Note

Identification and quantitation of Hb Olympia [β20(B2)Val → Met] and Hb San Diego [β109(G11)Val → Met] by high-performance liquid chromatography

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Hb Olympia and Hb San Diego are β-chain abnormal hemoglobins with an identical substitution of Val → Met but at positions β20 and β109, respectively[1,2]. The two variants have a high affinity for oxygen and cause an erythrocytosis in the heterozygote. Electrophoretic and chromatographic methods fail to separate the variants from the normal Hb A, thus complicating their isolation and quantitation[1–4]. Stamatoyannopoulos *et al.*[1] and Nute *et al.*[3] estimated that about 40% of the β-chains of the hemoglobin of the Hb Olympia heterozygote had a methionyl residue in the βT-3 peptide while Harkness *et al.*[4] assumed that the blood of the patient with Hb San Diego contained approximately 50% of mutant hemoglobin.

Recently, we had the opportunity to study these two variants by high-performance liquid chromatography (HPLC). With this method it was possible to separate the abnormal βT-3 peptide of Hb Olympia and the abnormal βT-12A peptide of Hb San Diego from their normal counterparts, allowing a more accurate quantitation of the mutant hemoglobins.

EXPERIMENTAL

The hemoglobins of red cell lysates were analyzed by various electrophoretic methods at both acidic and alkaline pH. Globin was prepared by the method of Anson and Mirsky[5] and the βA- + βX-chains were separated from the δ- and α-chains by CM-cellulose chromatography[6]. Aminoethylation (AE-) was effected by the procedure of Jones[7]. The isolated β- or AE-β-chains were digested with trypsin (TPCK-trypsin, Worthington) for 4 h at room temperature and at pH 8.5. The resulting tryptic peptides were separated by HPLC using an ammonium acetate–acetonitrile buffer system[8]. Amino acid analyses were carried out with a fully automated Beckman amino acid analyzer while the ultra-microsequencing procedure of Chang *et al.*[9] was used to determine the sequence of a few selected peptides.

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* Contribution No. 0740.

RESULTS

Hb Olympia or $\alpha_2\beta_2$ 20(B2)Val → Met

    The patient was a 45-year-old white male with erythrocytosis (Hb 18.6 g/dl, PCV 53.5 1/1, RBC 6.38 · 10^{12}/1) and a high blood oxygen affinity (patient, P_{50} = 17.7 mmHg; control, P_{50} = 26.7 mmHg). The chains (*i.e.*, a mixture of β^A- and β^X-chains) of the total globin were isolated by CM-cellulose column chromatography[6] and digested with TPCK-trypsin. The soluble peptides were separated by HPLC using the solvent program described before[8].

    The top chromatogram in Fig. 1 illustrates the separation of the peptides. Two βT-3 peptides were observed, which had amino acid compositions as listed in Table I. Sequence analyses confirmed the suspected Val → Met substitution in the second βT-3 peptide at a position that corresponds to position 20 of the intact β-chain. These results identified this variant as Hb Olympia[1]. The relative amount of the β-Olympia chain in the original mixture was established by measuring the weights of appropriate cut-outs (*i.e.*, the two βT-3 zones) of photocopies of the chromato-
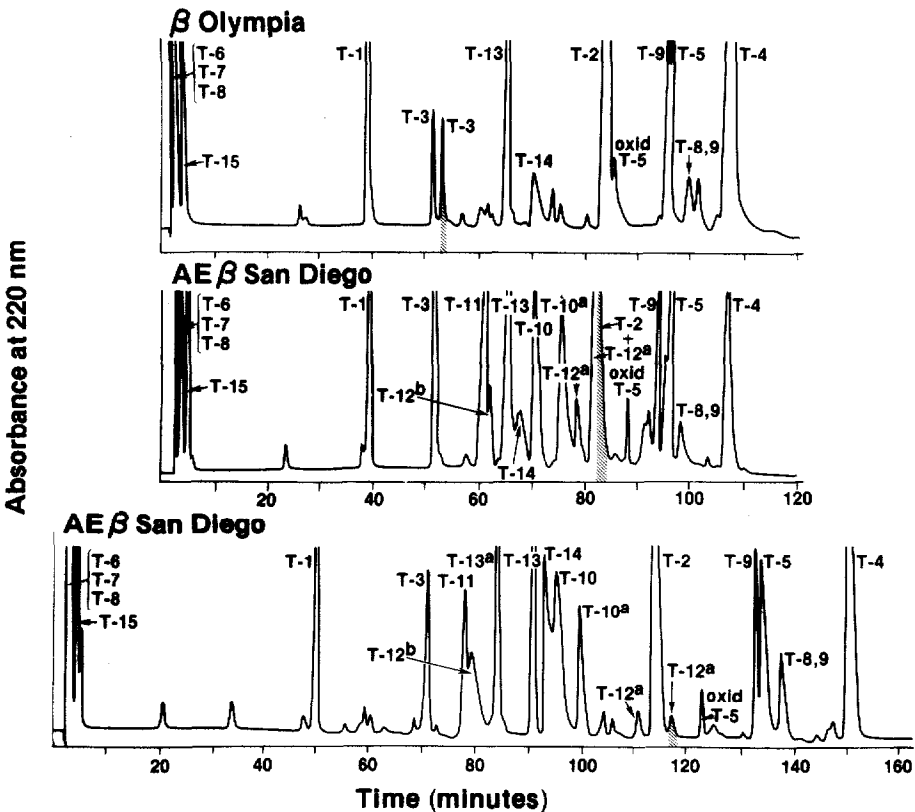

Fig. 1. Separation of tryptic peptides by HPLC. Top: mixture of βA and β-Olympia chains. Middle: mixture of the aminoethylated βA and β-San Diego chains. The two chromatograms were developed as described in ref. 8. Bottom: same as middle chromatogram except for the use of a slower gradient, which is listed in the text. The shaded areas mark the locations of the abnormal peptides.

TABLE I

AMINO ACID COMPOSITION OF SELECTED PEPTIDES ISOLATED FROM THE β^x CHAINS OF Hb OLYMPIA (PATIENT I) AND Hb SAN DIEGO (PATIENT II)

Residue	Patient I		Patient II	
	βT-3 normal	βT-3 abnormal	$\beta T12$-A normal	$\beta T12$-A abnormal
Asp	1.96(2)	2.08(2)	1.18(1)	1.30(1)
Glu	2.14(2)	2.02(2)		
Gly	3.34(3)	3.18(3)	1.34(1)	1.50(1)
Ala	1.06(1)	1.08(1)		
Val	2.74(3)	*1.66(3)*	1.80(2)	*1.00(2)*
Met	0 (0)	*0.94(0)*	0 (0)	*0.74(0)*
Leu	0.94(1)	0.99(1)	2.95(3)	3.01(3)
AE-Cys			0.72(1)	0.95(1)
Arg	1.15(1)	1.07(1)		

* The numbers in parentheses are the expected numbers of corresponding normal peptides.

gram. The value observed was 55.6%, which was considerably higher than the 40% suggested before[1,3].

Hb San Diego or $\alpha_2\beta_2$ 109(G11)Val → Met

This patient was a 43-year-old Japanese man with erythrocytosis (Hb 21.4 g/dl, PCV 62 l/l, RBC 6.46 × 10^{12}/l) and a high blood oxygen affinity (patient, P_{50} = 17.5 mmHg; control, P_{50} = 26.0 mmHg)[10]. The patient is the first Japanese detected with this particular Hb type, and detailed clinical and biochemical data have been published elsewhere[11].

Again, the β-chains of the whole globin were isolated by CM-cellulose column chromatography[6]. The isolated β- (*i.e.*, β^A + β^X) chains were aminoethylated and the AE-β-chains digested with trypsin. The tryptic peptides were separated by HPLC using the same program system as described above as well as by a slightly modified approach. This modified solvent program using a Perkin-Elmer Series 3 HPLC system was T-1, 10 min (1%), gradient curve 0; T-2, 180 min (1–60%), gradient curve 1; and T-3, 20 min (60–90%), gradient curve 1 (for details, see ref. 8).
2 peptide. The slower gradient used in the modified program allowed the complete separation of the two βT-12A peptides and the βT-2 peptide (bottom chromatogram in Fig. 1).

The amino acid compositions of the two βT-12A peptides listed in Table I suggested that the abnormal fragment was eluted behind the βT-2 fragment and the normal βT-12A in front of the peptide. Sequence analyses confirmed the substitution at a position in the peptide corresponding to position 109 of the β-chain, which identified the variant as Hb San Diego. Quantitation of the β^X-chain by the same method used for Hb Olympia estimated the percentage of β^X-chain as 42.1%, which was lower than 50% reported earlier[4] and agreed with the 30–50% suggested by the cyanogen bromide method[11].

DISCUSSION

HPLC is rapidly becoming one of the most important methods for the separation of peptide fragments, and various approaches have been described that are useful for the evaluation of structural abnormalities in hemoglobin variants including those with "silent" mutations[8,12-14]. The analyses described here provide additional examples and show that peptides in which a valyl residue is replaced with a methionyl residue are eluted considerably slower with the ammonium acetate–acetonitrile buffer system than their normal counterparts. This observation greatly facilitates the identification of the two high oxygen affinity Hb variants.

The use of HPLC for the determination of the relative amount of the variant β^X-chain should be viewed with caution despite the fact that reasonable numbers have been obtained. The quantitation can be influenced considerably by variation in the recovery of the peptide, by its relative purity when it emerges as a single zone from the column, and by possible differences in molecular extinction coefficients of the normal and abnormal fragments at the wavelength used (220 nm). Despite these uncertainties, this simple new approach may have considerable advantages and continued analyses using blood samples from additional patients seem indicated.

ACKNOWLEDGEMENTS

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