ing functions contributes to decreased resistance to infection in diabetics. Although our conclusion is tempered by our use of non-human antibodies, it is not unreasonable to expect that human antibodies would respond in like manner. Furthermore, our conclusion is consistent with the clinical observation that diabetic response to infection improves promptly on rectification of hyperglycemia (1), which ought not to be the case were glycated immunoglobulins impaired.

References

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Analysis for Carboxyhemoglobin by Gas Chromatography and Multicomponent Spectrophotometry Compared

Hendrik J. Vreman,1 David K. Stevenson,1,2 and Albert Zwart3

Measurements of carboxyhemoglobin (HbCO) by gas chromatography (GC) were compared with those obtained by multicomponent spectrophotometric analysis (MCA). Correlation was good for HbCO ranges (proportion of total hemoglobin) of 1.5 to ≤5% (GC = 0.94 MCA + 0.37%, n = 25, r = 0.98); 5.0 to 10.0% (GC = 0.89 MCA + 0.63%, n = 18, r = 0.93); >10% (GC = 1.01 MCA – 0.02%, n = 22, r = 0.99); and >20% (GC = 1.01 MCA – 0.10%, n = 95, r = 1.00). The correlation is lower for the clinically normal range of 0 to ≤1.5% (GC = 0.65 MCA + 0.24%, n = 29, r = 0.87). The gas-chromatographic method is linear for HbCO proportions of 30% to as little as 0.15%. Re-analysis of blood samples after two-month storage at 4°C showed that samples remained stable under these conditions.

Additional Keyphrases: neonates · pediatric chemistry · control materials · assessing exposure to CO

The carboxyhemoglobin (HbCO) content of blood is measured to assess the effects of exposure to carbon monoxide from environmental sources (1, 2). In addition, this determination is used to assess hemolysis in newborns, a process that yields endogenous CO (3, 4). For routine HbCO measurements, several methods are available, based mainly on spectrophotometry (5, 6). However, gas chromatography (GC) is, perhaps, the method of choice for determining very low proportions of HbCO (<1.5% of total hemoglobin). Furthermore, samples to be assayed by GC are more stable than those used for spectrophotometry, and the required sample volume is smaller (7). Thus GC is particularly suitable for use with blood samples from infants and in cases where immediate on-site analysis is not possible.

Recently, a sensitive GC method (7) and a multicomponent analysis spectrophotometric (MCA) method (8) have been reported for determination of HbCO. Although the GC method was not validated against other, non-GC methods, the MCA HbCO determination has been validated with a chemical titrimetric method (9). We undertook the present study to compare the HbCO values measured in whole blood by GC with those measured by the MCA method. Furthermore, we investigated the linearity of the GC method for HbCO proportions down to approximately 0.15%. Finally, because no assayed quality-control samples exist at present for measurement of HbCO by GC, we describe a method for preparing whole-blood control samples for internal quality-control purposes.

Materials and Methods

Subjects and Samples

Blood samples were obtained from specimens sent to the routine clinical laboratory for analysis for hemoglobin derivatives. Samples were from both smokers and nonsmokers. Most of the samples with HbCO proportions >5% were obtained by tonometry of patients’ blood with pure CO (1, 5).

Preparation of Control Samples

HbCO control samples were prepared from outdated whole blood in a blood bag obtained from the Stanford University Medical Center Blood Bank. Per 500 mL of blood, we added 500 mg of saponin (Sigma Chemical Co., St. Louis, MO) dissolved in 5 mL of distilled water. This was mixed well, then centrifuged for 10 min at 3000 x g. The “low control” hemolysed blood in the supernate was transferred to pediatric HbCO sample tubes (see below) with a syringe. For the “high control” sample, we mixed 0.6 mL of CO gas (purity 999 mL/L) with 100 mL of the hemolysate supernate (total hemoglobin concentration approx. 126 g/L) in a glass syringe. After 2 h of incubation, with periodic

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4 Nonstandard abbreviations: HbCO, carboxyhemoglobin; GC, gas chromatography; MCA, multicomponent analysis spectrophotometry; CvHb, the total concentration of hemoglobin in blood.

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mixings, we purged the hemolysate three times for 15 min each with 100 mL of nitrogen gas. We then transferred aliquots of this blood to pediatric sample tubes. These controls were stored in the dark at 4 °C and analyzed at weekly intervals.

The sample tube originally described (7) had too small a diameter for effective routine mixing. Therefore, we now used a standard-size tube measuring 155 × 3.0 mm (o.d.) × 1.7 mm (i.d.) and 360 μL of sample when sufficient blood is available to fill the tube. We also used a pediatric sample tube (76 mm × 3.0 mm (o.d.) × 1.7 mm (i.d.)) to collect and store smaller samples (up to 160 μL) of blood from neonates. We coat the interior of the standard-size and pediatric sample tubes with approximately 10 and 5 μL, respectively, of a solution containing 100 mg of saponin (Sigma) and 1000 USP units of sodium heparin (Sigma) per milliliter of distilled water. The tubes are positioned horizontally and the water is evaporated, either at room temperature or in an oven at 50 °C.

The saponin/heparin-treated tubes are filled with blood to within 2 cm of the top. The blood entry end is sealed with a "Critocap K" micro-Natelson tube closure (Fisher Scientific Co., Pittsburgh, PA). A 13 × 1.2 mm stainless-steel bar is inserted into the other end, which is then also sealed with a closure. Blood and reagent are thoroughly mixed by inverting the tube or shaking it horizontally at least 10 times until the contents become clear.

Gas-Chromatographic Analysis

The GC method for HbCO determination is performed as described elsewhere (7). Blood is incubated with K3Fe(CN)6 in a closed reactor at 0 °C. The liberated CO is separated from other volatile and reducing compounds on a standard molecular sieve column in the GC. The CO exiting the GC column enters the detector and passes through a heated (275 °C) mercuric oxide reaction bed to yield CO2 and Hg gas (10). The high molar absorptivity of Hg gas at 254 nm accounts for the sensitivity of the instrument.

Total-Hemoglobin Determination

Total hemoglobin (C+Hb) in the blood samples to be analyzed for HbCO by GC was determined with a cyanmethemoglobin kit (no. 525, Sigma) (11).

Calculations

For clinical purposes, concentrations of HbCO are expressed as a percentage, calculated as follows:

\[
\text{HbCO}(\%) = \frac{\text{vol CO}}{\text{vol CO}(\text{C+Hb} \times 1.368)} \times 100
\]

where "vol CO" is the milliliters of CO (under standard conditions of temperature, pressure, and dew point) bound to 1000 mL of blood, "C+Hb" is total concentration of the hemoglobin in blood (g/L), and 1.368 is the CO-binding capacity of hemoglobin in milliliters per gram (9).

Data are presented as mean ± SD.

Linearity Determination

We tested the linearity of the relation between sample volume and CO content as measured by the GC method for HbCO in the range of approximately 0.15 to 4.5% by pipetting 0.2 to 1.0 μL of low (0.75%) or high (4.5%) HbCO control samples into reaction vials and determining the CO content of each aliquot in nanoliters. The sample C+Hb concentration was the same (126 g/L) for both samples.

Sample Stability

The stability of samples for HbCO determination by GC was investigated by re-analyzing samples after at least two months of storage at 4 °C. In addition, we re-analyzed other samples after storage for six months at ambient temperatures or for three days at 40 °C.

Spectrophotometric MCA

MCA was used for determination of the five clinically relevant hemoglobin derivatives: deoxyhemoglobin, oxyhemoglobin, carboxyhemoglobin, methemoglobin (or hemoglobin), and sulfohemoglobin (5, 8).

With the help of a computer program, a combination of the five standard spectra of five pure hemoglobin derivatives was calculated, with which the measured absorption spectrum of the sample was compared. C+Hb was calculated by adding the concentrations of the five measured hemoglobin derivatives. The dyshemoglobins (carboxy-, met-, and sulfhemoglobin) were expressed as a percentage of C+Hb.

Results

Table 1 shows the results of analysis of blood samples by MCA and GC, grouped on the basis of clinically relevant HbCO concentrations. The 0–1.5% range represents the normal HbCO range; the other ranges represent different degrees of abnormal exposure to CO. Because the blood hemoglobin concentration is an integral part of the value obtained for % HbCO, the C+Hb values are also listed in Table 1. This also affords a comparison of the determination of blood C+Hb by MCA and the cyanmethemoglobin method. Figure 1 depicts the linearity of the GC method in the HbCO range below 4.5%. The amount of CO generated is directly proportional to the volume of pipetted blood. When 33 blood samples with HbCO concentrations up to 6.5% were re-analyzed after at least two months of storage at 4 °C, the mean HbCO concentration did not change significantly: 2.14 ± 1.69% vs 2.19 ± 1.68%. More extreme storage conditions, 40 °C for three days, did not significantly affect the pre- and post-treatment HbCO concentrations of 12 samples in the range of 0 to 5% HbCO (1.59 ± 1.59% vs 1.62 ± 1.47%, respectively). However, even though the % HbCO values were not changed by this treatment, this was the result of a proportional increase in both C+Hb (14.40 ± 10.8 vs 161.9 ± 25.4 g/L) and the CO content (3.0 ± 2.8 vs 3.3 ± 2.5 mL/L). This effect was not observed for the above samples stored at 4 °C or for samples stored at ambient temperatures for up to five months.

Discussion

Results of the method-comparison study show that the GC method correlates very well with the MCA method for the entire range of HbCO concentrations up to 30%. As Table 1 shows, the correlation coefficient (r) for different HbCO ranges is least (0.87) for the concentration range of 0 to 1.5%, which is the HbCO range for normal human adults. Further differentiation to the 0–1% range yields an r of 0.80, with a slope of 0.52 and a y-intercept of 0.32 (not shown). The decreased correlation could possibly be explained, in part, by the fact that the MCA method is based on five standard spectra, obtained by averaging spectral data from 10 to 20 different human subjects. Especially in the low HbCO range (<1.5%), scatter of the HbCO values, on the order of 0.1%, can be expected, owing to inter-subject variation in spectral absorptivity curves. However, it is
Table 1. HbCO Analysis by GC and MCA Compared for Various Ranges of HbCO Concentrations

<table>
<thead>
<tr>
<th>HbCO range, %</th>
<th>n</th>
<th>HbCO range, %</th>
<th>n</th>
<th>C*Hb</th>
<th>HbCO</th>
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<td></td>
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<tr>
<td>0&lt;HbCO&lt;1.5</td>
<td>29</td>
<td>145 ± 14</td>
<td>147 ± 14</td>
<td>0.97</td>
<td>0.96</td>
</tr>
<tr>
<td>1.5&lt;HbCO&lt;5.0</td>
<td>25</td>
<td>149 ± 13</td>
<td>152 ± 14</td>
<td>0.96</td>
<td>1.04</td>
</tr>
<tr>
<td>5&lt;HbCO&lt;10.0</td>
<td>19</td>
<td>152 ± 12</td>
<td>156 ± 12</td>
<td>0.95</td>
<td>0.88</td>
</tr>
<tr>
<td>HbCO&gt;10.0</td>
<td>22</td>
<td>138 ± 22</td>
<td>141 ± 18</td>
<td>0.89</td>
<td>0.75</td>
</tr>
<tr>
<td>HbCO&gt;15.0</td>
<td>95</td>
<td>146 ± 16</td>
<td>149 ± 16</td>
<td>0.93</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Fig. 1. Linearity test of HbCO measurements in blood by gas chromatography for samples with HbCO contents of 0.75 (C) and 4.5% (R). Each data point is the mean of three measurements. Sample volume routinely is 1 μL of blood

doubtful that further differentiation of values in the normal reference interval have pathophysiological meaning, though for some scientific studies accuracy in this range may be desirable.

Analysis for HbCO by the GC method of course depends, of course, on accurate measurement of C*Hb. However, we measured C*Hb with a small error, 2% (day-to-day variation, n = 56). The correlation between the two methods of determination in all ranges, except for HbCO values >10%, is also excellent. However, the cyanmethemoglobin method consistently yielded somewhat higher values (3.0 g/L or 2.0%).

The MCA method has been verified against a titrimetric method down to 1.0% HbCO (8). The linearity for the GC method at low HbCO concentrations is now also established for the range of 0.15 to 4.5% HbCO.

The effect of time and temperature on the stability of HbCO blood samples was also examined, particularly as these conditions relate to possible conditions during transport and storage of samples. We purposely elected to first analyze the fresh blood samples by spectrophotometry, followed by GC analysis after transport because values for samples analyzed by GC are stable (7). The results of the time- and temperature-stability study indicate that samples intended for HbCO analysis by GC are stable under most common conditions of transport and storage. Because the proportions of HbCO in blood samples remain stable over extended periods of time, blood can be used as control material. The prepared control samples in sample tubes and glass-sealed ampules were found to remain stable for HbCO at 4°C for at least five months. When these same samples were kept at ambient temperature, the HbCO increased slightly from 0.73 to 0.76% and from 4.56 to 4.61% for the low and high control samples, respectively, during five months. These control samples also showed spectrophotometric stability with respect to HbCO when measured by MCA. Therefore, it may be interesting to perform further investigations to determine whether these HbCO control samples are suitable as quality-control material in other clinical-chemical methods for measuring HbCO.

In clinical situations, HbCO is frequently determined spectrophotometrically by means of a commercial instrument, the IL-282 CO-Oximeter (Instrumentation Laboratory, Inc., Lexington, MA). However, especially for use with neonates, one should realize that falsely high HbCO values are measured when fetal hemoglobin is present (6). The cause of this artifact is that oxygenated and carboxygenated derivatives of fetal hemoglobin have slightly different absorption spectra as compared with hemoglobin of adults (12). This consideration may also apply to other spectrophotometric instruments for measurement of hemoglobin derivatives. In the MCA method, however, the entire absorption spectrum from 480 to 650 nm is used, so that these minor spectral differences are eliminated (9). The GC technique for HbCO is not affected by the presence of fetal hemoglobin, and the cyanmethemoglobin C*Hb method measured it without error.

In conclusion: this gas-chromatographic method for determination of HbCO concentrations in blood samples is sensitive, accurate, and reproducible. It may be the method of choice when proportions less than 1.5% HbCO must be measured accurately. The method's sensitivity makes it also particularly useful when only small blood samples are available. In addition, the stability of HbCO concentrations in the present sample containers obviates problems of shipping, and control samples can be easily prepared to aid in daily quality control.

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Oxybuprocaine and Five Metabolites Simultaneously Determined in Urine by Gas Chromatography and Gas Chromatography–Mass Spectrometry after Extraction with Extrelut®

Fumiyu Kasuya, Kazuo Igarashi, and Miyoshi Fukui

We describe a gas–liquid chromatographic (GC) method for determination of oxybuprocaine, and a gas chromatographic–mass spectrometric (GC-MS) method for simultaneous determination of four of its nine metabolites in urine. We used an Extrelut® column to simply and rapidly extract oxybuprocaine and its metabolites from urine. For the GC-MS analyses, we monitored the characteristic fragment ions at m/z 353, 395, 369, 411, and 235 for 3-butoxy-4-aminobenzoic acid (metabolite 2, M-2), 3-butoxy-4-aminobenzoic acid (M-3), 3-hydroxy-4-aminobenzoic acid (M-4), 3-hydroxy-4-aminobenzoic acid (M-5), and methaqualone (internal standard), respectively. We quantified the glucuronide of M-2 after enzymatic treatment. The assay's selectivity and reproducibility (within-day and between-day CVs <8% for all metabolites) make it applicable to determine oxybuprocaine and its metabolites in human urine. Mean 9-h urinary excretion of oxybuprocaine and its five metabolites from four healthy volunteers was 89.2% after a 100-mg oral dose.

Oxybuprocaine, a local anesthetic, is similar in structure to procaine. Its metabolism in humans is extensive, nine metabolites as well as the parent drug being detectable in urine (1).

Further to elucidate the metabolism of oxybuprocaine, we used GC1 and GC-MS to measure oxybuprocaine and its five principal metabolites in urine specimens from healthy volunteers who had received 100 mg of oxybuprocaine hydrochloride orally.

We also prepared the samples by passing them through 1-mL Extrelut® columns; this extraction generates no emulsions, and so speeds the procedure and provides a high clean-up efficiency (2–6).

Materials and Methods

Reagents: Oxybuprocaine (Santen Pharmaceutical Co. Ltd.) was obtained as the hydrochloride salt. Extrelut® (a wide-pore kieselguhr of grainy structure) was purchased from E. Merck, Darmstadt, F.R.G.; the derivatizing reagent for gas chromatography, N,O-bis(trimethylsilyl)acetamide (BSA) from Tokyo Kasei, Tokyo, Japan; and methaqualone from Eiwei Co., Tokyo, Japan. Other reagents and solvents were of A grade.

The authentic metabolites (1) of oxybuprocaine and procaine (7, 8) were prepared in the authors' laboratory.

Instrumentation: For the GC analyses we used a Shimadzu Model GC-1A chromatograph equipped with a hydrogen flame-ionization detector. The 1 m × 3 mm (i.d.) glass column was packed with 2% OV-101 on Chromosorb WHP (100–120 mesh; Hewlett-Packard, Avondale, PA). The column temperature was programmed from 180 to 220 °C at 10 °C/min, then held at 220 °C. The flow rate of the nitrogen carrier gas was 50 mL/min. The injection and the detector temperatures were both 250 °C.

We acquired mass spectra with a Hitachi M-5201 gas chromatograph coupled via a jet separator to a Hitachi M-60 mass spectrometer. The 1 m × 2 mm (i.d.) glass column was also packed with 2% OV-101 on Chromosorb WHP (100–120 mesh). The oven temperature was increased from 160 to 200 °C at 10 °C/min, then held at 200 °C. The injection and the interface temperatures were 230 and 260 °C, respectively. The flow rate of the helium carrier gas was 50 mL/min. The accelerating voltage was 3.0 kV; the ionizing current, 100 μA; and the ionizing potential, 20 eV. The magnet was scanned from m/z 160 to 430 in 8 s, with 1 s allowed for field stabilization between scans. Peak areas were calculated by a computer system, Hitachi 002B Datalizer.

Sample collection: Healthy volunteers were given an oral dose of 100 mg of oxybuprocaine hydrochloride, and urine collected at various intervals up to 9 h after the dose was stored at −20 °C until analyzed.

Pretreatment of the Extrelut column: We used 1-mL Extrelut columns. For assay of the parent drug, we washed the columns with diethyl ether/isopropanol (85/15 by vol). For the acidic metabolites, we loaded 1.0 mL of 0.1 mol/L HCl onto the columns before applying the samples; after 10 min, we eluted the acidic solution retained in the columns by washing with saturated sodium chloride solution. We then washed the columns with methanol, followed by diethyl ether/isopropanol (85/15 by vol).