found that both gabapentin and the internal standard are recovered from plasma in a 90–98% yield after extraction with 1-mL C\textsubscript{18} extraction columns. The recovery of gabapentin is drastically reduced when the sample passes through the C\textsubscript{18} sorbent at a relatively fast rate. However, endogenous amino compounds present in plasma are more polar than gabapentin and are not retained by C\textsubscript{18} sorbent even when the sample is passed at a slow rate.

Different authors have used different compositions of OPA derivatizing agent, a mixture of a methanolic solution of OPA, a thiol, and borate buffer, but the fluorescent derivatives are produced in similar yields. The presence of water in the reagent makes the OPA derivative unstable, and the stability can be improved by decreasing the water content (16). In our described procedure, the derivatizing reagent does not contain borate buffer. The methanolic eluate obtained from the C\textsubscript{18} extraction column contains traces of borate buffer, which is adequate to initiate the reaction. The resulting OPA derivatives are stable for at least 4 h at room temperature and for at least 24 h when stored at 4–8 °C.

Gabapentin OPA derivatives have been detected at excitation wavelengths of 230–250 or 330–350 nm with a common emission wavelength of 430–450 nm. Excitation at 230–250 nm provides ~2.5 times the sensitivity of that observed at 330–350 nm with the same attenuation, slit width, and emission wavelength. However, the baseline is unstable at excitation wavelengths of 230–250 nm, and the detector must be set at a higher attenuation to decrease the noise. The procedure presented here is extremely sensitive even at an excitation wavelength of 350 nm. α-Aminocyclohexanepropionic acid (cat. no. 43,805-7; Aldrich) behaved similar to gabapentin and its analog for solid-phase extraction. It produced a fluorescent OPA derivative that eluted close to the gabapentin analog for solid-phase extraction and gas chromatography mass spectrometry for therapeutic drug monitoring. J Anal Toxicol 1999:23:1–6.

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References


Hemoglobin Wayne in a British Family: Identification by Electrospray Ionization/Mass Spectrometry, Tim M. Reynolds, Tim C. Harvey, Brian N. Green, Adrian Smith, and Andrew J. Hartland (1) Clinical Chemistry Department, Queen’s Hospital, Burton-on-Trent, Staffordshire DE13 0RB, and Division of Clinical Sciences, Wolverhampton University, Wolverhampton WV1 1SB, United Kingdom; (2) Diabetes & Endocrinology Department, Manor Hospital, WalsallWS2 9PS, United Kingdom; (3) Micromass UK Ltd., Wythenshawe, Manchester M23 9LZ, United Kingdom; (4) Haematology Department, Queen’s Hospital, Burton-on-Trent, Staffordshire DE13 0RB, United Kingdom; (5) Clinical Chemistry Department, Northwick Park Hospital, London HA1 3UJ, United Kingdom; address correspondence to this author at: Clinical Chemistry Department, Queen’s Hospital, Belvedere Rd., Burton-on-Trent, Staffordshire DE13 0RB, United Kingdom; fax 44-1283-593064, e-mail Tim.Reynolds@Queens.Burton-tr.wmids.nhs.uk)

Hemoglobin (Hb) Wayne (an elongated α-chain frameshift variant that exists as 2 forms, Hb Wayne Asn and Hb Wayne Asp) was first described in 1976 in a Caucasian neonate in Pike County, Central Georgia, whose grandparents originated from Birmingham, Alabama (1). Two other families have been reported, one a Caucasian family from Michigan (2), and the other from Canada (3). Clinically, Hb Wayne leads to an increased oxygen affinity Hb with noncooperativity and a markedly reduced Bohr effect. Hb Wayne Asn and Hb Wayne Asp exhibit differences in their carbon monoxide-binding properties (4).
We report the identification of Hb Wayne in a 28-year-old Caucasian diabetic female patient whose blood was undergoing analysis for glyco-Hb. Her initial pathologic investigations showed no abnormalities (144 g/L Hb; erythrocytes, $5.09 \times 10^{12}$/L; packed cell volume, 0.437 L/L; mean cell volume, 85.8 fl; mean cell Hb, 28.3 pg; mean cell Hb concentration, 330 g/L). Analysis of a hemolysate for glyco-Hb using a HA-8140 analyzer (Menarini Diagnostics Ltd.) revealed a slight shoulder on the glycated Hb peak with an estimated HbA1c concentration of 13.8% [Diabetes Control and Complications Trial (DCCT)-aligned]. This analyzer uses HPLC on a methylacrylic acid/methylacrylate ester column (HSV-II-V column; Sekisui Chemical Co Ltd.) with a sequence of three eluants producing a HbA1c result every 4 min. Other common hemoglobins (HbA 2, HbC, HbF, HbS, and metHb) were excluded because their retention times on the analyzer are characteristic and different from that of the unusual peak. A sample was referred for electrospray ionization/mass spectrometry (electrospray ionization/MS); Ref. (5)]. The mass spectrometrically estimated HbA1c was 61 g/L.

Electrospray ionization/MS analysis of intact $\alpha$- and $\beta$-chains (5) using a Micromass Quattro Ultima tandem mass spectrometer showed that the abnormal Hb contained an $\alpha$-chain with a mass ($15617.54 \pm 0.05$ Da; $n = 4$) 491.16 Da heavier than the normal chain (Fig. 1), suggesting the variant Hb Wayne. Tandem MS (MS/MS) analysis of the intact variant and normal chains showed that the altered sequence lay at the C-terminal end of the $\alpha$-chain and gave supporting evidence for Hb Wayne. Final confirmation of the sequence was obtained from a tryptic digest, followed by MS/MS of the variant peptides ($\alpha$T12–13 and $\alpha$T14).

All of these results were fully consistent with the sequence of Hb Wayne except the mass of the variant $\alpha$-chain. This was substantially higher (0.60 Da) than predicted from the sequence of Hb Wayne (15616.94 Da), but would occur if there were partial (60%) deamidation of Asn$^{139}$ to Asp$^{139}$. Such partial deamidation has already been described for this variant (2–4), in which the proportions of the Asn$^{139}$ and Asp$^{139}$ species were approximately equal. MS analysis cannot resolve these species because their mass difference is only 1 Da and measures their abundance weighted mean mass. Hence, the predicted mass for the combined species, assuming equal proportions, is 0.50 Da higher than the sequence mass of the Asn$^{139}$ species and is within experimental error of the measured mass. Therefore, all the mass spectral data were consistent with the Hb Wayne frameshift mutation, lead-

![Fig. 1. Electrospray mass spectrum of blood from the patient (1:500 dilution). A second $\alpha$-chain peak can be seen, 491.1 Da heavier than the normal $\alpha$-chain.](image-url)
ing to replacement of the three C-terminal amino acids, α139–141, by Asn\textsuperscript{139}–Thr-Val-Lys-Leu-Glu-Pro-Arg\textsuperscript{146} together with partial deamidation of Asn\textsuperscript{139} (40%) to Asp\textsuperscript{139} (60%). The patient was heterozygous for the variant, which was present at 18% of total α-chains.

The previously reported cases of Hb Wayne were identified by biochemical methods. In electrospray ionization/MS, ions are formed from intact polypeptides by adding one or more protons (H) (5). MS measures the mass to charge ratio (m/z) of these ions. Because the number of charges (n) is known, the molecular mass (M\textsubscript{r}) in daltons (Da) is deduced using M\textsubscript{r} = n(m/z – H), and software converts the original m/z data into a mass spectrum as in Fig. 1 of the data supplement (available with the online version of this Technical Brief at http://www.clinchem.org/content/vol48/issue12/). In effect, MS/MS combines the purification and sequencing of polypeptide mixtures, previously undertaken by the use of several procedures into one operation without chromatography. The technique uses an instrument consisting essentially of two mass spectrometers in series. The first spectrometer purifies the polypeptide mixture by selecting ions with a specific m/z. These ions are then fragmented by collisions with a gas in a cell situated between the two spectrometers. The resulting fragment ions are then analyzed according to their m/z in the second spectrometer to give sequence information. Mainly, fragment ions result from cleavages between the amino acid residues in the polypeptide chain. The skill in MS lies in identifying diagnostic peaks in complex spectra with the aid of computer programs. The complete MS analysis of the variant Hb was carried out and results were returned to the referring laboratory within 4 working days, a substantial reduction compared with biochemical techniques.

This is the third abnormal Hb we have reported that has been analyzed by this technique (6–8), and we recommend it as a rapid method for identification of unknown variants, although we caution that interpretation requires considerable expertise.

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Different Kinetics of Bone Markers in Normal and Delayed Fracture Healing of Long Bones, Markus Herr-
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No clinical tests can predict delayed fracture healing. Early knowledge about the individual prognosis of a fracture could help to prevent severe complications (e.g., pseudoarthrosis), and enable the physician to modify therapy. Plain radiography remains the standard method to monitor fracture healing, but it documents delayed healing only late in the course. Biochemical bone markers might provide earlier information. Osteocalcin (OC) and bone alkaline phosphatase (ALP), both markers of bone formation, as well as serum type I collagen C-terminal telopeptide (β-CTx), a marker of bone degradation, are used mainly to monitor bone-consuming diseases and antiresorptive therapies (1–9). Few studies have analyzed the behavior of these markers after fracture. We evaluated their ability to distinguish patients with normal and delayed fracture healing before radiologic evidence of delay is available.

We investigated 14 patients with a traumatic crural (n = 13) or femoral (n = 1) shaft fracture (Table 1). All patients underwent operative therapy and were monitored for 1 year. No patient suffered from diseases or had been administered medication known to interfere with bone metabolism. Blood samples for measurement of OC, bone ALP, and β-CTx were taken within 24 h of fracture and after 7, 14, 28, 42, 60, 90, 180, and 365 days. Serum was separated from whole blood and stored at −20 °C until measurement. We also evaluated a clinical score of fracture healing. At the end of the study, patients were classified as having normal or delayed fracture healing. The rate of healing was considered normal if the fracture healed completely after 6 months (score 4) and score 2 was reached on day 60. Otherwise, fracture healing was classified as delayed.

The clinical score was defined as follows: (0), no weight bearing, strong inflammation, pain at rest; (1), mobilization with crutches, partial weight bearing (less than