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Allelic discrimination by denaturing high-performance liquid chromatography

Peter J. Oefner*

DNA Sequencing and Technology Center, Stanford University, 855 California Avenue, Palo Alto, CA 94304, USA

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Abstract

Ion-pair reversed-phase high-performance liquid chromatography on alkylated non-porous poly(styrene–divinylbenzene) particles allows the resolution of single-stranded DNA molecules of identical size (<100 nucleotides) that differ in a single base. Allelic discrimination is obtained by injecting short DNA amplicons containing the genetic variants of interest into an adequately preheated mobile phase that results in the instantaneous complete denaturation of the PCR products. All possible transitions and transversions other than C→G can be typed accurately. The method complements the discovery of single-nucleotide polymorphisms by means of HPLC based heteroduplex detection under partially denaturing conditions and allows their rapid genotyping without the need of adding a reference chromosome.

Keywords: Allelic discrimination; Denaturing high-performance liquid chromatography; Polymerase chain reaction; Oligonucleotides

1. Introduction

The large-scale identification of single-nucleotide polymorphisms (SNPs) in the human as well as other model genomes such as yeast and *Arabidopsis thaliana* by fluorescence-based sequencing [1], hybridization high-density variation-detection DNA chips [2,3], and denaturing high-performance liquid chromatography [4–7] is well on its way and has already resulted in the identification of thousands of SNPs. For this reason, the development of simple and inexpensive technology for the genotyping of SNPs has become of great interest as the ability to discriminate between allelic forms of SNPs is increasingly seen as fundamental to future molecular genetic analysis of disease [8–10].

There are a number of available methods for SNP genotyping such as restriction endonuclease digestion [11], allele-specific hybridization [12,13], nick translation PCR [14,15], ligase chain reaction [16,17], allele-specific polymerase chain reaction [18,19], T_m-shift genotyping [20], and minisequencing [21–29]. The latter method, which is based on the annealing of a primer immediately upstream or downstream from the polymorphic site and its extension by one or more bases in the presence of the appropriate dNTPs and ddNTPs, has become very popular. It has been combined with a variety of techniques for detecting the extension products, including radiolabeling [21], luminous detection [22], colorimetric ELISA [23], gel-based fluorescent

*Tel.: +1-650-812-1926; fax: +1-650-812-1975.
E-mail address: oefner@genome.stanford.edu (P.J. Oefner)

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detection [24], mass spectrometry [25,26], capillary electrophoresis [27], fluorescence polarization [28], and most recently high-performance liquid chromatography [29]. All of the aforementioned genotyping techniques share the polymerase chain reaction as the initial sample pretreatment step. Some of the methods can be done in a single step in a single tube, partly requiring expensive fluorescent dye-labeled oligonucleotide probes [14,15]. Others require additional steps such as hybridization or primer extension. The latter technique also requires prior purification of the PCR product from unincorporated dNTPs and oligonucleotides by either solid-phase extraction or enzymatic treatment with Shrimp Alkaline Phosphatase and Exonuclease I. For these reasons, genotyping is still a far more costly undertaking than the initial SNP discovery. This constitutes a severe limitation in the application of SNPs to genetic studies.

The present study explores the ability of high-performance liquid chromatography to resolve the single-stranded components of short PCR products (<100 bp) differing in a single base on alkylated non-porous poly(styrene–divinylbenzene) particles upon proper modification of the chromatograph. It complements the discovery of SNPs by DHPLC and by making use of the same instrument it provides low cost analysis of biallelic markers in hundreds of samples.

2. Experimental

2.1. Sequences and reagents

The sequences investigated are listed in Table 1. The position and chemical nature of the SNP are given in brackets and the priming sites are written in lower case. All oligonucleotide primers were obtained from Life Technologies (Rockville, MD, USA). Phosphorylated and dephosphorylated oligodeoxyadenylic acids, oligodeoxycytidylic acids, oligodeoxyguanylic acids and oligodeoxythymidylic acids, 12–18 bases in length, were purchased from Amersham Pharmacia Biotech, Inc., (Piscataway, NJ, USA). All PCR reagents and 2 *M* triethylamine acetate buffer were obtained from PE Biosystems, Foster City, CA, USA. HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA), tetrasodium ethylenediamine–tetraacetic acid from Sigma (St. Louis, MO, USA).

2.2. Polymerase chain reaction

Polymerase chain reactions were performed in a 50 μl volume containing 10 m*M* Tris–HCl, pH 8.3, 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 50 μ*M* dNTPs, 0.2 μ*M* of each primer, 50 ng of genomic DNA, and 1 unit of AmpliTaq Gold. The PCR cycling regime carried out in a Perkin-Elmer 9600 thermal cycler comprised

Table 1
List of sequences amplified to genotype the single-nucleotide polymorphisms given in brackets[a]

SNP No.	5′–3′ Sequence	Amplicon size
SNP1	aaaccacattctgagcatacccCC[C/A]AAAAATTtcatgccgaagctgtggtc	51bp
SNP2	caacttaatcagatttaggacacaaaagc[A/T]actacataatgaaaaagagagctggtga	58bp
SNP3	gaaacggcctaagatggttgaaT[G/C]ctctttatttttctttaatttagacatgttcaaa	58bp
SNP4	gacttttttgtacccaccatttgtGGAACTAAATT[A/G]Tatcagtacaaaaagggctacattc	60bp
SNP5	agacagttcttcaggaaaacaccT[C/T]CTTTGGACTCACAccatgtgttttccattcaaatta	61bp
SNP6	cccaaacccattttgatgctT[G/T]ACTTAAaaggtcttcaattattattttcttaaatattttg	62bp
SNP7	ccattgaggaacaacatacagcTTCTGTTCG[G/A]cctcggctgtgggctc	48bp
SNP8	aataaacctttacggggctaagcCT[C/T]agacctgcaagctgcttgttatg	50bp
SNP9	agacatctgactcccagcatgaa[C/T]GGTCccaactcctctctaacaaaaggtaa	53bp
SNP10	tttgttcatacggtcaatattcgat[A/T]CTCTCAGtcctcactgctggtccttacg	54bp
SNP11	cgaaaaagaagatggtgagttcacTTTT[T/C]acctcaataaaaccctttacataaa	54bp
SNP12	gctccatttgaaggttctataactgAAACTAGAATAC[C/A]TAAgctatggggaactaaactctgaat	65bp
SNP13	gataagccatatgatccagcaggATTAATTCCTTTTAC[C/T]GTTTAATTAgtcgtagatactcaagacagaccgt	73bp
SNP14	tgtcctttagtttctatttggttttATATATTATCATATGAACTATAAAGAAG[G/A]Ttgaagcaaagaacagccaaataat	79bp
SNP15	tggagtatttctctagcttgctgAAATAATG[C/G]CAAATTTTATAATATGATACTAGCAACACAAATATTTAgctaaaattacgttgcattaaaaa	94bp

[a] Priming sites are written in lower case.

an initial denaturation step at 95°C for 10 min to activate AmpliTaq Gold. Subsequent denaturing steps were 94°C for 20 s and extension steps of 72°C for 60 s; annealing temperatures were lowered over the first 14 cycles in 0.5°C decrements from 63 to 56°C, followed by 20 cycles at 56°C for 45 s each. Following a final extension step at 72°C for 10 min, samples were chilled to 6°C and stored in a refrigerator until DHPLC analysis.

2.3. Denaturing high-performance liquid chromatography (DHPLC)

Initial experiments to evaluate the effect of temperature on the separation of oligonucleotides were performed on a chromatograph consisting of an on-line vacuum degassing system (Alltech, Deerfield, IL), an automatic sampling system equipped with a biocompatible injection valve and a 20-μl titanium sample loop (Model AS-100 T HRLC®, Bio-Rad, Hercules, CA), a high precision low pressure gradient pump (Model 480, Gynkotek, Germering, Germany), a column oven (Model CH-150, Eldex), a UV–Vis detector (Model Spectra 100, Thermo Separation Products, Riviera Beach, FL), a multichannel interface (Dual Channel Interface HP 35900E, Hewlett-Packard, Mountain View, CA), and a PC-based data system (G1304A, Version A.02.05, Hewlett-Packard). For the preheating of the mobile phase an 80-cm PEEK tubing, 0.01-inch I.D., which had been encased in a tin-alloy block (Part No. 330-HX, Timberline, Inc., Boulder, CO), was used.

Allelic discrimination studies, on the other hand, were carried out on automated HPLC instrumentation from Varian (Walnut Creek, CA, USA). All liquid contact parts were constructed of titanium, fluorocarbon, sapphire, and polyetheretherketone (PEEK), to avoid contamination of the stationary phase with metal cations that cause both a loss of recovery as well as decreased separation efficiency. In detail, the instrumentation consisted of an on-line degasser, two SD-200 high-pressure pumps, an electronic pressure module, a 600-μl dynamic mixer, a 6-port injection valve mounted into a MISTRAL column oven, an automated sample injector (AI-1A), a DYNAMAX UV-absorbance detector (254 nm), and a PC-based system controller and data analysis package. The aforementioned 80-cm PEEK tubing of 0.01-inch I.D. was placed immediately before the sample loop in the column oven to thermally precondition the mobile phase.

The stationary phase consisted of 2-μm non-porous alkylated poly(styrene–divinylbenzene) particles [30] packed into 50×4.6-mm ID columns, which are commercially available (DNASep™, Transgenomic, San Jose, CA, USA). The mobile phase was 0.1 *M* triethylammonium acetate buffer at pH 7.0, containing in addition 0.1 m*M* Na_4EDTA. Crude PCR products were eluted with a linear acetonitrile gradient at a flow-rate of 0.8–1.0 ml/min. The start- and end-points of the gradient were adjusted according to the size of the single-stranded DNA sequences.

3. Results and discussion

3.1. Impact of instrument configuration on resolution of oligonucleotides

Previously, it has been described that optimum resolution of oligonucleotides is obtained at a column oven temperature of 50°C [30]. However, as can be seen from the data presented in Fig. 1, resolution of oligodeoxythymidylic acids can be improved significantly at temperatures higher than 50°C provided that both the sample loop and an 80-cm coil of PEEK tubing are placed in the column oven. Positioning only the coil in the oven, but not the injection loop, resulted in neither an improvement nor a decrease in resolution of the oligonucleotides. The importance of preconditioning DNA prior to its contact with the column matrix had been already recognized for the successful resolution of homo- and heteroduplex species under partially denaturing conditions [31,32]. It proved impossible to detect heteroduplices when the DNA sample was injected directly into the column. However, resolution of hetero- from homoduplices improved increasingly with increasing length of the tubing placed between the injector and the column. The same effect can be obtained by placing the coil in front of the injector with the sample loop being mounted inside the oven. The observed improvement in the resolution of oligonucleotides is also reflected in a significant increase in the number of theoretical plates. The

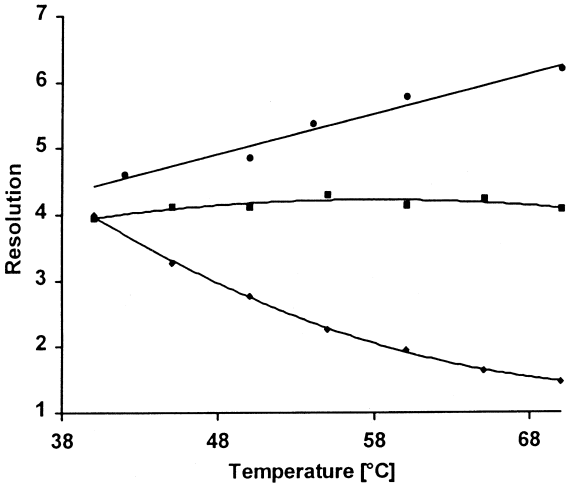


Fig. 1. Impact of instrument configuration on the resolution of oligodeoxythymidylic acids: (♦) sample loop and 80-cm preconditioning coil outside the oven, (■) only 80-cm preconditioning coil in the oven, and (●) both sample loop and 80-cm preconditioning loop placed inside the oven. Column: DNASep™, 50×4.6 mm I.D.; mobile phase: 0.1 *M* TEAA, pH 7.0; linear gradient: 5–10% acetonitrile in 10 min; flow-rate: 1 ml/min; temperature: 42 to 72°C; detection: UV, 254 nm; sample: $pd(T)_{14/15}$, 0.2 μg each.

number of theoretical plates increased from 4.58×10^5 to 8.50×10^5 and 3.75×10^5 to 6.33×10^5 plates/m, respectively, for a phosphorylated and dephosphorylated hexadecamer of oligodeoxythymidylic acid upon increase of the column oven temperature from 40 to 80°C.

Using the improved instrument configuration, it became possible to resolve oligodeoxyguanylic acids that had not been separated previously by means of ion-pair reversed-phase HPLC. This is due to the ability of guanine-rich single-stranded DNA sequences to form strong intra- and intermolecular interactions [33,34]. Therefore, thermal denaturation obviates the need of adding formamide to the sample [35] or working under highly alkaline pH conditions as shown for the separation of oligonucleotides on a strong anion-exchanger [36]. Fig. 2 shows the simultaneous separation of homooligonucleotides in the size range of 12–18 bases. In agreement with a previous study of homotetramers [37], the homooligomers eluted in the order G<C<A<T.

Intra- and intermolecular interactions are also observed in the case of oligodeoxyadenylic acids, although they have been reported to be significantly less than those of guanine-rich sequences [34]. This explains why in contrast to oligodeoxythymidylic acids, which have not been observed to interact with each other, no linear increase in resolution is observed with increasing temperature for oligodeoxyadenylic acids (Fig. 3). Only at temperatures above 70°C a resolution similar to that of oligodeoxythymidylic is obtained. This applies to both phosphorylated and dephosphorylated oligonucleotides.

Intra- and intermolecular interactions also explain the non-linear reaction isochores observed in Van't Hoff plots for oligodeoxyadenylic acids (Fig. 4). In

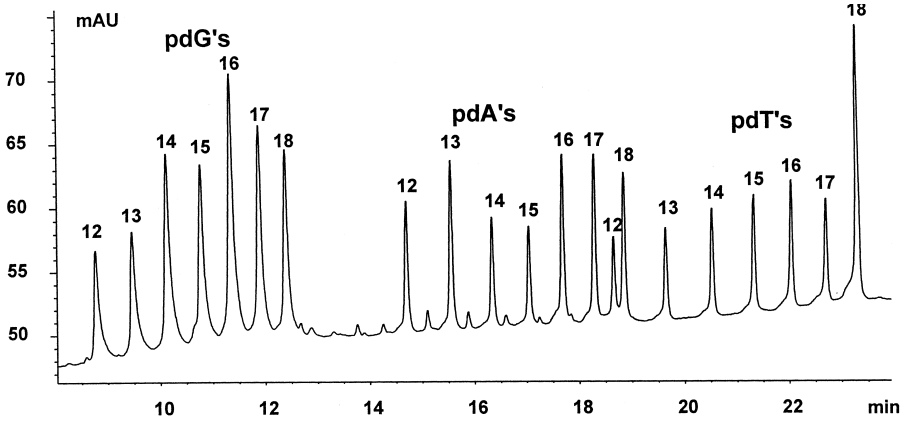


Fig. 2. Separation of phosphorylated homooligonucleotides under thermally denaturing conditions. Column: DNASep™, 50×4.6 mm I.D.; mobile phase: 0.1 *M* TEAA, pH 7.0; linear gradient: 0–10% acetonitrile in 30 min; flow-rate: 0.8 ml/min; temperature: 80°C; detection: UV, 254 nm; sample: $pd(G)_{12-18}$, $pd(C)_{12-18}$, $pd(A)_{12-18}$ and $pd(T)_{12-18}$, 0.2 μg each.

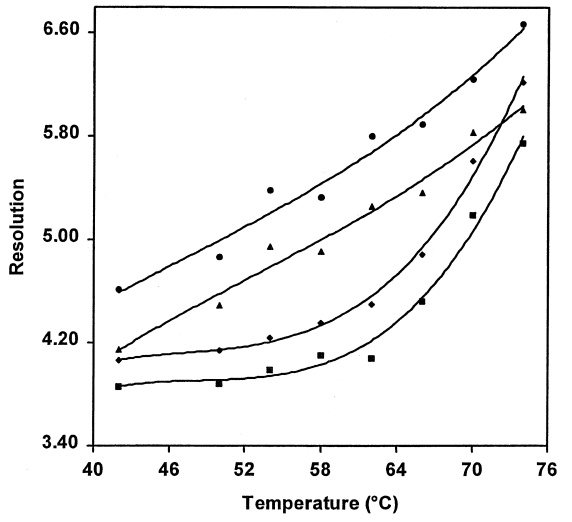


Fig. 3. Effect of temperature on the resolution of dephosphorylated oligodeoxyadenylic acids and phosphorylated oligodeoxythymidylic acids. Column: DNASep™, 50×4.6 mm I.D.; mobile phase: 0.1 *M* TEAA, pH 7.0; linear gradient: 3.75–8.75% acetonitrile in 10 min for $d(A)_{14/15}$ and $d(A)_{15/16}$, 5–10% acetonitrile in 10 min for $pd(T)_{14/15}$ and $pd(T)_{15/16}$; flow-rate: 1 ml/min; temperature: 42–74°C; detection: UV, 254 nm; sample: (■) $d(A)_{15/16}$, (♦) $d(A)_{14/15}$, (▲) $pd(T)_{15/16}$, and (●) $pd(T)_{14/15}$, 0.2 μg each.

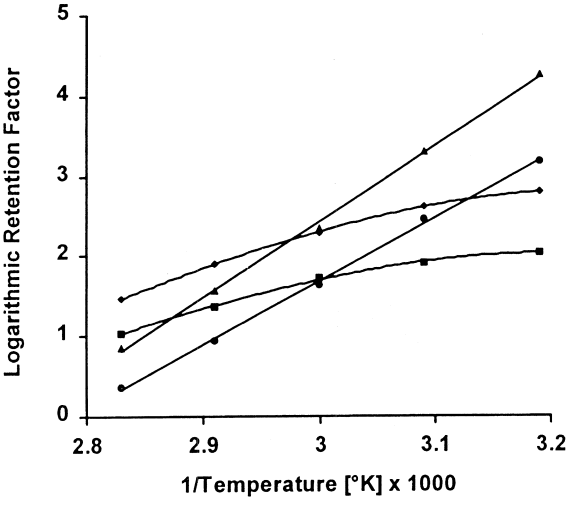


Fig. 4. Van't Hoff plots illustrating the dependence of the logarithmic retention factors of homooligonucleotides (●, $pd(T)_{16}$; ▲, $d(T)_{16}$; ■, $pd(A)_{16}$; ♦, $d(A)_{16}$) on the reciprocal absolute temperature. Due to intra- and intermolecular interactions oligodeoxyadenylic acids do not yield linear reaction isochores. The respective adsorption enthalpies for $d(T)_{16}$ and $pd(T)_{16}$ in 7.25% acetonitrile were −81.44 and −59.36 kJ mol^{-1}.

contrast, linear isochores were obtained for phosphorylated and dephosphorylated oligodeoxythymidylic acids the geometry of which does not allow the formation of atypical Watson–Crick base pairs [34]. The plots clearly demonstrate the dependence of the logarithmic retention factors of $d(T)_{16}$ and $pd(T)_{16}$ on the reciprocal absolute temperature. In a mobile phase of 100 m*M* TEAA, pH 7, and 7.25% acetonitrile the adsorption enthalpies of the two oligodeoxythymidylic acids were determined to be −81.44 and −59.36 kJ mol^{-1}, respectively. Similar adsorption enthalpies were determined for the two phosphorylated 18-mer heterooligonucleotides 5′TGTAAAACGACGGCCAGT and 5′CAGGAAACAGCTATGACC, that were also found to yield linear reaction isochores. The respective values in 100 m*M* TEAA and 5.25% acetonitrile were −65.23 and −63.80 kJ mol^{-1}. This indicates that phosphorylation at the 5′-base has the greatest effect on the enthalpies due to the hindered hydrophobic interaction between the former and the column matrix. The difference in adsorption enthalpies between $d(T)_{16}$ and $pd(T)_{16}$ amounts to >25%.

3.2. Allelic discrimination by denaturing HPLC

It has been shown that 22-mer oligonucleotides differing in a single base at the 3′-end elute in the order of C<G<A=T [30]. The elution order was attributed to the fact that cytosine is the least hydrophobic base, followed by guanine which has more polar groups (carbonyl and amino) than adenine (only amino). Hence, it can be speculated that retention of isomeric oligonucleotides that exhibit roughly the same degree of electrostatic interaction with the ion-pairing reagent is controlled by differences in the hydrophobicity of the bases located at the 3′-end and by their hydrophobic interaction with the stationary phase. The originally observed elution order is corroborated by the present study for a set of four 16-mer heterooligonucleotides differing again in a single base at the 3′-end, with the elution order being C<G<A<T (Fig. 5). Interestingly, resolution of the latter two oligonucleotides improves significantly with an increase in column temperature from 50 to 80°C, while that of the former two decreases slightly. More importantly, an increase in column temperature also allowed the almost com-

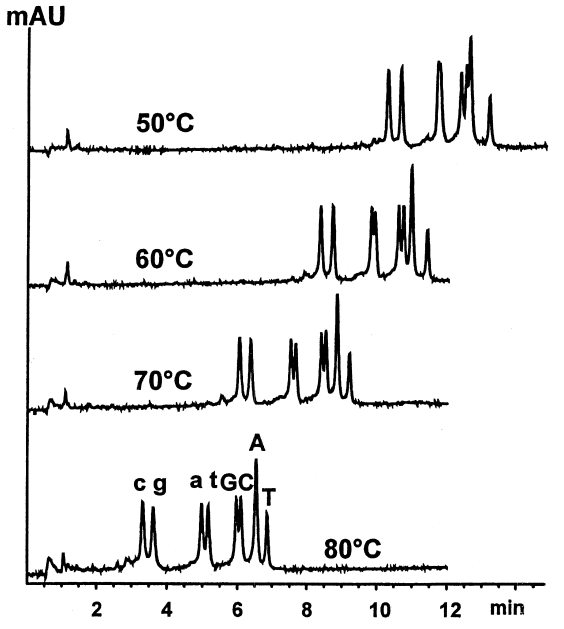


Fig. 5. Impact of temperature on the separation efficiency of 16- and 22-mer heterooligonucleotides that differ in a single base at either the 3′-end or in the center of the molecule. Column: DNASep™, 50×4.6 mm I.D.; mobile phase: 0.1 *M* TEAA, pH 7.0; linear gradient: 3.75–6.25% acetonitrile in 15 min; flow-rate: 1 ml/min; temperature: 50–80°C; detection: UV, 254 nm; sample: 16- and 22-mer oligonucleotides, 0.15 μg each. Peak identification: c, TCCATGAATCACTCC**C**; g, TCCATGAATCACTCC**G**; a, TCCATGAATCACTCC**A**; t, TCCATGAATCACTCC**T**; G, GTGCTCAGTGT**G**GCCCAGGATC; C, GTGCTCAGTGT**C**GCCCAGGATC; A, GTGCTCAGTGT**A**GCCCAGGATC; and T, GTGCTCAGTGT**T**GCCCAGGATC.

plete baseline resolution of four isomeric heterooligonucleotides identical in sequence except for a single base substitution at the 12th nucleotide from the 5′-end (Fig. 5). At present, it remains unclear why the elution order of the latter set of heterooligonucleotides corresponds to that of homooligonucleotides (see Fig. 2), while substitution of the base at the 3′-end results in a reversal of the elution order of cytosine and guanine.

The successful resolution of heterooligonucleotides differing only in a single base irrespective of the location of the substitution prompted the present investigation whether high-performance liquid chromatography under completely thermally denaturing conditions could be used for direct allelic discrimination without prior addition of a known homozygous reference as required for high-performance liquid chromatography under partially denaturing conditions [4,31]. It had been already established that this concept would not work for amplicons typically used for the discovery of SNPs, the size of which ranges from 200 to 700 base pairs. Short amplicons, however, are commonly used for other genotyping techniques, such as nick translation PCR with fluorogenic probes [15] and allele-specific hybridization on high-density oligonucleotide arrays [6]. In order to evaluate the feasibility of using denaturing HPLC for genotyping short amplicons, the primers of which flank the polymorphic site of interest and the bases in its immediate vicinity, we generated a number of amplicons 51–62 bp in size (SNP1–6). They contained biallelic sites of different chemical nature, specifically the two transitions C→T and A→G, as well as the four transversions C→A, C→G, T→A, and T→G. From Fig. 6, it is apparent that all but the C→G transversion could be discriminated successfully. Particularly striking is the case of the T→A transversion, which cannot be discriminated by assays such as T_m-shift genotyping [19] because the substitution only results in the replacement of a T→A in one chromosome to A→T in the other chromosome. This surprising observation may be attributed to the fact that retention is governed not only by the substituted base but also by the immediate sequence context. However, this was not a unique observation as confirmed subsequently by the successful genotyping of SNP10. Further, as expected, the complementary strands of an amplicon are usually resolved well, resulting in the observation of usually two peaks in case of a homozygous sample, and four peaks in case of a heterozygous sample. As evident from Fig. 5, temperature can be used to optimize resolution. For instance, two isomeric single-stranded DNA molecules that differ in a single adenine or thymine are resolved somewhat better at a lower temperature, e.g. 70°C (SNP2). Other mismatches are discriminated more clearly at 80°C. Generally, even an amplicon very rich in GC base pairs will be denatured completely at 70°C due to the presence of acetonitrile in the mobile phase. Only the C→G transversions investigated, namely SNP3 and SNP15, could not be genotyped successfully, although partial resolution was observed at a

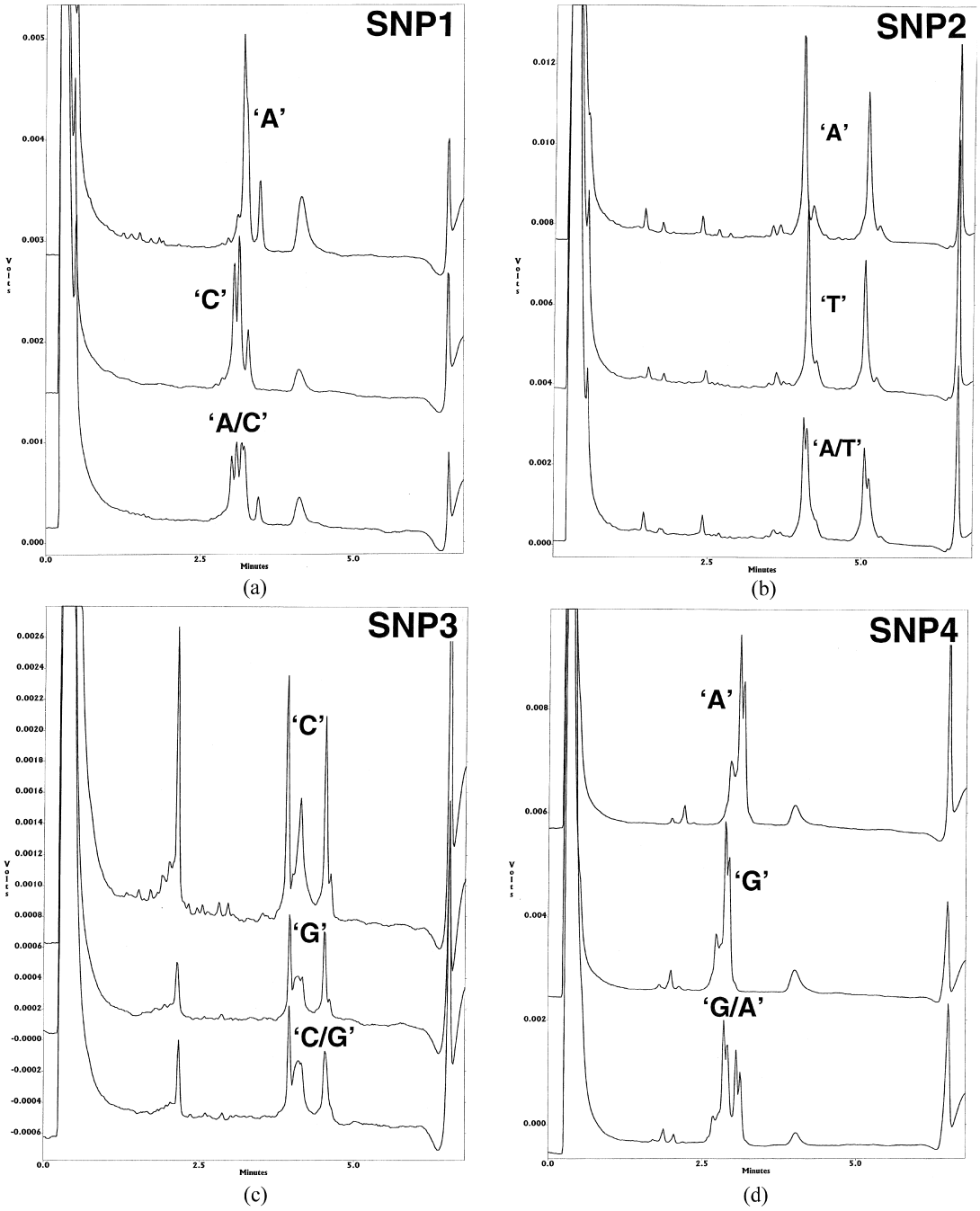
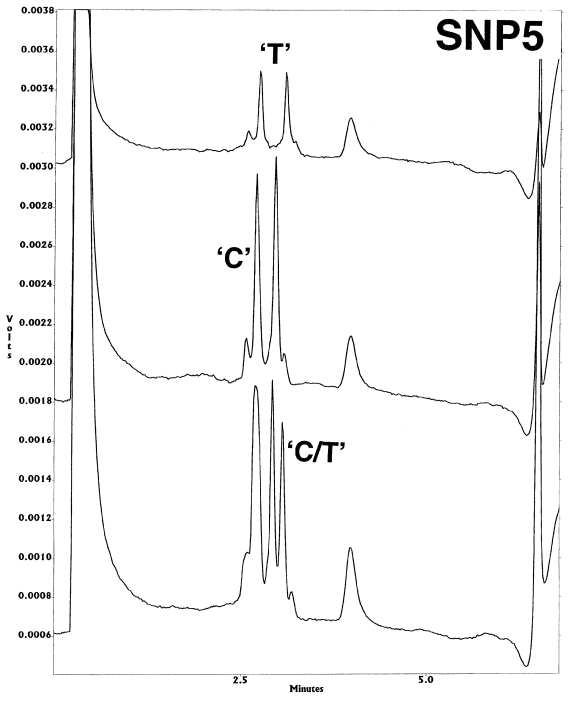
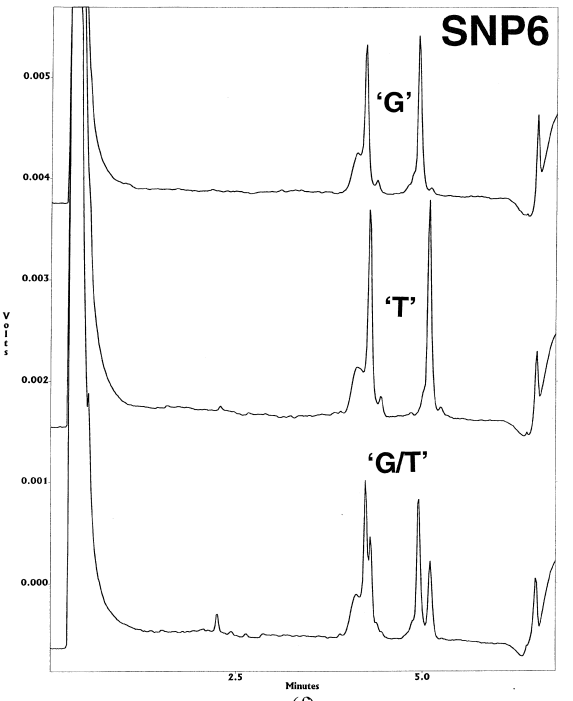


Fig. 6. Allelic discrimination of all possible transitions and transversions except C→G by high-performance liquid chromatography under completely denaturing conditions. Column: DNASep™, 50×4.6 mm I.D.; mobile phase: 0.1 *M* TEAA, pH 7.0; linear gradient: 7–10% acetonitrile in 5.5 min; flow-rate: 0.9 ml/min; temperature: SNP1, 75°C; SNP2, 70°C; SNP3–6 and SNP13, 80°C; detection: UV, 254 nm. The void peak comprises unincorporated nucleotides and excess primers. The peak eluting at approximately 4 min is a system peak. The greater than expected number of peaks for SNP13 indicates amplification of a second locus due to the presence of a close paralog in the genome.



(e)



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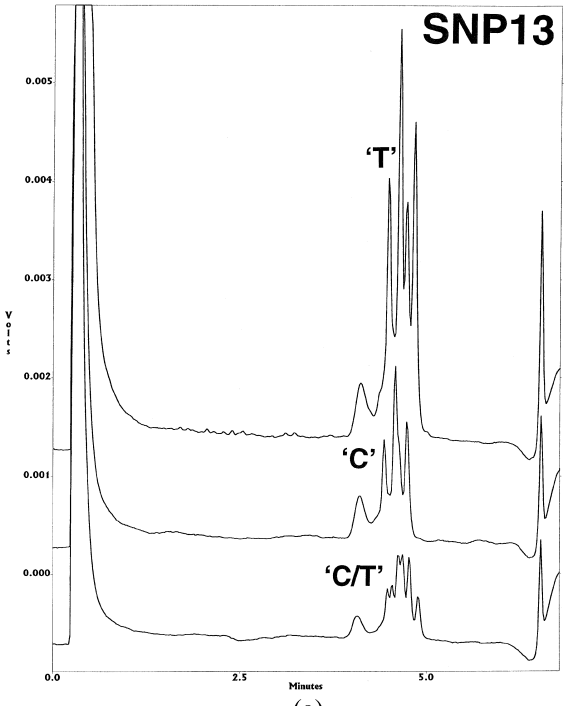

(g)

Fig. 6 (*continued*).

concentration of 50 m*M* triethylammonium acetate in the mobile phase. Hence, it may be worthwhile to investigate whether the use of a different ion-pairing reagent may eventually allow the successful allelic discrimination of C→G transversions by HPLC. In this context, it is important to remember that C→G and G→C transversions comprise only 9.5% [38] of all substitutions observed in mammalian genes due to a significant bias towards C→T (21.0%) and G→A (20.7%) transitions which are readily discriminated by HPLC.

The reliability of the present genotyping approach is dependent to a certain extent on the reproducibility of retention times. In order to assess the reprobucibility of elution profiles, we repeated the genotyping of SNP2 and SNP6 14 and 17 times, respectively. The coefficients of variation for the absolute retention times of the four major product peaks ranged from 0.4 to 0.6% for SNP2 and 0.3–0.4% for SNP6, respectively. An even more reliable measure is the ratio of the retention times of the two complementary strands. In case of SNP2, the ratios for allele A and allele T were 0.794±0.003 (mean±SD, C.V.=0.39%) and 0.814±0.003 (C.V.=0.41%), respectively, with the values ranging from 0.787 to 0.797 and 0.806–8.818, respectively. Comparing the two means of the ratios of retention times using a *t*-test, they were found to differ significantly from each other (t_s=17.638, $t_{0.001\ [26]}$=3.707). The same was true for SNP6: the arithmetic means and standard deviations for the T allele and G allele were 0.845±0.002 (0.842–0.850, C.V.=0.27%) and 0.857±0.002 (0.853–0.860, C.V.=0.22%), respectively. Again, the *t*-test was highly significant (t_s=17.493, $t_{0.001\ [32]}\approx$3.6). The high reproducibility of retention times also corroborates the excellent chemical and physical stability of poly(styrene–divinylbenzene) particles at high temperature with more than 600 analyses having been performed over a period of 10 days without any noticeable deterioration in separation efficiency. Ultimately, it would be advantageous to couple HPLC to mass spectrometry to confirm the identity of the peaks. Past problems with the use of triethylammonium acetate that was found to reduce drastically ion formation during electrospray ionization have been overcome recently by replacing it with triethylammonium bicarbonate without affecting the proven separation efficiency of ion-pair reversed-phase HPLC. Further, in combination with acetonitrile added as a sheath liquid to the column effluent, analyte detectability in the femtomol range has been accomplished for even large oligonucleotides [39].

In addition to the six SNPs depicted in Fig. 6, we tested nine SNPs (SNP7–15) for which genotyping information had been obtained recently by dye terminator sequencing. The HPLC based genotyping results were found to be in complete accordance with those determined by sequencing (except SNP15 that could not be genotyped at all because of the nature of the substitution, see above). In this context, however, it is important to realize that potential genotyping discrepancies may not be the result of technical errors but rather that of the fairly common presence of pseudogenes or close paralogs. Primers used for the discovery of SNPs are typically located in the flanking non-coding regions of exons that show a higher degree of sequence divergence than the coding regions hence rendering it more likely that only the gene copy of interest is being amplified. This specificity may get lost upon redesign of the primers in order to generate a shorter amplicon amenable to direct genotyping. Such an example is SNP13 (Fig. 6). DHPLC and sequence analysis of the original 325-bp PCR product yielded no evidence of duplication. However, re-amplification of the polymorphic site with a new pair of primers resulted in the appearance of more than two major product peaks in the homozygous state due to the co-amplification of another region of the genome similar in sequence.

Although it may appear disappointing at first that we failed to discriminate all alleles, it needs to be emphasized that similar limitations also apply to other genotyping technologies. For instance, in a recent study evaluating the use of high-density oligonucleotide arrays for the purpose of genotyping biallelic markers it was observed that only approximately 60% of a total of 487 biallelic markers proved amenable to allelic discrimination by this approach [6]. Therefore, the demonstrated ability of HPLC to discriminate all substitutions with the exception of C→G and G→C transversions constitutes a significant achievement.

4. Conclusions

The ability of ion-pair reversed-phase HPLC to resolve at elevated column temperatures the single-stranded components of short PCR products even when they differ only in a single base expand the utility of high-performance liquid chromatography in genetic studies. It complements the proven ability of denaturing HPLC to detect single-base mismatches in amplicons as long as 1 kb and constitutes an inexpensive and readily automated approach to the scoring of biallelic markers in disease association studies and gene mapping by means of linkage disequilibrium.

Acknowledgements

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