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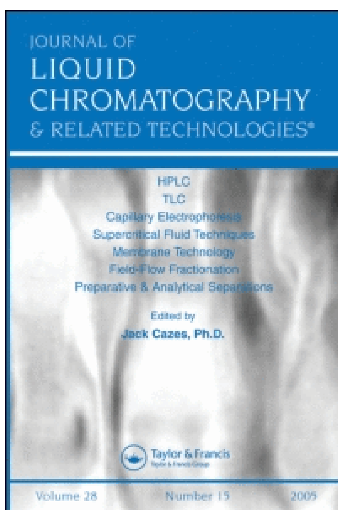

Journal of Liquid Chromatography & Related Technologies

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Online publication date: 27 August 2010

To cite this Article Huuskonen, K. H. , Kunnas, T. A. , Tanner, M. M. , Kortelainen, M. L. and Nikkari, S. T.(2009) 'Application of Denaturing HPLC for Detection of G/C Polymorphism in the Cyclo-Oxygenase-2 Gene', Journal of Liquid Chromatography & Related Technologies, 32: 2, 201 — 209

To link to this Article: DOI: 10.1080/10826070802602973

URL: http://dx.doi.org/10.1080/10826070802602973

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Journal of Liquid Chromatography & Related Technologies®, 32: 201–209, 2009
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ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070802602973

Application of Denaturing HPLC for Detection of G/C Polymorphism in the Cyclo-Oxygenase-2 Gene

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Abstract: Denaturing high performance liquid chromatography (DHPLC) is a fast and sensitive method to detect gene mutations. The aim of this study was to examine whether DHPLC is an appropriate method to detect a G/C transversion in the promoter region of human cyclo-oxygenase-2 gene. A fragment of 228 bp was amplified from 336 subjects for DHPLC analysis. Results were confirmed by restriction endonuclease analysis. Considering that detection of G/C transversion is quite challenging by DHPLC, the specificity was high (94,3%). Nonetheless, resolution significantly decreased after 400 samples, while it should be possible to analyze 1000 samples per column.

According to our findings, DHPLC is not the most adequate method to detect G/C transversion in the gene because of the relatively rapid loss of column resolution. This should be kept in mind when genomic DNA is screened for unknown mutations.

Keywords: Cyclo-oxygenase-2 gene, Denaturing HPLC, DHPLC, G/C Polymorphism

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INTRODUCTION

DHPLC (denaturing high performance liquid chromatography) was developed in 1995.[1,2] The method enables the rapid analysis of PCR amplified genomic DNA and it is suitable for detection of point mutations, small insertions, and deletions independent of their location in the DNA under analysis. DHPLC requires neither specific primers, reagents, or enzymes, nor has the PCR product to be purified before analysis.[3] The specificity and sensitivity of the method is high: mutations can be detected at the accuracy of 92%,[4] or even with 100% accuracy.[5] Thus, it can be considered as an alternative to more traditional methods, such as restriction endonuclease analysis and sequencing.[3]

Cyclo-oxygenase (COX) catalyzes the first steps of prostanoid production from arachidonic acid to many prostaglandins such as prostacyclin and thromboxane. The enzyme has three known isoforms of which COX-2 expression is primarily induced in response to inflammatory stimuli by growth factors, mitogens, and cytokines.[6] A functional G/C polymorphism located 765 base pairs upstream from the transcription start site (– 765G/C) has been identified in the promoter region of the human COX-2 gene, C allele leading to decreased promoter activity in vitro.[7] Recently, there have been several studies considering C allele and its role, for instance, in the incidence of myocardial infarction and stroke,[8] cancer,[9,10] bronchial asthma,[11] and sarcoidosis.[12]

The aim of the study was to evaluate the suitability of DHPLC in detection of G/C transversion in the COX-2 promoter and determine the optimum parameters for analysis.

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EXPERIMENTAL

Subjects

DNAs collected from the Medical School students of Tampere University during 2003 (n = 105) and 2004 (n = 105) were used in the study. DNAs were extracted from buccal swab samples by a QIAamp DNA Mini Kit (Qiagen). The second material consisted of a series of autopsies performed at the Department of Forensic Medicine, University of Oulu in 1995–2004 (n = 126). Cases were women under fifty years old. DNAs were extracted from coronary arteries by the QIAamp DNA Mini Kit.

COX-2 Amplification

Primers were designed based on the sequence of the human COX-2 gene (NCBI/U04636, gi: 496975) using the Primer3 software (http://frodo.

wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The 25 μL reaction, which was pipetted directly to a 96 well plate, was composed of 50 pmol of each primer (forward 5′-CATTAACTATTTACAGGGTAACTG CTT-3′; reverse 5′-TGCAGCACATACA TACATAGCTTTT-3′), 200 μM of each dNTP, and 2,5 U HotStarTaq DNA Polymerase in 1 × PCR buffer (Qiagen). PCR conditions consisted of 15 min of an initial polymerase activation step at 94°C, followed by 35 cycles of 94°C 30 s, 56°C 30 s, 72°C 30 s, and a final extension at 72°C for 5 min. The primers produced fragments of 228 bp.

DHPLC

The differentiation of various genotype forms is based on their distinct retention in the column when temperature is near the melting temperature of the PCR product. Heteroduplexes are less attached to the column and are, therefore, eluted earlier.[3] The analysis is performed in two phases. First, heterozygotes are screened out of homozygotes. Then, a known amplified homozygote product is added to homozygote samples, which are denatured and re-annealed. Samples are interpreted as mutant or wild type homozygotes, based on the signal they give in this second run.[13] If the sample and known homozygote represent the same form, the sample gives a homozygote signal (one peak). If the sample is different from the homozygote added to the sample, the signal appears like a heterozygote signal (at minimum two peaks). The signal can be interpreted by comparing it to known controls.[14]

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DHPLC Equipment and Reagents

DHPLC analyses were directly performed from 96 well plates using Agilent 1100 HPLC-machinery (Agilent Technologies, Palo Alto, CA, USA) with a monolithic Helix DVB column for double strand DNA (CP28355, 3 × 50 mm, Varian Analytical Instruments, Walnut Creek, CA, USA). Machinery was controlled with ChemStation software. Helix BufferPak A and B buffers were used as reagents. Buffer A consisted of 100 mM triethylammoniumacetate (TEAA) and 0.1 mM EDTA (pH 7.0). Buffer B contained 100 mM TEAA, 0.1 mM EDTA and 25% v/v acetonitrile (pH 7.0). Eluted samples were detected with UV at 260 nm.

Standards

A pUC18 HaeIII plasmid digest (Sigma, St. Louis, MO, USA) was used to evaluate the column resolution. DYS271, a SNP (single nucleotide

Table 1. Sample protocol

Time (min)	A (%)	B (%)	Flow (mL/min)	Pressure (max) (bar)
0,5	47	53	0,400	350
6,0	35	65	0,400	350
6,5	35	65	0,600	350
7,0	0	100	0,600	350
8,0	0	100	0,400	350
8,5	50	50	0,400	350

polymorphism) standard was injected into the column to test its ability to detect mutations. A signal of four peaks indicated that the column was still viable. After these commercial standards, control samples (GG homozygote and heterozygote) were analyzed before and after the sample series. All plates included a null control.

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Samples

Prior to analysis, samples were denaturated at 95°C for 3 min after which the plate was left at room temperature for at least half an hour to enable strand re-annealing. Aliquots of 9 μL of PCR product were injected into the column. Before the second run, 9 μL of amplified GG product was added to the sample, which was subsequently denatured and re-annealed. Samples were run according to protocol presented in Table 1, with increasing linear acetonitrile gradient (2%/min) and temperature of 57–58°C, when the heterozygote differentiation was optimal. DHPLC Melt programme (http://insertion.stanford.edu/melt.html) was used to evaluate the optimal temperature.

Signal Confirmation

All heterozygote and CC homozygote signals in addition to 53 random GG homozygote signals were confirmed by digesting the fragments with SsiI (Fermentas, Vilnius, Lithuania) at 37°C overnight. The PCR product was cleaved into fragments of 168 bp and 60 bp if the G allele was present. Digested products were resolved with 2% MetaPhor (Cambrex, East Rutherford, NJ, USA) agarose gel electrophoresis and visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Signals

Homozygote samples mostly gave a signal of one sharp peak (Figure 1), but in some cases an additional flat peak was observed before the primary signal. Instead, heterozygotes gave various signals. At optimum resolution, three peaks could be detected (Figure 2a). However, the most common signal contained two peaks (Figure 2b). The heterozygote signal was more difficult to interpret as the resolution decreased (Figure 2). In fact, the column resolution was not sufficient to detect G/C transversion after 400 samples. Weakened resolution was evident as shown by flattened and widened homo- and heterozygote signals resembling each other.

During the second round, only one peak was observed if the unknown sample represented the GG genotype (homozygote signal) (Figure 1). When the sample was of CC genotype, two peaks were seen (heterozygote signal) (Figure 2b).

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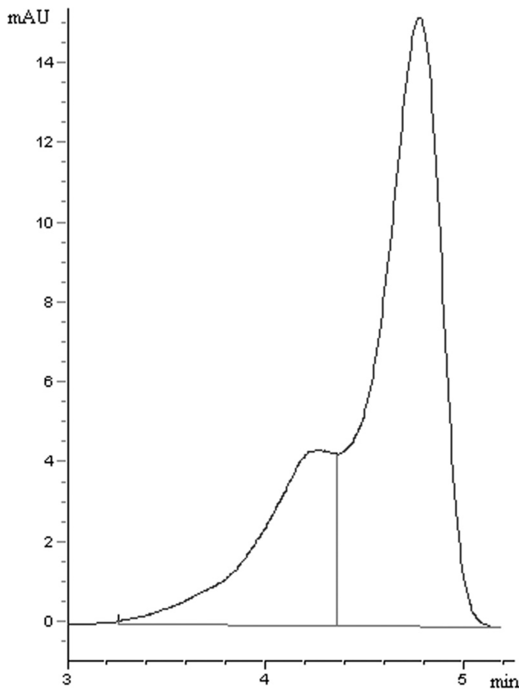

Figure 1. Homozygote signal.

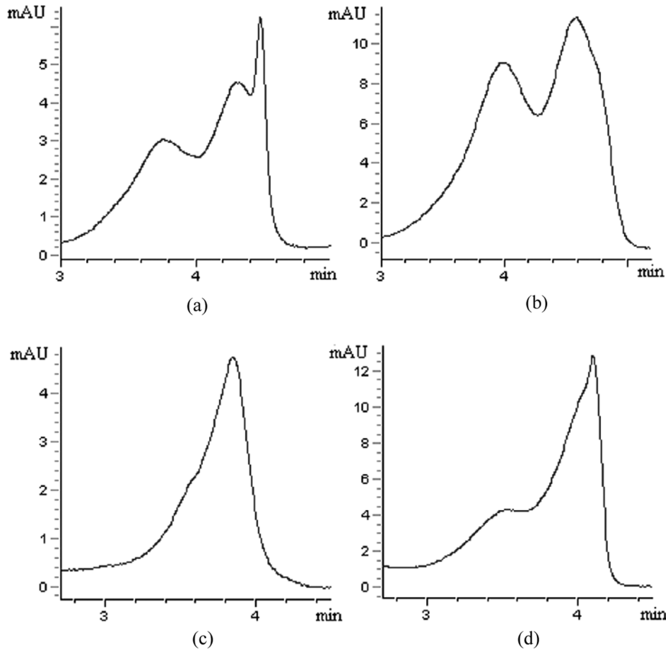

Figure 2. Heterozygote signals. As resolution is decreased, less distinct peaks are observed (a–d) and the signal may start to resemble the homozygote signal (cf. Figure 2d and Figure 1).

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DHPLC Specificity

DHPLC could detect sample genotypes correctly in 94.3% of cases. The sample was regarded unidentified if the signal was weak (under 1 mAU) or the genotype could not be interpreted based on controls.

Resolution

G/C transversion is the most challenging mutation to be detected with DHPLC because the melting temperatures of the different forms are very close to each other. Of every sample, some DNA is left in the column despite the washing procedures. Eventually, pores in the column are blocked up, its ability to bind DNA is decreased, and samples start to elute from the column with lower acetonitrile concentrations. This can be verified from peak widening, which results from the loss of specificity in elution conditions. Before noticing that resolution after 400 samples was not sufficient, we tried to utilize the whole 1000 sample capacity of

the column. In the beginning of the plate run, controls gave proper signals but soon resolution drastically decreased. We tried to sustain the level of differentiation by washing the column in the middle of the run but this did not improve the final result. Even if this would have restored the resolution, the major advantage of the method, automation, would have been lost: conditions would have to be optimized again after the heavy washing procedure, which slows down the analysis.

The key to a successful analysis is the selection of a suitable temperature based on controls. When temperature is too high, peaks from heterozygote control fuse together and DNA elutes prematurely as a consequence of extensive denaturation. When temperature is too low, peaks fuse again but elution time increases since no denaturation occurs. Despite the benefits the Melt programme can offer, temperature prediction is challenging. To achieve the best resolution of over 96%, controls must be tested with 1–2°C higher temperatures than Melt predicts.[15] Temperature suggested by Melt was 56°C. Samples were run with temperature of 57–58°C when the heterozygote differentiation was optimal. However, in our case specificity still remained under 96%. This can be partly due to the fact that temperatures calculated by Melt are column temperatures, not oven temperatures. Thus, the temperature in the oven must be adjusted to a higher value in order to obtain the desired temperature in the column. The temperature prevailing in the column could possibly be monitored by a surface thermometer. The weakness of this method is the condition instability. The experiment may not be repeated with exactly the same parameters since the conditions must be optimized each time.

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False positive signals may occur if the fragment contains some other polymorphic site in addition to the site of interest. In our study, this is highly unlikely but, in theory, possible. In some cases, a flat extra peak was observed before the actual homozygote signal. The formation of this additional peak depends on the DNA under analysis. If the sequence contains palindromic areas, these may form hairpin structures during reannealing. Since these structures are unstable, strands containing them elute earlier from the column than strands that are properly paired. As a consequence, the homozygote signal may start to resemble the heterozygote signal as the first peak grows. Therefore, it is essential that in the homozygote signal the second peak is substantially higher and sharper than the first one (cf. Figure 1).

An alteration in the DHPLC signal indicates that there is a change in sample DNA. It offers no information about the nature or location of the mutation.[3] Therefore, an altered signal must be confirmed by sequencing or restriction endonuclease analysis. Since determining the whole fragment sequence was not necessary, we performed confirmation with restriction endonuclease analysis using an enzyme, which cleaves exactly

the polymorphic site. However, missing a positive signal is more probable than detecting false positive signals if conditions during the analysis are not carefully monitored.

CONCLUSIONS

DHPLC can be considered as an alternative to more traditional methods of genomic DNA analysis, such as restriction endonuclease analysis and sequencing. However, it is not the most adequate method to detect G/C transversions because under half of the column sample capacity can be utilized. After 400 samples, it is still possible to analyze some other, easier mutations. However, when DHPLC is used in genomic screening of unknown DNA it is possible that G/C transversions remain unobserved.

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ACKNOWLEDGMENT

This study has been supported by funds from Medical Research Fund of Tampere University Hospital.

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Received August 16, 2008
Accepted September 17, 2008
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