water (15:85 by volume). F$_2$-iPs were eluted by washing the cartridge with 2 mL of hexane–ethyl acetate–propan-2-ol (30:65:5 by volume). For comparing the analytical performance, the samples were also purified by the method described by Nourooz-Zadeh et al. (3) using two solid-phase cartridges.

F$_2$-iPs obtained by the purification procedures were analyzed by gas chromatography–negative ion chemical ionization–mass spectrometry (GC-NICI-MS). The eluting solution was evaporated under nitrogen before the derivatization. The extracted F$_2$-iPs in the samples were incubated with 25 µL of pentafluorobenzyl bromide (100 mL/L in acetone) and 25 µL of N,N-diisopropylethylamine (200 mL/L in acetone) at 60 °C for 10 min. Excess reagents were evaporated under nitrogen. The resulting pentafluorobenzyl ester was incubated with 50 µL of N,O-bis-(trimethylsilyl) trifluoroacetamide and 5 µL of N,N-diisopropylethylamine (200 mL/L in acetone) at 60 °C for 5 min. Excess reagents were removed under nitrogen. The pentafluorobenzyl–trimethylsilyl derivatives were reconstituted in 40 µL of isoctane and the samples analyzed on a GC8000 series gas chromatograph (in NICI mode) coupled to a MD800 mass spectrometer (Fisons Instruments) using methane as the reagent gas. Chromatography was carried out on an SPB-1701 column (30 m × 0.25 mm; film thickness, 0.25 µm; Supelco Inc.) using helium as the carrier gas. Injections (1 µL) were performed by an AS800 autosampler (Fisons Instruments). The temperature of the injector was kept at 280 °C. The oven temperature was increased from 150 °C to 235 °C at a rate of 15 °C/min, increased from 235 °C to 250 °C. The oven temperature was increased from 150 °C to 235 °C at a rate of 15 °C/min, increased from 235 °C to 250 °C at 10.3 °C/min, and then maintained at 280 °C for 10 min. The MD800 was programmed for optimal detection with the following settings: electron energy, 70 eV; emission energy, 250 µA; ion energy, 1.0 eV; and ion energy ramp, 1.0 mV per atomic mass unit. Selected ion monitoring was performed to monitor the carboxylate (M−181; loss of CH$_2$C$_3$F$_5$) at ions m/z 569 and 573 for F$_2$-iPs and the internal standard, respectively. Peak identification was based on the comparison of the relative retention indices with the internal standard. The concentration of F$_2$-iPs isomers was calculated using the ratio of the peak height of m/z 569 to that of m/z 573.

To evaluate the analytical performance of the present method, one of the F$_2$-iPs isomers, iPF$_2$α-III, was used in the quantification and subsequent calculation of recovery and precision performance. The results and the comparison with the method of Nourooz-Zadeh et al. (3) are presented in Table 1. A recovery of ~90% was achieved with our method compared with ~70% with the purification method of Nourooz-Zadeh et al. (3) with comparable data for intraassay and interassay imprecision and linearity. As shown in Fig. 1, the Oasis purification procedure led to identical GC-NICI-MS chromatograms for F$_2$-iPs in calibration solutions and plasma.

In summary, a substantially improved recovery for isolating F$_2$-iPs from biologic matrices can be achieved by our one-step purification procedure compared with previous methods. It is also simpler and takes only one-half the extraction time needed by the two-cartridge method (3). The procedure is also well suited as a preparatory step to isolate F$_2$-iP isomers in complex tissue matrices before applying specific purification procedures, e.g., immunoaffinity chromatography of iPF$_2$α-III.

References

Electrospray Mass Spectrometry: An Efficient Method to Detect Silent Hemoglobin Variants Causing Erythrocytosis, Dilip K. Rai, William J. Griffiths, Gunvor Alvelius, and Britta Landin

Erythrocytosis, which is characterized by high hematocrit (packed cell volume), is a common condition causing complications attributable to hyperviscosity of blood. According to modern nomenclature, patients can be subdivided into one group of “apparent erythrocytosis” and one of “absolute erythrocytosis” (1). The absolute-erythrocytosis group contains both genetic disorders, acquired conditions such as polycythemia, and secondary reactions attributable to chronic hypoxia or renal disease. The occurrence of hemoglobin (Hb) variants with increased oxygen affinity is a well-known cause of hereditary erythrocytosis, but in recent years interest has also been given to mutations in the erythropoietin receptor gene (2). Theoretically, functional studies of oxygen saturation curves would be the ideal method to screen for Hb variants that cause erythrocytosis, but such methods are not generally available and there are diverging opinions whether P$_{50}$...
measurements of stored or shipped samples can be used (3, 4). Of the nearly 200 Hb variants with increased oxygen affinity described, fewer than one-half have been found in conjunction with significant erythrocytosis (3). Several of the variants are attributable to amino acid substitutions that do not affect the net charge, i.e., the substitutions are “silent” when investigation is performed by conventional electrophoretic techniques. In contrast, almost all Hb variants in this group can readily be found by mass spectrometry exploiting the change in molecular weight of the variant globin.

Using electrospray mass spectrometry (ESMS), we have screened 70 consecutive samples from patients with unexplained erythrocytosis that had showed normal Hb pattern on isoelectric focusing (IEF) (5) and HPLC (6, 7) analysis. The investigation was approved by the Ethics Committee at Huddinge University Hospital. In three cases, we detected β-globin variants. This report illustrates how the information from electrophoretic methods and the knowledge of the wild-type globin gene sequence can facilitate the interpretation of mass spectral findings.

The database of human Hb variants found at the Globin Gene Server home page (8) was searched for Hb variants associated with erythrocytosis. By this means, 89 different Hb variants associated with erythrocytosis were found. [Details of the results of the database search are available as a data supplement at Clinical Chemistry Online (http://www.clinchem.org/content/vol47/issue7/)]. Of these variants, 33 were neutral, i.e., the substitution involved amino acids of the same group (neutral, basic, or acidic). In 9 of the 89 variants, the mass of the variant did not differ from that of the wild-type globin by >6 Da. Of this group of variants, which as the intact protein would not be expected to be resolved from wild-type globin by ESMS on quadrupole instruments, only one neutral Hb variant (Hb Linköping, β36 Pro → Thr) was found. Although this variant cannot be separated from Hb Aβ by conventional acid electrophoresis, IEF has shown excellent separation (9).

ESMS analysis was performed on two different instruments capable of resolving intact Hb masses that differ by >6 Da with the aid of a deconvolution program. The initial screening of the series of whole-blood samples was carried out using a Quattro I mass spectrometer (Micromass), and the trypsin-digested (Sigma-Aldrich) samples were analyzed on a quadrupole time-of-flight mass spectrometer (Micromass) as described previously (10). A 1612-bp DNA fragment including all three exons of the β-globin gene was amplified by PCR using primers 5′-ATAAGTACGGGCGAGCCATCTAT-3′ and 5′-TAGTGGCAAGATCCAGTCGAAAG-3′. Nucleotide sequencing of exons 1 and 2 was performed using a sequencing primer (5′-GGAATTCATTTGGCTGACAACT-3′) and Big Dye™ terminator (PE Applied Biosystems).

The mass spectra of intact globins displayed peaks for the wild-type α (15 126 Da) and β (15 867 Da) chains and their common adducts (Na⁺, K⁺, heme, and glutathione). ESMS analysis of the first case showed the presence of a β variant (β3) with a mass shift of +32 Da from the wild-type β chain (Fig. 1A). The variant constituted ~54%. Several possible amino acid substitutions correspond to a +32 Da mass difference: Ala→Cys, Asp→Phe, Met→Tyr, Pro→Glu, and Val→Met. Only the Val→Met substitution, however, results from a single-base substitution. This is also a neutral substitution. In the ES mass spectrum of the tryptic digest, an intense doubly protonated peak of wild-type βT3 (m/z 657.8) was observed as well as a variant peak (m/z 673.8) 16 Thompson above (Fig. 1A, inset). This corresponds to a +32 Da shift in the uncharged molecule. The location of a variant peptide βT3 was further supported by observation of a peak corresponding to the singly protonated variant peptide (m/z 1367.3) 32 Thompson above the wild-type βT3 peptide peak (m/z 1347). To pinpoint which of the three valine residues in the βT3 peptide (VNDEVGGEALGR) was substituted, the doubly protonated wild-type and variant peptides were subjected to tandem MS (MS/MS). The MS/MS spectra for both peptides showed almost complete series of singly charged Y ions (Y1–Y12) as well as complementary B ions (B2–B6, Fig. 1B). The observation of the same m/z value for the Y8 ion in the MS/MS spectra of both the wild-type and variant peptides rules out substitution of the valine, which is the eighth residue from the COOH terminus. The mutation was localized by the Y11 and B3 fragment ions, which were shifted by +32 Thompson from the corresponding Y11 and B3 ions of the wild-type peptide. The effect of this m/z change attributable to the Val→Met substitution on the consecutive fragments was demonstrated by an equivalent m/z shift in Y12 and were supplemented by an equal m/z shift in B3 to B6 ions. The Val→Met substitution located by Y11 and B3 corresponds to position 20 in the β-globin chain, indicating the substitution β20Val→Met. Thus, the variant is identified as Hb Olympia (β20Val→Met) (11). Sequence analysis of amplified DNA confirmed the expected mutation G→A in codon β20. In a similar way, a second case of Hb Olympia was identified in this investigation.

The ES mass spectrum of the intact globin chains from the third case showed a β variant (β5) 14 Da larger than the wild-type β-globin (Fig. 1C). The variant constituted ~51%. There are eight possible amino acid exchanges that correspond to the mass shift of +14 Da: Asn→Gln/Lys, Asp→Glu, Gly→Ala, Ser→Thr, Thr→Asp, and Val→Leu/Ile. The Asn→Gln and Asn→Lys substitutions are not possible as single point mutations and thus were ruled out. Of the remaining possibilities, a Thr→Asp substitution would be expected to alter the intrinsic charge of the Hb molecule, which was not compatible with the IEF pattern. Considering the remaining five possible substitutions and starting with the already described Hb variants associated with erythrocytosis (see on-line data supplement), we directed our interest to tryptic peptides βT11 and βT12, both of which represent inner core peptides of the Hb molecule. Because the core peptides often are difficult to characterize by ESMS (12),
DNA sequencing was performed. Heterozygosity for a mutation T→A was found, corresponding to β99 Asp (GAT)→Glu (GAA). We then performed ESMS to confirm this mutation on the protein level, our interest being focused on the βT11 peptide (LHVDPENFR). Analysis of the tryptic digest demonstrated doubly protonated wild-type (m/z 563.8) and variant (m/z 570.8) peptides as shown in the inset of Fig. 1C. The 7-Thompson change in m/z in the doubly protonated peptide is equivalent to a change of 14 Da in the intact β chain.

MS/MS analysis of the doubly protonated wild-type and the variant peptides localized the amino substitution to the Y6 and B4 ions corresponding to residue 99 in the wild-type β chain (Fig. 1D). Both the ESMS and DNA sequencing conclusively indicated the substitution β99Asp→Glu, which is known as Hb Coimbra (13) or Hb Ingelheim (14).

In this study, three cases of Hb variants known to cause erythrocytosis were found by ESMS but missed by the IEF method. In two cases, the diagnosis of heterozygous Hb Olympia (β20 Val→Met) was achieved solely by the use of ESMS. This could be accomplished because the array of possible substitutions was limited by the assumptions that the neutral variant had been described previously and was attributable to a point mutation. In the third case, the initial mass spectral findings indicated a variant β-chain and provided preliminary data. Because these were compatible with several substitutions in the inner core of the Hb molecule, DNA sequencing was performed and revealed heterozygosity for Hb Coimbra (β99 GAT→GAA). The DNA result was later confirmed on the protein level by ESMS and MS/MS experiments.

Although Hb variants are relatively seldom found to be the cause of high Hb concentrations in unselected patient groups, the possible occurrence of a Hb variant often is considered early in the investigation of patients with high

Fig. 1. ESMS analysis of hemolysate for the first case (Hb Olympia; A and B) and the third case (Hb Coimbra; C and D).

(A), deconvoluted mass spectrum demonstrating the presence of variant β chain at 15 899 Da, i.e., 32 Da larger than the mass of the wild-type β chain (15 867 Da). Shown in the inset is the portion of the mass spectrum from tryptic digest showing the singly and doubly protonated forms of wild-type (monoisotopic m/z 1346.7 and 673.8) and variant (monoisotopic m/z 1346.8) peptides. Shown in the top right corner is the proposed amino acid sequence and Y and B fragment positions. The amino acid substitution is localized by Y6 and B4 ions, confirming that the mutation occurs at residue 4 in peptide βT11 and position 99 in the β chain.
Hb concentrations. Standard techniques commonly used for screening for Hb variants, such as electrophoresis, IEF, or ion-exchange HPLC, are not sufficient because 33 of the 89 different Hb variants associated with erythrocytosis do not induce any major change in net charge of the Hb molecule. The additional use of ESMS is encouraged because combination of this technique and IEF will increase the sensitivity for Hb variants that cause erythrocytosis to 100%.

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Quantification of Aromatic Hydrocarbon Receptor (AhR) and Related Genes by Calibrated Reverse Transcription-PCR in Blood Mononuclear Cells, Cristina Dassi, Paolo Brambilla, Stefano Signorini, Piermario Gerthoux, Pietrapaola Molteni, Raffaella Sala, and Paolo Macarelli* (1 University Department of Laboratory Medicine, University of Milano-Bicocca, Hospital of Desio, Via Mazzini 1, 20033 Desio, Milan, Italy; 2 Department of Laboratory Medicine, Hospital of Merate, Largo Mandic 1, 23807 Lecco, Italy; *author for correspondence: fax 39-036-2383464, e-mail mocarelli@uds.unimib.it)

The aromatic hydrocarbon (Ah) gene battery (1) comprises Ah receptor (AhR), its nuclear translocator (ARNT), and the genes that encode the detoxifying enzymes of phase I (e.g., cytochrome P4501A1, -1A2, and -1B1) and phase II. These enzymes and growth regulatory factors such as interleukin 1-β (IL-1β) are coordinately induced by polychlorinated dibenzo-p-dioxins and polycyclic aromatic hydrocarbons, toxic chemicals that are ubiquitous in ambient air and in cigarette smoke, via AhR (2–4).

Quantification of the regulation of AhR and related genes could be useful to monitor the degree of polycyclic aromatic hydrocarbon exposure and to understand the pathways to chemical carcinogenesis and atherosclerosis (5). The expression of AhR and related genes has been studied using Northern blot analysis (6) or quantitative reverse transcription-PCR (RT-PCR) using labeled primers (7), which are low-sensitivity techniques; competitive RT-PCR (8), which is more sensitive but quite laborious; and real-time PCR (9), which is expensive.

An interesting technique, proposed for the first time by Tsai and Wiltbank (10) and considered later by Bor et al. (11), is called “calibrated” RT-PCR; it is a competitive RT-PCR based on the addition of a fixed amount of internal standard (IS) to the sample and to a set of calibrators. The labor, time, and costs for this method are lower than those for classic competitive RT-PCR. We present calibrated RT-PCR assays to study the expression of AhR, ARNT, cytochrome P4501B1 (CYP1B1), and IL-1β for the AhR gene battery and of β-actin (β-Act) as housekeeping gene in peripheral blood mononuclear cells from 32 healthy subjects.

Total RNA was extracted according to the method of Chomczynski and Sacchi (12) from 1 × 10⁷ peripheral blood mononuclear cells in suspension isolated by step-gradient centrifugation on Histopaque. RNA was suspended in 50 µL of sterile water, quantified by measuring the absorbance at 260 nm, diluted to 100 mg/L with sterile water, and stored in liquid nitrogen.

The ISs, designed to be smaller than the corresponding target sequences, were homologous recombinant cDNAs obtained by oligonucleotide-mediated mutagenesis (13) from AhR, ARNT, CYP1B1, and β-Act sequences inserted in pGEM plasmid. A more recently developed and simple method (14, 15) was adopted to obtain the IL-1β IS. Table 1 shows the primers used for the amplification and the oligonucleotides used to produce corresponding ISs. Forward and reverse primers were chosen on differ-