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Analysis of Polymerase Chain Reaction Products by On-Line Liquid Chromatography−Mass Spectrometry for Genotyping of Polymorphic Short Tandem Repeat Loci

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Capillary ion-pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) was used to separate and purify DNA fragments amplified by the polymerase chain reaction (PCR) prior to their characterization by electrospray ionization mass spectrometry (ESI-MS). The investigation by ESI-MS of single- or double stranded species could be effortlessly selected by chromatography of the nucleic acids under either nondenaturing or denaturing conditions, which were realized by proper adjustment of the column temperature. ESI-MS detection sensitivity was improved by a factor of 10 upon replacement of 25 mM triethylammonium bicarbonate as ion-pair reagent by 25 mM butyldimethylammonium bicarbonate because of the applicability of higher acetonitrile concentrations to elute the DNA from the monolithic, poly(styrene/divinylbenzene)-based capillary columns. For fragments ranging in size from 67 to 84 base pairs, the mass accuracies and mass reproducibilities were typically better than 0.02 and 0.008%, respectively, which enabled the characterization and identification of the PCR products with high confidence. The hyphenated method was applied to the genotyping of polymorphic short tandem repeat (STR) loci from the human tyrosine hydroxylase gene (humTH01). The different alleles both in homo- and heterozygotes were identified on the basis of the masses of the single-stranded amplicons and were in full accordance with the alleles identified by conventional capillary electrophoretic sizing.

Since its invention in the early 1980s, the polymerase chain reaction (PCR) has advanced to be one of the most important tools in practically all areas of research and experimentation involving nucleic acids. Applications of this method include forensic DNA typing, clinical diagnosis, DNA amplification for cloning or sequencing, construction of cDNA libraries, and detection of mutations.[1] The great value of PCR rests within the possibility to rapidly and specifically amplify by a cyclic, enzyme-catalyzed process a target DNA sequence using minute quantities of starting material.[2] The currently applied techniques for detection and identification of PCR products, such as electrophoresis,[3] chromatography, fluorescence spectrometry,[4] or sequence-specific hybridization,[5] enable the generation of analytical data at different information levels. Size-dependent separation by slab-gel electrophoresis offers positive identification of PCR products on the basis of electrophoretic migration.[6] However, the whole process is labor- and time-intensive, and quantitation upon staining of the gels is prone to error. These disadvantages are alleviated by the use of capillary electrophoresis (CE)[7] or high-performance liquid chromatography (HPLC),[8] which are fully automatable and allow reliable quantitation of the separated PCR fragments utilizing on-line UV-[7,9] or fluorescence detection.[10,11] Mass spectrometry (MS) has added another dimension to nucleic acid detection and identification,[12] because in contrast to electrophoretic migration or chromatographic retention, the molecular mass represents an intrinsic property of a molecule that is independent of the physicochemical environment present in the course of its determination.

The applicability of MS to the structural investigation of nucleic acids has been triggered by the introduction of matrix-assisted laser desorption/ionization (MALDI)[13] and electrospray ionization (ESI),[14] which allow the ionization and mass analysis of nucleic acids ranging in molecular mass from a few hundred to several

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million.[15] Nevertheless, mass spectrometric approaches to the analysis of DNA fragments amplified by PCR are faced with several problems, because the target sequences are contaminated with relatively high amounts of mononucleoside triphosphates, oligonucleotide primers, DNA polymerase, detergents, and ionic buffer constituents. Adduction of nucleic acids with cations has been shown to result in poor signal-to-noise ratios and shifts of the mass signals to higher m/z,[16,17] and the presence of salts and an excess of reagents significantly contributes to the chemical background. Additionally, analyte ionization is inhibited because of competition with other ionic species contained in the electrosprayed solution. Hence, purification of PCR products prior to mass spectrometric investigation is indispensable. Multiple ethanol precipitation,[18] solid-phase extraction,[19] microdialysis,[20] affinity purification,[21] and combinations thereof[22−25] are commonly applied to the purification of PCR products but they are time-consuming, require considerably high amounts of sample, and incur significant sample losses.

We recently demonstrated that capillary ion-pair reversed phase HPLC (IP-RP-HPLC) is highly suited for the rapid, on-line desalting and purification of femtomole amounts of single- and double-stranded nucleic acids ranging in size from a few nucleotides to several hundred base pairs prior to ESI-MS.[26,27] We now have selected amplicons related to polymorphic short tandem repeat (STR) loci of forensic relevance as a reference system in order to explore the applicability of the hyphenated system to the analysis of PCR products. STRs are DNA segments typically found in noncoding regions, which are composed of repeating units of di- to pentanucleotide motifs. Because the number of repeat units is polymorphic in human populations, STRs are very useful for identity testing and genetic mapping.[28] Although the detection and identification of the length variation of STR-containing PCR products are predominantly accomplished by capillary gel electrophoresis,[29] both MALDI-MS[30−32] and ESI-MS[24,33] have been successfully applied to characterize STRs by utilizing the sample preparation protocols described above. In an attempt to automate and simplify sample preparation, to reduce the amount of sample required for analysis to a level that is routinely available from PCR, and to increase cost-effectiveness, we investigated the usefulness of on-line sample purification by IP-RP-HPLC in monolithic capillary columns as the separation medium. The performance characteristics of the IP-RP-HPLC−ESI-MS system are critically evaluated and compared to the results obtained by conventional capillary gel electrophoretic analysis.

EXPERIMENTAL SECTION

Chemicals and Materials. Acetonitrile (HPLC gradient-grade) and water (HPLC grade) were obtained from Merck (Darmstadt, Germany). Triethylamine (analytical reagent grade) and butyldimethylamine (analytical reagent grade) were purchased from Fluka (Buchs, Switzerland). A 0.50 M stock solution of triethylammonium bicarbonate (TEAB) or butyldimethylammonium bicarbonate (BDMAB) was prepared by passing carbon dioxide gas (AGA, Vienna, Austria) through a 0.50 M aqueous solution of the amines at 5 °C until pH 8.4−8.9 was reached. The synthetic oligodeoxynucleotide $(dT)_{24}$ (M_r 7 238.71) was ordered from Microsynth (Balgach, Switzerland) and used without further purification. Monolithic capillary columns measuring 60 × 0.20 mm i.d. were prepared according to the published protocol.[27]

Polymerase Chain Reaction. Buccal scrapes were taken from four volunteers, and DNA was extracted using the Chelex method.[34] PCR was performed in 0.5 mL microamp reaction tubes (Perkin-Elmer, Foster City, CA) by adding 2 ng of DNA to a total volume of 50 μL of PCR buffer II (Perkin-Elmer), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μM of each primer ($humTH01_f$ 5′-CCTGTTCCTCCCTTATTTCCC; $humTH01_r$ 5′-GGGAACACA-GACTCCATGGTG; the indexes f and r are used to distinguish between the forward and reverse strands, respectively) and 1.25 U TaqGold DNA polymerase (Perkin-Elmer). Amplification was carried out in a 9600 GeneAmp thermocycler (Perkin-Elmer) comprising 32 cycles of 94 °C denaturation for 30 s, 58 °C annealing for 15 s, and 72 °C extension for 30 s. Prior to amplification, the enzyme was activated by a 95 °C incubation for 11 min. Finally, samples were cooled to room temperature.

High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry. The HPLC system consisted of a low-pressure gradient micropump (model Rheos 2000, Flux Instruments, Basel, Switzerland) controlled by a personal computer, a vacuum degasser (Knauer, Berlin, Germany), a column thermostat made from 3.3-mm-o.d. copper tubing that was heated by means of a circulating water bath (model K 20 KP, Lauda, Lauda-Königshofen, Germany), and a microinjector (model C4-1004, Valco Instruments Co. Inc., Houston, TX) with a 500-nL internal sample loop. ESI-MS was performed on a Finnigan MAT LCQ quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ion source. The capillary column was connected directly to the spray capillary (fused silica,

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90-μm o.d., 20-μm i.d., Polymicro Technologies) by means of a microtight union (Upchurch Scientific, Oak Harbor, WA). A syringe pump equipped with a 250-μL glass syringe (Unimetrics, Shorewood, IL) was used for continuous infusion and for pumping sheath liquid. For analysis with pneumatically assisted ESI, an electrospray voltage of 3.4 kV and a nitrogen sheath gas flow of 40 arbitrary units were employed. The temperature of the heated capillary was set to 200 °C. Total ion chromatograms and mass spectra were recorded on a personal computer using LCQ Navigator software, version 1.2 (Finnigan). Mass calibration and preliminary tuning were performed in the positive ion mode by direct infusion of a solution of caffeine (Sigma, St. Louis, MO), methionylarginylphenylalanylalanine (Finnigan), and Ultramark 1621 (Finnigan). Fine-tuning for ESI-MS of oligodeoxynucleotides in the negative ion mode was performed by infusion of 3.0 μL/min of a 20 pmol/μL solution of $(dT)_{24}$ in 25 mM aqueous TEAB containing 20% acetonitrile (v/v). A sheath flow of 3.0 μL/min of acetonitrile was added through the triaxial electrospray probe. Cations present in $(dT)_{24}$ were removed by on-line cation exchange using a 20 × 0.50 mm i.d. cation-exchange microcolumn packed with 38−75 μm Dowex 50 WX8 particles (Serva, Heidelberg, Germany).[35]

Capillary Gel Electrophoresis. For capillary electrophoretic allele determination, 5′-FAM-labeled forward primers were used in the PCR. Amplification products were combined with the internal size standard Genescan-500 ROX (Perkin-Elmer) in deionized formamide, heat-denatured at 94 °C for 2 min and snap-cooled on ice prior to loading on a CE310 genetic analyzer (Applied Biosystems, Foster City, CA). Electrophoretic determination of size was carried out using POP-4 as polymer under standard conditions according to the manufacturer's recommendations. Analysis of the data was performed using the software packages GeneScan Analysis Version 2.1 and Genotyper Version 2.5 (Applied Biosystems).

RESULTS AND DISCUSSION

Analysis of Double- and Single-Stranded PCR Products. By virtue of its high resolving capability and the applicability of mobile phases comprising only volatile components, IP-RP-HPLC is the chromatographic mode of choice for nucleic acid fractionation[8] in on-line combination with ESI-MS.[26,27,36] In IP-RP-HPLC, retention of nucleic acids is effected upon electrostatic interactions between charged analytes and the ion-pair reagent, which is adsorbed from the mobile phase onto the surface of the nonpolar stationary phase.[37] Additional solvophobic interactions between hydrophobic structure elements of the solutes and the hydrophobic surface of the stationary phase may contribute to retention.[37] Elution of the nucleic acids is accomplished in IP-RP-HPLC by the reduction of the amount of adsorbed ion-pair reagent that is induced by increasing the concentration of an organic modifier, typically acetonitrile.

Figure 1 illustrates a chromatogram of the components of a PCR mixture analyzed in a monolithic, poly(styrene/divinylbenzene)-based 200-μm-i.d. capillary column using mass spectrometric detection. The target PCR product was an 82-bp fragment amplified from the noncoding region of the human tyrosine hydroxylase gene (humTH01, allele 9.3; for a detailed discussion of the different alleles and allele nomenclature see below). Mononucleotides, salts, and low-molecular-mass buffer components passed the column in the void volume and were, therefore, efficiently removed from the DNA oligomers. The two single-stranded oligonucleotide primers and the amplified, double-stranded DNA fragment eluted at retention times of 7.3, 8.1, and 8.4 min, respectively (Figure 1a). The difference in retention times between the two 21-mer primers having the same length was relatively large compared to the rather small difference in retention between the forward primer and the double-stranded product having a size of 82 bp. This observation is consistent with the structural differences between single- and double-stranded nucleic acids. Whereas electrostatic interaction is the predominant retention mechanism with double-stranded DNA fragments having a helical structure, in which the hydrophobic nucleobases are enclosed by the hydrophilic sugar−phosphate backbone, additional solvophobic interactions involving the nucleobases play an important part in the retention of single-stranded nucleic acids.

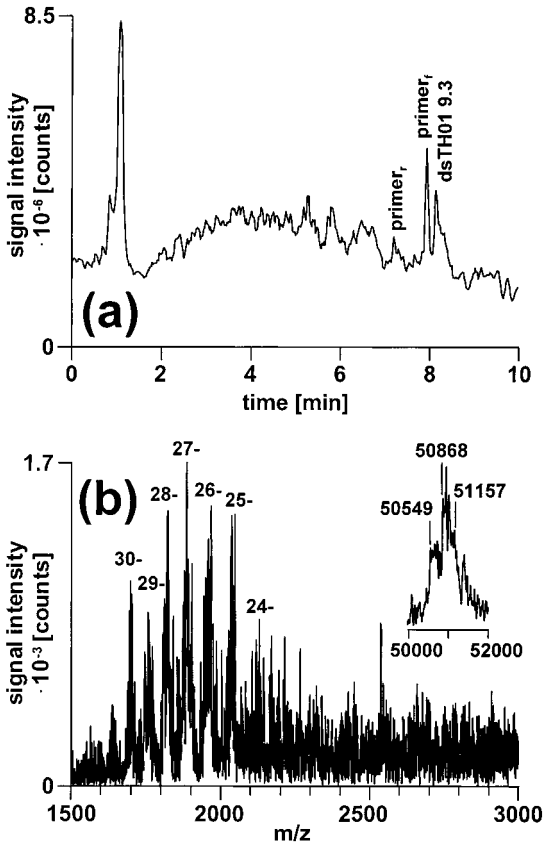

Figure 1. IP-RP-HPLC−ESI-MS analysis of the double-stranded PCR amplicon from the humTH01 9.3 allele under nondenaturing conditions. Column, monolithic PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM TEAB, pH 8.40, (B) 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 10−60% B in 10 min; flow rate, 3.0 μL/min; temperature, 45 °C; scan, 1000−3000 amu; sheath liquid, 3 μL/min acetonitrile; sample, humTH01 allele 9.3, ∼100 fmol.

The reverse- and forward primers were readily identified by their molecular masses of 6480.0 and 6210.3, respectively (spectra not shown). Figure 1b illustrates the ESI mass spectrum of the double-stranded PCR product, where multiply charged ions carrying 24−30 negative charges were found. Deconvolution of

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the raw spectrum yielded a mass peak with three maxima at 50549, 50868, and 51157 (see inset in Figure 1b). With a mass deviation of only 0.025%, the first mass correlates well with the theoretical mass of 50 536.35 of the target PCR product, and the other two measured masses indicate the addition of one and two 2′-deoxyadenosines with theoretical mass differences of 313 and 626, respectively. Such nontemplate additions of 2′-deoxyadenosines by *Taq* DNA polymerase are commonly observed[29,38] and could be suppressed by the use of *Pfu* DNA polymerase.[39] However, *Pfu* is not very practical as amplification enzyme for routine PCR, because cycle times have to be increased considerably in order to permit the proof-reading activity of the enzyme.

Because ESI is a very gentle process, double-stranded DNA fragments longer than 25−50 bp usually do not dissociate during ionization and can be investigated as intact molecules by MS. However, base substitutions are difficult to identify in double-helical DNA by mass measurements, because A−T and a G−C base pairs have very similar average masses of 615.4 and 616.4, respectively, and A/T or C/G substitutions do not cause any mass shift at all. Hence, denaturation of double-stranded PCR products into the corresponding single strands prior to their mass spectrometric characterization may be advantageous because of the reduction in mass of the detected species and the possibility of identification of base substitutions. The isolation of single strands is feasible by labeling of one of the PCR primers with biotin followed by affinity solid-phase extraction of the labeled strand with magnetic streptavidin particles[23] or by selective digestion with λ exonuclease upon labeling of one of the strands with 5′-terminal phosphate groups.[38] However, these methods are laborious and add to the cost and complexity of sample preparation. In IP-RP-HPLC, the occurrence of nucleic acids as single- or double-strands can be effortlessly adjusted by appropriate selection of the column temperature. Depending on the length of the DNA strand and the ionic strength of the mobile phase, DNA fragments are completely denatured at temperatures between 50 an 80 °C, but the double-helical structure usually persists at temperatures below 50 °C.[40]

The chromatographic separation of the 82-bp PCR product under denaturing conditions is illustrated in Figure 2. Both single strands were completely separated, with the forward strand eluting after the reverse because of the higher proportion of relatively hydrophobic thymidines in the forward strand (Figure 2a). Figure 2b depicts the mass spectrum extracted from the peak at 7.3 min. Deconvolution yielded two mass peaks at 25 781 and 26 096, which correspond to the masses of the reverse strand having a theoretical mass of 25 783.20 and the reverse strand with an additional 2′-deoxyadenosine having a theoretical mass of 25 096.42, respectively. A mass spectrum of equivalent quality was extracted from the peak at 8.9 min (spectrum not shown) and enabled the identification of the forward strand at a mass of 24 754 and of the forward strand plus 2′-deoxyadenosine at 25 063. In contrast to the products of the reverse strand, in which the ratio of the signal intensities of nonadenylated to monoadenylated amplicons was approximately 3:1, the signal intensities of both of the products were almost identical in the products of the forward stand. This leads to the assumption that *Taq* polymerase attaches nontemplate 2′-deoxyadenosines preferentially to the forward strand.

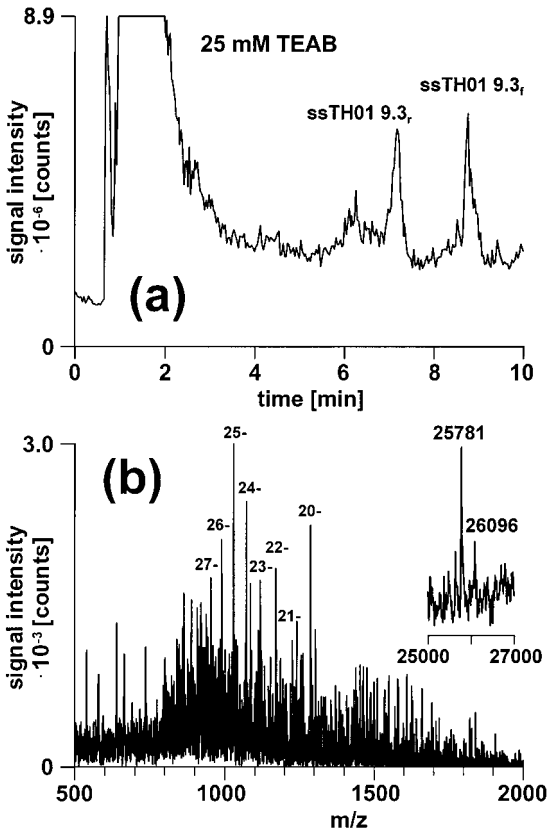

Figure 2. IP-RP-HPLC−ESI-MS analysis of the single-stranded TH01 9.3 allele using triethylammonium bicarbonate (TEAB) as the ion pair reagent under denaturing conditions. Column, monolithic PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM TEAB, pH 8.40, (B) 25 mM TEAB, pH 8.40, 20% acetonitrile; linear gradient, 10−60% B in 10 min; flow rate, 3.0 μL/min; temperature, 50 °C; scan, 500−2000 amu; sheath liquid, 3.0 μL/min acetonitrile; sample, humTH01 allele 9.3, ∼500 fmol.

Influence of Ion-Pair Reagent on Detection Sensitivity. Many of the published sample preparation protocols for MS experiments are based either on two-stage PCR[41] or on the pooling of several 50- or 100-μL PCR reactions before purification and preconcentration procedures can be successfully applied.[24,25,38] To get satisfactory signal-to-noise ratios utilizing the triethylammonium ion as an ion-pair reagent for IP-RP-HPLC−ESI-MS (see chromatogram and spectrum in Figure 2), we had to perform a 5-fold preconcentration step by means of reducing the volume of one 50-μL PCR to 10 μL through lyophilization. However, one of the most important prerequisites for the applicability of MS to the routine analysis of PCR products is the possibility of directly examining PCR reactions without prior preconcentration procedures. From the theory of the electrospray process, it is known that a reduction in the surface tension of the electrosprayed solution enables the formation of smaller droplets, resulting in more efficient ionization.[42] Therefore, acetonitrile was added postcolumn as the sheath liquid through the triaxial electrospray

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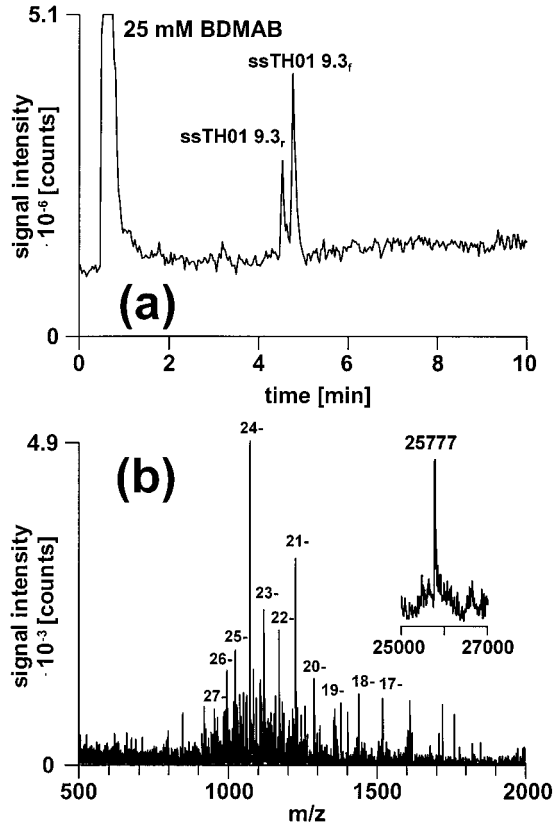

Figure 3. IP-RP-HPLC−ESI-MS analysis of the single-stranded humTH01 9.3 allele using butyldimethylammonium bicarbonate (BDMAB) as the ion pair reagent. Column, monolithic PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM BDMAB, pH 8.40, (B) 25 mM BDMAB, pH 8.40, 40% acetonitrile; linear gradient, 15−70% B in 10 min; flow rate, 3.0 μL/min; temperature, 70 °C; scan, 500−2000 amu; sheath liquid, 3.0 μL/min acetonitrile; sample, humTH01 allele 9.3, ~100 fmol.

probe, which enabled a 10-fold improvement in detection sensitivity for nucleic acids.[43]

In an attempt to further improve the sensitivity of ESI-MS detection, we were looking for chromatographic conditions that would allow us to increase the concentration of acetonitrile in the mobile phase. Because retention in IP-RP-HPLC critically depends on the amount of adsorbed ion-pair reagent, an increase in the concentration of acetonitrile has to be accompanied by an increase in the hydrophobicity of the ion-pair reagent. Consequently, we selected butyldimethylammonium ion as an alternative and more hydrophobic ion-pair reagent instead of the triethylammonium ion. The IP-RP-HPLC−ESI-MS analysis of 100 fmol of a PCR product without any preconcentration procedure utilizing 25 mM butyldimethylammonium ion as the ion-pair reagent is illustrated in Figure 3a. Gradients of 2−12% (Figure 2a) and 6−28% acetonitrile (Figure 3a) were required to elute the DNA from the column with triethylammonium and butyldimethylammonium, respectively, representing a more than 2-fold increase in applicable acetonitrile concentration with the more hydrophobic ion-pair reagent. When compared to the chromatogram shown in Figure 2a, the signal-to-noise ratio was enhanced by a factor of approximately 2, which, together with the 5-fold decrease in sample concentration, corresponds to an improvement in detection sensitivity by a factor of 10. Better spectral quality can be deduced from the mass spectrum shown in Figure 3b in which a clear series of multiply charged ions was apparent and chemical noise was considerably reduced, as compared to the spectrum illustrated in Figure 2b. This example demonstrates that injection of 500 nL of a PCR reaction is sufficient for direct IP-RP-HPLC−ESI-MS characterization of the target DNA fragment without the necessity of any other sample preparation procedure.

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Table 1. Determination of Alleles from the Human TH01 Locus by IP-RP-HPLC−MS

allele	theor mass	meas mass	rel dev, %
TH01 10_f	25 057.35	25 056	−0.0054
TH01 10_r	26 096.42	26 095	−0.0054
TH01 10 + A_f	25 370.56	25 363	−0.0298
TH01 10 + A_r	26 409.63	26 407	−0.0100
TH01 9.3_f	24 753.15	24 754	0.0034
TH01 9.3_r	25 783.20	25 781	−0.0085
TH01 9.3 + A_f	25 066.37	25 063	−0.0134
TH01 9.3 + A_r	26 096.42	26 096	−0.0016
TH01 9.0_f	23 846.56	23 845	−0.0065
TH01 9.0_r	24 836.58	24 836	−0.0023
TH01 9.0 + A_f	24 159.77	24 157	−0.0115
TH01 8.0_f	22 635.77	22 637	0.0055
TH01 8.0_r	23 576.74	23 575	−0.0074
TH01 8.0 + A_f	22 948.98	22 948	−0.0043
TH01 8.0 + A_r	23 889.95	23 885	−0.0207
TH01 7.0_f	21 424.98	21 424	−0.0046
TH01 7.0_r	22 316.90	22 315	−0.0085
TH01 7.0 + A_f	21 738.19	21 736	−0.0101
TH01 6.0_f	20 214.19	20 213	−0.0059
TH01 6.0_r	21 057.06	21 057	−0.0003
TH01 6.0 + A_f	20 527.40	20 526	−0.0068
TH01 6.0 + A_r	21 370.27	21 366	−0.0200

Determination of Different Alleles. The human TH01 locus is located in intron 1 of the human tyrosine hydroxylase gene and has a tetrameric repeating unit of the sequence AATG.[28] The nomenclature of the different alleles is based upon the number of repeating units, the most frequent alleles observed in white caucasian populations being 6, 7, 8, 9, 9.3, and 10. Allele 9.3 is a common variant of allele 10 that has a 2′-deoxyadenosine deletion in the seventh repeat. By designing primers that anneal near the repeat region, smaller PCR products were amplified than are normally used with standard capillary electrophoretic studies. Small DNA fragments hold several advantages in a chromatographic, mass spectrometric, and forensic context: (1) Chromatographic resolution in IP-RP-HPLC is highest for oligomers up to 100-mers, enabling single-nucleotide resolution over the whole size range.[44] (2) Because fewer charge states are detected in the mass spectra of smaller fragments, the total ion current is distributed among fewer species, resulting in better signal-to-noise ratios and higher mass accuracy. (3) PCR efficiency typically improves with smaller amplicon size; thus, shorter cycle times may be used. (4) Highly degraded forensic DNA samples can be examined.

With our selected set of primers, PCR products ranging in size from 67 (allele 6) to 83 bp (allele 10) were amplified and subsequently analyzed by IP-RP-HPLC−ESI-MS. The results of the mass determinations and the identification of the different allele types, as well as the relative mass deviations, are sum-

(44) Huber, C. G.; Stimpfl, E.; Oefner, P. J.; Bonn, G. K. *LC-GC Int.* **1996**, *14*, 114−27.

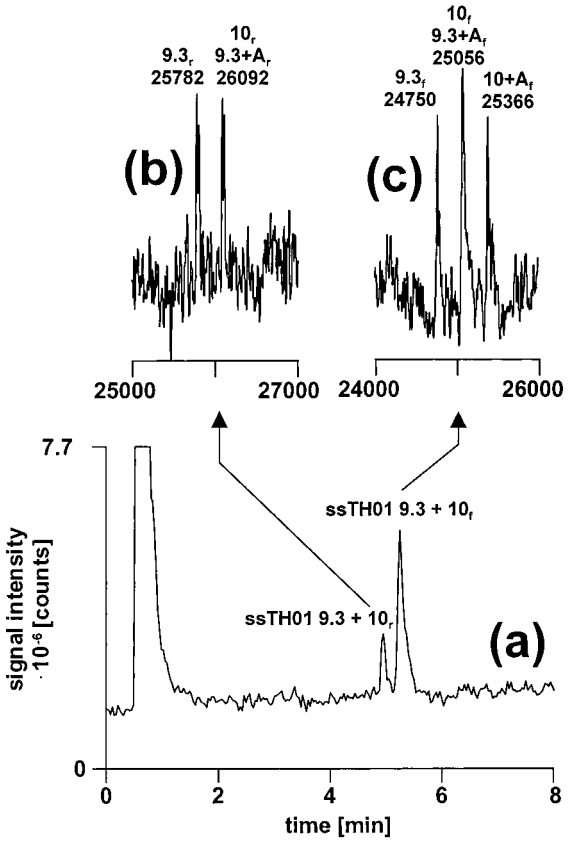

Figure 4. Determination of heterozygous alleles. Column, monolithic PS-DVB, 60 × 0.20 mm i.d.; mobile phase, (A) 25 mM BDMAB, pH 8.40, (B) 25 mM BDMAB, pH 8.40, 40% acetonitrile; linear gradient, 15−70% B in 10 min; flow-rate, 3.0 μL/min; temperature, 70 °C; scan, 500−2000 amu; sheath liquid, 3.0 μL/min acetonitrile; sample, mixture of the humTH01 alleles 9.3 and 10, ∼50 fmol each.

marized in Table 1. It is seen that for all alleles, at least three, and in most cases, four, allele-specific PCR products were detected, two coming from the reverse and the forward strand and two from the adenylated species of both strands. Although it adds to the complexity of data interpretation, this multiplex information about allele size significantly increases the confidence of allele identification, which is particularly important in forensic casework. To demonstrate the excellent accuracy and reproducibility of mass determination by IP-RP-HPLC−ESI-MS, data from multiple injections of the PCR mixture from allele 6 were statistically evaluated. From six analyses of the amplified products under denaturing conditions, the masses of the four single stranded species were determined. The relative deviations between the measured masses and the masses calculated from the base sequences ranged from −0.0134 to −0.0018%, and the pooled relative standard deviation of all 24 mass measurements was only 0.0074%, which is equivalent to 1.5 mass units absolute mass difference. A mass difference of about 150 mass units (half of a nucleotide) from the expected value would be required to incorrectly assign alleles differing by one base pair (e.g., alleles 9.3 and 10). These data validate the high accuracy and precision of mass measurements by IP-RP-HPLC−ESI-MS, which can also be utilized as confident verification that the correct sequence has been amplified by the PCR.

Comparison of IP-HPLC−ESI-MS and CE for Allele Determination. Fully automated analysis of PCR-amplified STR systems by CE is an established tool in forensic science.[29] Because

Table 2. Blind Analysis of Different HumTH01 Alleles by IP-RP-HPLC−ESI-MS and CE

individual	meas mass	allele identification by IP-RP-HPLC−MS	PCR fragment size determined by CE, nt	allele identification by CE
A	21055	6_r		
	23574	8_r		
	20213	6_f		
	20526	$6 + A_f$	62.38	6 + A
	22636	8_f		
	22948	$8 + A_f$	69.72	8 + A
B	24833	9_r		
	25802	9.3_r		
	23845	9_f		
	24159	$9 + A_f$	73.74	9 + A
	24753	9.3_f		
	25064	$9.3 + A_f$	76.74	9.3 + A
C	25780	9.3_r		
	26095	10_r; $9.3 + A_r$		
	24750	9.3_f		
	25064	$9.3 + A_f$	76.47	9.3 + A
	25064	10_f		
	25368	$10 + A_f$	77.52	10 + A
D	22314	7_r		
	23574	8_r		
	21424	7_f		
	21736	$7 + A_f$	65.96	7 + A
	22635	8_f		
	22948	$8 + A_f$	69.78	8 + A

the electrophoretic mobility is variable under different electrophoretic conditions, multiple color fluorescence detection systems combined with DNA sizing standards or allelic ladders as internal standards are necessary for reliable genotyping of STR markers. Although most tetranucleotide STR alleles differ in size by four nucleotides, some alleles do exist that differ in size by only a single nucleotide relative to other alleles, such as alleles 9.3 and 10 of humTH01. Accurate allele assignment for these alleles depends on adequate sizing precision and peak resolution of the CE separation systems. The high degree of sizing accuracy of IP-RP-HPLC−ESI-MS permits reliable genotyping of STR loci without the use of an allelic ladder. Figure 4 illustrates, as an example, the analysis of a heterozygous sample in which alleles 9.3 and 10 were readily identified on the basis of the measured mass values. Distinction of these two alleles was previously not possible by IP-RP-HPLC with UV detection, because the amplified 160−176-bp PCR fragments were too long to enable single basepair resolution.[45] In IP-RP-HPLC−ESI-MS however, both alleles were effortlessly differentiated, because in contrast to UV detection, MS can identify coeluting species on the basis of their different masses, as shown in the deconvoluted mass spectra of Figure 4b,c.

Finally, the performance and allele-calling accuracy both of CE and IP-RP-HPLC−ESI-MS were evaluated by blind analysis of unknown samples from four volunteers. In CE analysis with fluorescence detection, DNA fragment size was calculated from the migration times of the single-stranded PCR products and the DNA sizing standards using the local Southern method. Fragment sizes were converted to allele sizes upon comparison with an allelic

(45) Devaney, J. M.; Girard, J. E.; Marino, M. A. *Anal. Chem.* **2000**, *72*, 858−64.

(46) Oberacher, H.; Oefner, P. J.; Parson, W.; Huber, C. G. *Angew. Chem.* **2001**, *113*, in press.

Table 3. Comparison of CE and IP-RP-HPLC−ESI-MS for the Sizing of PCR-Amplified Short Tandem Repeat Loci

parameter	CE	IP-RP-HPLC−ESI-MS
amt genomic DNA req for assay	1−5 ng	1−5 ng
size range for single nucleotide resolution	up to 250 nt[29]	up to 500 nt[46]
calibration	allelic ladder plus in-lane size standard in each run	external mass calibration every few months
sizing accuracy	±0.16 nt with internal sizing standard	±1.5 mass units (±0.0038 nt) with external mass calibration
analysis time	30 min for single capillary system, 165 min for 96 capillary system	5−10 min per run
multiplexing capability	1−96 samples per run	1 sample per run

ladder in which the correct alleles were previously identified by DNA sequencing. IP-RP-HPLC−ESI-MS analysis was performed under the conditions given in Figure 2, and the allele sizes were deduced from the masses of the single-stranded PCR products. The data summarized in Table 2 reveals that all of the test individuals were heterozygous and that the allele combinations 6/8, 9/9.3, 9.3/10, and 7/8 were identified by both methods. The accordance between the results obtained by CE and IP-RP-HPLC−ESI-MS was 100 percent, which proves that both methods are equivalently suitable for routine characterization of STR markers.

CONCLUSIONS

IP-RP-HPLC is highly suitable for the on-line sample preparation and purification of lower femtomole amounts of PCR products for subsequent investigation by ESI-MS. The method is applicable to the routine analysis of amplicons related to polymorphic STR loci and represents a fully automatable alternative to established capillary electrophoretic analysis protocols. At present, CE with laser-induced fluorescence detection holds the advantages of lower detection limits and higher sample throughput as a result of the possibility of multiplexing. IP-RP-HPLC−ESI-MS, on the other hand, offers higher information content, better sizing accuracy and reproducibility without the need for alleleic ladders, and the possibility of directly analyzing unlabeled PCR products. Multiplexing through application of mass tags or multicapillary arrays and a reduction in analysis times upon application of step gradients will allow a considerable increase in the sample throughput of IP-RP-HPLC−ESI-MS. The major advantages and disadvantages of both methods are summarized in Table 1.

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