vitro studies have shown a correlation between estrogens and carcinogenesis [1]. The basis for this effect is believed to be their ability to induce cell proliferation and neoplastic changes via interactions with estrogen receptors [1].

Recent studies have challenged the assumption that interactions with the estrogen receptors are the only mechanism by which steroids affect cellular growth. In particular, Diaz-Perez et al. have shown that estrogens and other steroids affected the extent of DNA replication in a cell-free system obtained from progesterone and estrogen receptor negative HeLa cells [2,3]. The results suggested that steroids could express their activity by direct interaction with DNA

* Corresponding author at: Bioanalytical and Drug Discovery Unit, National Institute on Aging/National Institutes of Health, Gerontology Research Center, 5600 Nathan Shock Drive, Baltimore, MD 21224-6825, USA. Tel.: +1 410 558 8294; fax: +1 410 558 8409.
E-mail address: moaddelru@grc.nia.nih.gov (R. Moaddel).
✠ Deceased.
[2] Present address: Gerontology Research Center, National Institute on Aging/NIH, Baltimore, MD, USA.

1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2005.03.030

or by binding to the protein machinery catalyzing DNA replication.

The primary regulated step of DNA replication is at initiation, where multiprotein complexes control the unwinding of the DNA template to trigger the initiation of DNA synthesis [4] Mutation of short regions of helically unstable DNA (DNA unwinding elements, DUEs) eliminates replication initiation in yeast or human cells [5,6]. Using the DUE of the human c-myc replication origin region as bait in a yeast one-hybrid screen a DNA unwinding element binding protein, DUE-B, was identified [7]. DUE-B is a nuclear protein of approximately 24 kDa (209 aa) that is bound to the c-myc DUE in vivo. Analysis of the predicted DUE-B amino acid sequence revealed that the central region of the protein, amino acids 115–174, displayed 53% similarity to the human androgen receptor.

The role that DUE-B plays in DNA replication is illustrated by the fact that downregulation of DUE-B expression using siRNA inhibits the entry of cells into the S-phase of the cell cycle. In addition, immunodepletion of DUE-B in an Xenopus egg extract inhibited replication, and the addition of (his_6)-tagged DUE-B protein isolated from a transfected HeLa cell extract restored this activity [7].

The current study was designed to test whether the results obtained in the initial studies of steroids in the cell-free replication system could result from interactions with the DUE-B protein. The objective of the project was the development of an immobilized DUE-B protein liquid chromatographic stationary phase, which could be used to determine whether the binding of several steroids to the DUE-B protein correlated with their effects on in vitro DNA replication.

DUE-B is a soluble protein and a number of approaches have been utilized to immobilize non-membrane bound proteins on silica-based liquid chromatographic columns. The most successful of these techniques is covalent tethering, which has been utilized for such diverse proteins as human serum albumin [8,9] and α-chymotrypsin [10]. An alternative approach that did not require in vitro chemical modification of DUE-B was afforded by the expression of DUE-B protein fused to a carboxy terminal hexahistidine (his_6) tag from a baculovirus vector in insect cells [7]. The resulting protein was isolated using Ni^{2+}-nitrilotriacetic acid (Ni-NTA) chromatography performed on an agarose support. Ni-NTA and Ni-IDA, Ni^{2+}-iminodiacetic acid, chromatography are widely used purification techniques that utilize coordination complexes between histidine residues on the expressed protein and Ni^{2+} with additional interactions between Ni^{2+} and NTA or IDA used to anchor the complex to the chromatographic support [11,12].

Since the (his_6)-tagged DUE-B protein was active following chromatographic purification [7], it was expected to retain its activity after immobilization on the Ni-NTA or Ni-IDA columns, as long as the chromatography was performed under non-denaturing conditions. This suggests that metal complexation chromatography can be used to develop an immobilized DUE-B column. However, since the desired on-line screen required increased efficiency and stability, relative to the performance of Ni-NTA and Ni-IDA, a commercially available high performance liquid chromatography chiral ligand exchange column was used in these studies. Chiral ligand exchange chromatography has been extensively used for the enantioselective separations of amino acid enantiomers [13,14]. The technique employs an immobilized chiral selector, such as proline, and utilizes Cu^{2+} as the coordinating metal ion. In general, these phases are stable and efficient.

In these studies, the commercially available CLC-L copper ligand exchange phase (ASTEC) was used as the chromatographic support and the Cu^{2+} was replaced with Ni^{2+} as the coordinating metal ion. The data from the study indicate that the DUE-B protein containing a His_6 tag was successfully immobilized on the column and that the immobilized protein differentially bound members of a test group composed of steroids. In addition, the magnitudes of the steroid-immobilized DUE-B interactions, reflected by the observed retention times, correlated to the effect of the steroids in the cell-free replication system, i.e. the longer the retention, the greater the increase in DNA replication. The results suggest that these phases can be used for on-line pharmacological studies and as rapid screens for the isolation and identification of lead drug candidates from complex biological or chemical mixtures.

2. Materials and methods

2.1. Chemicals

The steroids used in this study are listed in Table 1 and their structures are presented in Fig. 1. Glycerol, ammonium acetate and other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS 1×, pH 7.4) was purchased from Biosource (Camarillo, CA, USA).

2.2. Preparation of the DUE-B protein

The preparation and characterization of the recombinant DUE-B protein has been previously described [7]. In brief, the cDNA encoding the DUE-B protein with a C-terminal $(his)_6$ tag was amplified by PCR, inserted into the pBlue-Bac baculovirus expression vector (Invitrogen) and expressed in Sf9 cells. The hexahistidine tagged protein was purified by Ni^{+}-NTA agarose chromatography under non-denaturing conditions. The resultant protein migrated as a single band in a silver stained gel (Fig. 2).

2.3. Preparation of the DUE-B chromatographic column

2.3.1. Chromatographic column

A 4 mm i.d. × 2 cm column containing the CLC-L copper ligand exchange stationary phase was used in this study and was provided by Advanced Separations Technologies (Whippany, NJ, USA).

Table 1
Compounds used in this study

#	Compound	Chemical name	Supplier
1	Estriol	1,3,5(10)-Estratrien-3,16α,17β-triol	Sigma[a]
2	Estrone acetate	1,3,5(10)-Estratrien-3,16α,17α-triol	Sigma[a]
3	Progesterone	4-Pregnen-3,20-dione	Sigma[a]
4	Prednisolone	1,4-Pregnadien-11β,17,21-triol-3,20-dione	Sigma[a]
5	Testosterone	4-Androsten-17β-ol-3-one	Sigma[a]
6	16-Hydroxy estrone	1,3,5(10)-Estratrien-3,16α-diol-17-one	Sigma[a]
7	2-Hydroxy estrone	1,3,5(10)-Estratriene-2,3-diol-17-one	Sigma[a]
8	E2602	1,3,5(10)-Estratrien-3,16α,17β-triol-16-acetate	Steraloids[b]
9	E2695	1,3,5(10)-Estratrien-3,16α,17β-triol-3-hemisuccinate	Steraloids[b]
10	E2710	1,3,5(10)-Estratrien-3,16α,17β-triol-3-mehtyl ether	Steraloids[b]
11	E3120	1,3,5(10)-Estratrien-3,16α,17β-triol-6-one 6-*O*-carboxymethyl oxime	Steraloids[b]
12	E982	1,3,5(10)-Estratrien-3,17β-diol-7-carboxy thioether-*N*-hydroxy succinimide	Steraloids[b]

[a] Sigma Chemical Co., St. Louis, MO, USA.
[b] Steraloids, Newport, RI, USA.

2.3.2. *Immobilization of DUE-B protein*

The column was conditioned by passing 15 ml of water through the column, followed by 540 ml of a 5 mM solution of Ni_2SO_4 hexahydrate and then 15 ml of water, all at a flow rate of 0.5 ml/min. A solution containing 40 μg of the DUE-B protein in 40 ml PBS 1× was then passed through the primed column at a flow rate of 0.2 ml/min followed by 5 ml of PBS 1×. When not in use, the column was stored with 0.1% NaN_3 at 4 °C.

2.3.3. *Determination of the amount of immobilized protein*

A protein standard series was prepared with BSA in the concentration range of 0–20 μg/ml. The protein content was determined following the instructions provided in the Pierce BCA protein assay kit in which 2.5 ml of reagent A and 2.4 ml of reagent B were mixed with 0.1 ml of reagent C in a Falcon tube at 4 °C. Aliquots (100 μl) of the DUE-B protein solution prior to immobilization and post-immobilization and standards were added in triplicate to a 96-well plate and 100 μl of the mixed BCA reagent was added to each well. The plate was incubated at 37 °C for 2 h and the absorbance values measured at λ = 570 nm with a microplate reader (Bio-Rad, San Diego, CA, USA). The amount of protein in the DUE-B solutions was calculated using Microsoft Excel running on a personal computer and the amount of DUE-B protein immobilized on the CLC-L column was determined by the difference between the protein content of the pre-immobilzation solution less the protein content of the post-immobilization solution.

2.3.4. *Regeneration of the column*

The column was washed with 60 ml of 250 mM imidazole, followed by 40 ml of water, 30 ml of 20% ethanol and finally

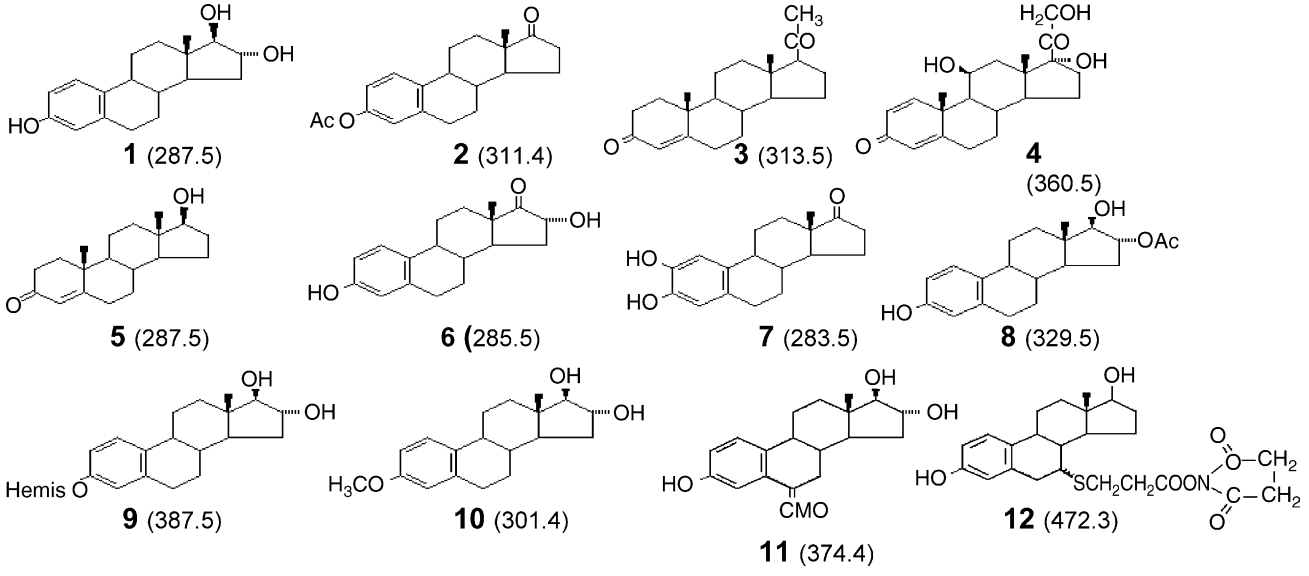

Fig. 1. The molecular structures of the compounds that were used in this study and the specific molecular ions that were monitored by negative-ion mass spectrometry (*m*/*z*) for each compound.

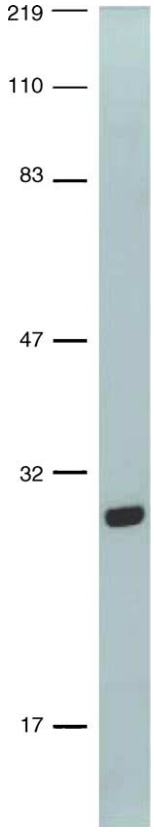

Fig. 2. The hexahistidine tagged protein was purified by Ni^{+}-NTA agarose chromatography under non-denaturing conditions. The resultant protein migrated as a single band in a silver stained gel.

20 ml of water all delivered at a flow rate of 1 ml/min. The DUE-B protein could then be immobilized as described in Section 2.3.2.

2.4. Chromatographic studies

2.4.1. Chromatographic system

The DUE-B column was placed in a chromatography system consisting of a LC-10AD isocratic HPLC pump (Shimadzu, Columbia, MD, USA), connected on-line to the front end of a PE SCI-EX API-100 single quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA).

2.4.2. Chromatographic conditions

The running buffer was composed of ammonium acetate [1 mM, pH 7.4], the flow rate was set at 0.1 ml/min and the experiments were carried out at ambient temperature. A 50 μl aliquot of each ligand was injected onto the column at the following concentrations: 5 μM (compound **12**); 10 μM (compounds **8** and **11**); 20 μM (compounds **1**, **4**–**7**); 30 μM (compounds **2** and **9**); 40 μM (compounds **3** and **10**).

2.4.3. Mass spectrometer settings

Mass spectrometry experiments were performed in negative ion electrospray mode, data was recorded using Sample Control 1.1 and Biomultiview software. The Ion Spray was set at 4.8 kV. The mass spectrometer drying gas temperature was 350 °C, with nitrogen supplied as the nebulising gas (6 l/min). Single ion monitoring (SIM) modes were employed for these experiments. The *m*/*z* values used in the study are presented in Fig. 1.

2.4.4. Displacement studies

Either estriol [100 μM] or testosterone [100 μM] was added to the running buffer, and the chromatographic experiments were carried out as described above.

2.5. In vitro replication assay

The effects of the test compounds on DNA replication were determined using a previously described assay [2,3]. In brief, water (positive control) or the test compounds (10 nM) were added to HeLa cell extracts (S3 nuclei and cytosol, purchased from Cellex Biosciences, Minneapolis, MN, USA) and the solution was incubated at 30 °C for 15 min. To this solution was added a mixture composed of plasmid pX24 containing origin of replication from hamster dihydrofolate reductase (DHFR) locus, an ATP regenerating system, the nucleotides ATP, CTP, GTP, UTP, dATP, dGTP, dTTP and dCTP (Sigma Chemical Co.) and 10 μCi of [α-^{32}P] dCTP and [α-^{32}P] dTTP (NEN-DuPont, Boston, MA, USA). The resulting mixture was incubated at 30 °C for 1 h. The reaction was terminated, the DNA was isolated and purified and the extent of semiconservative DNA replication was measured using the Dpn I resistance assay [15].

3. Results and discussion

3.1. Characterization of the DUE-B/CLC-L column

Based upon the results of the BCA protein assay, ∼30 μg of DUE-B were immobilized onto the stationary phase. Since a marker ligand for the DUE-B had not been identified and characterized, frontal displacement chromatography or zonal displacement chromatography techniques could not be used to compare the binding affinities of the non-immobilized and immobilized protein. However, previous studies using an in vitro replication system had demonstrated that estrogens could enhance DNA replication in an estrogen receptor-free system [2,3]. Since it was hypothesized that the interaction of the steroids with the DUE-B protein was responsible for the observed replication, a series of 12 steroids were used to characterize the binding activity of the DUE-B/CLC-L column.

A subset of six of the 12 test compounds was chromatographed on the CLC-L column before the DUE-B protein was immobilized and after the synthesis of the DUE-B/CLC-L column. For each compound, the observed retention times were greater on the DUE-B/CLC-L column than on the CLC-L column itself, with the increases ranging

Table 2
Observed retention times (RT) for six steroids on the CLC-L column before immobilization of the DUE-B protein and after immobilization to form the DUE-B/CLC-L column

Compound	CLC-L RT (min)	DUE-B/CLC-L RT (min)	Δ (min)
1	6.0	22.3	16.3
3	4.9	13.9	9.0
4	5.8	8.9	4.1
5	5.9	8.6	2.7
7	3.4	7.6	4.2
8	7.5	14.5	7.0

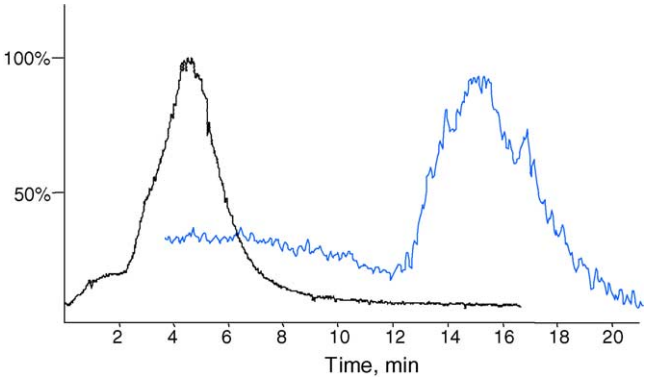

Fig. 3. Chromatographic traces from the chromatography of 40 μM progesterone on the CLC-L column (black trace) and on the DUE-B/CLC-L column (blue trace), see text for experimental details.

from 45% to 271% (Table 2). The chromatographic traces obtained on the CLC-L column were relatively symmetrical, while those obtained on the DUE-B/CLC-L column were broad and asymmetric, which was consistent with on-column ligand–protein interactions (Fig. 3).

Increased chromatographic retentions were observed for up to 4 weeks, indicating that ligand-DUE-B binding activity was retained on the column during this period. The stability of the binding activity was followed using the retention times of compounds **3** and **4**, and when these retention times fell to ∼8 min and ∼5 min, respectively, the DUE-B/CLC-L column was deemed to be inactive.

Once the DUE-B/CLC-L column was deemed to be inactive, the column was regenerated. The similar retention times observed for compounds **3** and **4** (a variation of only 10% and 4%, respectively) after two regenerations of the column indicate that the DUE-B/CLC-L columns were reproducible (Table 3).

Table 3
The retention times (RT) observed for the test compounds progesterone (**3**) and prednisolone (**4**) on regenerated DUE-B/CLC-L columns

Column	Compound **3** RT (min)	Compound **4** RT (min)
1	13.9	8.9
2	14.0	9.5
3	16.6	9.6
Average	14.8 ± 1.5	9.3 ± 0.4

In an attempt to determine if one of the test ligands could be used as a competitive displacer of the others, 100 μM concentrations of compound **1** or **5** were added to the mobile phase and the remaining 11 compounds were chromatographed. Compounds **1** and **5** were chosen based upon earlier studies which had demonstrated that in the cell-free system, compound **1** produced a 363% increase in DNA replication while compound **5** had no effect on DNA replication [2,3].

In these studies, the addition of these compounds to the mobile phase resulted in no significant displacement of any of the test compounds (data not shown). However, the addition of compound **5** to the mobile phase produced a significant increase in the retentions of compound **1**, 22–37 min, and compound **10**, 10–16 min, while the addition of compound **1** to the mobile phase produced a significant increase in the retention of compound **3**, 14–24 min, and a slight increase in the retention of compound **5**, 9–11 min.

In an immobilized protein-based liquid chromatographic stationary phase, an increase in retention produced by the addition of a second compound to the mobile phase has been attributed to a cooperative allosteric interaction [16]. The observation of such an interaction in the DUE-B/CLC-L system suggests that the protein has at least two binding areas that can interact through ligand induced conformational changes and that the immobilized protein retained this conformational mobility. This conclusion is consistent with results showing that baculovirus expressed DUE-B crystallized as a homodimer, and that endogenous DUE-B in human cell extracts or Xenopus egg extracts chromatographs as a homodimer on sizing columns [7].

3.2. DNA replication studies

The calculated effects of the test compounds on DNA synthesis in the cell-free system ranged from 99% of the control to 600% (Table 4). The observed effects of compounds **1**–**3** and **5**–**7** were consistent with previous data [2,3]. While the data can be ranked according to their quantitative effect, a more logical approach is qualitative, whereby a compound

Table 4
The observed retention time (RT) of the test compounds on the DUE-B/CLC-L column and the effect of the test compounds on DNA replication in a cell-free system, presented as %DNA synthesized relative to control

Compound	RT (min)	%DNA
1	22.3 ± 4.1	363
2	10.9 ± 5.0	111
3	13.9 ± 1.8	208
4	8.9 ± 1.1	140
5	8.6 ± 0.5	99
6	8.8 ± 0.9	166
7	7.6 ± 0.4	156
8	14.5 ± 4.4	200
9	4.7 ± 0.9	100
10	10.2 ± 1.0	150
11	5.5 ± 0.4	100
12	74.0 ± 6.9	600

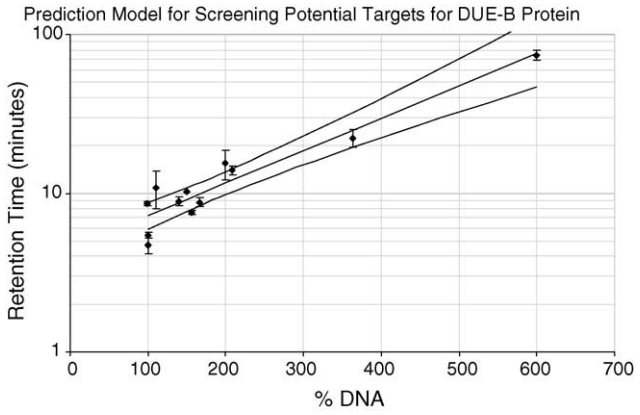

Fig. 4. Correlation between the chromatographic retention of the steroid compounds used in this study expressed as natural logs (ln) of the retention times observed on the DUE-B/CLC-L column and the effect of the steroid compounds on DNA replication in a cell-free system expressed as %DNA replicated relative to control; where a 95% confidence band has also been computed. See text for experimental details.

can be deemed to have no significant effect of DNA replication when the %DNA synthesized relative to control is <200%, while a result of >200% would reflect a significant enhancement of DNA synthesis. Thus, compounds **1**, **3**, **8** and **12** would fall into the latter category.

3.3. Correlation of chromatographic retention and effect on DNA replication

The chromatographically determined retention times and the %DNA replication determined in the cell-free system were correlated using a non-linear regression program found in Microsoft Excel (Fig. 4). A least squares regression, weighting each point on the basis of the expected standard error of the average of the replicates, produced the following relationship, providing an adequate fit to the data:

$$\ln(\text{retention time}) = \exp(a + b \times \%\text{DNA})$$

where $a = 1.50 \pm 0.12$, and $b = 0.0047 \pm 0.0005$.

A 95% confidence band around the fitted curve, which encloses the region in which the true relationship is expected to lie, was also computed and is presented in Fig. 4.

The coefficient of determination for the correlation between %DNA replication activity and retention time was 0.9694, indicating a strong relationship. However, as indicated above, the relationship between the test compounds and %DNA replication is essentially qualitative. Thus, in the initial screening of steroids for compounds that interact with the DUE-B protein and which would enhance DNA replication, the more logical approach would be to use a qualitative measure in which a retention time of 14 min would be the cut-off between potentially inactive and active compounds. This observation will need to be expanded using a larger cohort of steroids and by including other classes of compounds. The results of these studies will be reported elsewhere. In addition, in this study, it was necessary to use different concentrations of the test ligands due to the lack of sensitivity of the mass spectrometer. As only qualitative assessments are being made, we are confident that the differences in the concentration will not effect the conclusions of the study. This issue will also be addressed in the next series of studies.

The correlation between the observed retention times and %DNA synthesized was determined without correcting for any non-specific retention produced by the CLC-L backbone. The initial comparison of the retention of six of the compounds on both the CLC-L and DUE-B/CLC-L columns had demonstrated the average retention time on the CLC-L column was 5.6 ± 1.4 min (Table 2). To check if a correction for non-specific retention was necessary, 5.6 min was subtracted from each of the retention times presented in Table 4 and the resulting data was correlated with %DNA replication (data not shown). There was no change in the qualitative relationship, i.e. a retention time of greater than 8 min indicated a 200% or greater increase in DNA replication, and there was a liner relationship between the chromatographic and replication data, $r^2 = 0.9046$ ($p < 0.0001$) {with compound **12**}; $r^2 = 0.8694$ ($p < 0.0001$) {without compound **12**}. The results indicate that a single chromatographic experiment on a characterized DUE-B/CLC-L column should suffice to qualitatively screen a series of steroids.

4. Conclusions

The results of this study demonstrate that a DUE-B protein with a C-terminal $(his)_6$ tag has been successfully immobilized on a commercially available CLC-L liquid chromatographic ligand exchange column using Ni^+ as the coordinating metal ion. The data indicate that the resulting DUE-B/CLC-L column differentially retained the members of a series of steroids and that the extent of retention correlated with the effect of the steroids on DNA replication in a cell-free system. The results suggest that the DUE-B/CLC-L column can be used to screen compounds for their binding affinity to the DUE-B protein and for their effect on cellular replication. In addition, the data also suggest that His-tagged proteins can be directly immobilized on the CLC-L stationary phase and the resulting columns used as on-line rapid screens for the isolation and identification of small molecule or protein ligands from complex biological or chemical mixtures. Further studies are needed to investigate these possibilities.

Acknowledgements

The authors would like to thank Dr. Tom Beesley from Advanced Separations Technologies Inc., for his assistance and guidance. The work was supported in part by a grant to IWW from Replicor Inc.

References

[1] R. Clarke, L.A. Hilakivi-Clarke, B. Trock, Biologist 48 (2001) 21.

[2] M.J. Diaz-Perez, I.W. Wainer, M. Zannis-Hadjopoulos, G.B. Price, J. Cell. Biochem. 61 (1996) 444.
[3] M.J. Diaz-Perez, M. Zannis-Hadjopoulos, G.B. Price, I.W. Wainer, J. Cell. Biochem. 70 (1998) 323.
[4] H. Nishitani, Z. Lygerou, Genes Cells 7 (2002) 523.
[5] S. Lin, D. Kowalski, Mol. Cell. Biol. 17 (1997) 5473.
[6] G. Liu, M. Malott, M. Leffak, Mol. Cell. Biol. 23 (2003) 1832.
[7] J. Casper, M. Kemp, M. Ghosh, G. Randall, A. Vaillant, M. Leffak, J. Biol. Chem. 280 (2005) 13071.
[8] E. Domenici, C. Bertucci, P. Salvadori, G. Felix, I. Cahagne, S. Motellier, I.W. Wainer, Chromatographia 29 (1990) 170.
[9] C. Bertucci, V. Andrisano, R. Gotto, V. Cavrini, J. Chromatogr. B 768 (2002) 147.
[10] P. Jadaud, S. Thelohan, G.R. Schonbaum, I.W. Wainer, Chirality 1 (1989) 38.
[11] E. Hochuli, Genet. Eng. 12 (1990) 97.
[12] J. Porath, I. Olsson, J. Carlsson, G. Belfrage, Nature 258 (1975) 598.
[13] V.A. Davankov, S.V. Rogozhin, J. Chromatogr. 60 (1971) 280.
[14] V.A. Davankov, S.V. Rogozhin, A.V. Semechkin, T.P. Sachkova, J. Chromatogr. 82 (1973) 203.
[15] K.W. Peden, J.M. Pipas, S. Pearson-White, D. Nathans, Science 209 (1980) 1392.
[16] J. Chen, D.S. Hage, Nat. Biotechnol. 22 (2004) 1445.