Methylene Unit Calibration for Analysis of Organic Acids by Gas–Liquid
Chromatography/Mass Spectrometry

Irvin L. Bromberg, James A. Heininger,1 and A. George Cherlan2

We describe a method for automatic computation of
"methylene unit" retention-time indices in real time on a
computer-controlled gas chromatograph/mass spectrometer system. The calculation is based on the expo-
nential relationship between the methylene unit values of
hydrocarbon reference standards and the oven tempera-
ture at their elution. Using this mathematical relationship,
we calculated calibration factors, which eliminates the
need to include a set of methylene unit reference standards
with each run and simplifies and speeds identification of
unknown peaks in chromatograms. Long-term stability and
good precision of the calibration factors were observed
during the six-month period of study.

Additional Keyphrases: heritable disorders • calibration
factors • organic aciduria • urine • elution temperature
markers

Detection and identification of organic acids in those inborn
ersors of metabolism characterized by organic aciduria require
sophisticated instrumentation such as a gas–liquid chromato-
graph and mass spectrometer (GC/MS) combination (1, 2).
The task of characterizing peaks and estimating retention
time indices in organic acid analysis is facilitated by using
straight-chain aliphatic hydrocarbon reference standards,
known as "methylene units" (3–7). With the GC/MS system
the methylene-unit (MU) values of peaks of unknown com-
ounds may be used as indices in a library of mass spectra that
have been previously ranked by MU values, for rapid
searching and peak matching (4, 7).

To aid in determination of the MU indices, it has been
suggested that the MU standards be mixed with the speci-
men for chromatography (4, 5); by linear interpolation between
standards that elute on either side of unknown peaks, the
unknown MU indices may be estimated (3). However, this
has certain disadvantages when the chromatogram shows
many peaks, some of which may overlap with the methy-
lene standards.

To avoid this overlap, we used to run the standards and
the unknown separately, place the chromatograms side by side,
and compare them visually. Because this manual method lacks
precision, however, we modified the "Peak Normalized
Spectra" computer programs of the Hewlett-Packard 5992B
GC/MS system such that MU standards and unknowns are
inter-related by the oven temperature at elution.

Materials and Methods

The 5992B GC/MS system (Hewlett-Packard Co., Palo
Alto, CA 94304) was used for the study. The column was
packed with 3% OV-22 on Chromosorb WHP 80/100 mesh
(Chromatographic Specialties, Brockville, Ontario, Canada).
Ultra-high-purity helium, flow rate 20 mL/min, was the carrier
gas. The analytical conditions were: starting temperature =
70 °C, holding time = 2 min, solvent elution time = 4 min,
total run time = 25 min, programmed rate of temperature rise
= 8 °C/min, maximum temperature = 250 °C.

The series of even-numbered methylene units C4–C24
(straight-chain aliphatic hydrocarbons) was purchased as a
These were dissolved in hexane at a concentration of 200 mg/L
each. We injected 2 μL as the standard MU mix into the
GC/MS and chromatographed this under the conditions
described above.

Noting the oven temperature at the moment of elution and
detection of each peak, we plotted the correlation between MU
and temperature at elution and used non-linear regression to
determine the mathematical relationship between them. We
then tested whether the resulting regression parameters could be
reliably used as calibration constants to permit real-time
calculation of MU values of unknowns during a run without
including MU standards.

Having established the method, we collected data for six
months to observe the long-term stability of the calibration
constants and the temperature at elution of each MU. Finally
we compared the actual oven temperature (as measured by
the instrument's built-in oven probe) with a prediction de-
derived mathematically, and selected the method of calculation
best suited to this MU calibration technique.

Results and Discussion

We found a simple exponential relationship between MU
and temperature at elution (Figure 1). The equation may be
computed for any GC column by analyzing a series of meth-
ylene standards and by using least-squares fitting (after
natural logarithmic transformation of the MU axis) to
determine the two parameters that describe the curve—the
intercept and the exponential factor (see caption to Figure 1).
A second-degree polynomial (quadratic function) that fits
the calibration curve closely may also be computed, but analysis
of variance and testing by the F statistic indicate that the
exponential fit is superior. Computation is considerably
simpler for the exponential regression, where two parameters
describe the curve, than for the quadratic fit, which requires
three parameters.

The standard MU mix described was run about every two
weeks for six months. The coefficient of variation (CV, %) of
the temperature at elution of each MU was very small, as
shown in Table 1. The longer the hydrocarbon chain length,
the better the precision: the CV ranged from a maximum of
0.67% for MU no. 14, to a minimum of 0.41% for MU no. 24.
The values of the calibration parameters were correspondingly
very constant: the mean intercept was 6.639 (CV 0.55%), and
the mean exponential factor was 0.005290 (CV 0.20%).

Thus the curve is stable and the parameters calculated
during a calibration can be used by the system for several days.
The computer need only retain the two calibration constants
in memory and use them in a subroutine to calculate the MU

CLIN. CHEM. 28/2, 349–351 (1982)
values while an analysis is in progress. It suffices to repeat the calibration of the system once every 10–14 days (more often if starting with a newly packed column or a fresh tank of carrier gas), and to include only MU no. 20 as an external standard with each patient’s sample, to monitor for unusual drift. MU 20 eluted at a mean temperature of 208.2 °C (CV 0.45%) and its mean calculated MU value was 20.00 (CV 0.41%) (n = 12). Taking two standard deviations as action limits, a problem with an analysis should be suspected if MU 20 elutes at a temperature ±0.9% from the mean or if its calculated MU value differs from 20.00 by ±0.8%.

We noted that any drift affects all the MU in parallel, and that the precisions (CV) of all the calculated MU values were similar, ranging from 0.40% to 0.45%.

One may run an unknown from a starting temperature higher than that used during calibration and still relate the temperature at elution to the MU. For most peaks, except for those eluting very close to the starting temperature, the computed MU value will still be valid.

The oven temperature at any moment in the run can be predicted from the following expression:

\[ T = T_1 + (RT - HT) \times R \]

where \( T \) is the predicted temperature at the current retention time (RT), \( T_1 \) is the starting temperature, \( HT \) is the holding time (time spent holding at the starting temperature before the temperature increase begins), and \( R \) is the preprogrammed rate of oven temperature increase.

For 60 unselected peaks from calibration runs performed during the six-month period, linear least-squares regression showed a nearly perfect correlation between the predicted temperature and the actual oven temperature. The regression of predicted \( y \) vs actual \( x \) temperature gave the equation \( y = 0.00728 + 1.0005x \) \( (r = 0.9999) \). The actual temperature recorded by the oven probe is subject to slight moment-to-moment fluctuations in the cooling/heating thermal control, and its resolution is not better than 0.1 °C, being limited by its transducer and the analog-to-digital converter. These fluctuations could introduce slight imprecision into the calculated MU values; however, because a substance during its passage through the column is subjected to the integrated effects of the fluctuations, they will tend to cancel out. Therefore it is preferable to use the predicted temperature instead of the actual temperature to prepare the calibration curve and to compute the MU values of unknowns.

The overall relationship for calculating the methylene unit value of an unknown becomes:

\[ MU = k \cdot e^{RT_1 + (RT - HT)R} \]

where \( T_1, RT, HT, \) and \( R \) are as defined previously, MU is the methylene unit value result, \( k \) is the intercept of the calibration curve, \( e \) is the Naperian constant (2.7183), and \( F \) is the exponential factor from calibration.

Although our application concerns the analysis of organic acids, this technique could be used in a wide variety of GC procedures (with or without a mass spectrometer) in such fields as toxicology and therapeutic drug monitoring. Other series of molecular standards, such as carboxylic acids, might serve well as elution temperature markers in different applications. The gas chromatograph must be controlled by a computer that is user-programmable, so that the subroutines required to perform the regression and interpolation calculations can be added.

In conclusion, the exponential relationship between MU value and temperature at elution may be used to compute, on a computer-controlled gas–liquid chromatograph, the real time MU value without including a complete series of reference standards with each run. Certain changes to the chromatographic temperature conditions may be made without having to recalibrate. The result is improved analytical throughput and simpler interpretation of chromatograms. The parameters expressing the relationship are very stable over time.

### References


Radioimmunoassay for Hepatitis B Core Antigen

Evangelista Sagnelli,1,3 Carlos Pereira,1 Giovanni Triolo,1,3 Salvatore Vernace,2 and Florenzo Paronetto1,3

Serum hepatitis B core antigen (HBcAg) is an important marker of hepatitis B virus replication. We describe an easy, sensitive radioimmunoassay for determination of HBcAg in detergent-treated serum pellets containing Dane particles. Components of a commercial kit for anti-HBc (1) are used, and HBcAg is measured by competitive inhibition of binding of 125I-labeled antibodies to HBcAg with HBcAg-coated beads. We assayed for HBcAg in the sera of 49 patients with hepatitis B surface antigen (HBsAg)-positive chronic hepatitis, 50 patients with HBsAg-negative chronic hepatitis, and 30 healthy volunteers. HBcAg was detected in 60% of patients with HBsAg-positive chronic hepatitis but not in patients with HBsAg-negative chronic hepatitis. Patients with chronic Hepatitis B (an antigen closely associated with the core of Dane particles) determined in the same sera by radioimmunoassay, was not detected in 50% of HBcAg-positive sera.

Additional Keyphrases: immobilized reagents • “kit” methods • comparison with microsolid-phase radioimmunoassay

The intact hepatitis B virus, also referred to as Dane particles, is a 42-nm diameter structure seen in the sera of patients acutely or chronically infected with this virus; it is composed of an outer surface (containing hepatitis B surface antigen, HBsAg) and an inner core (containing hepatitis B core antigen, HBcAg).4 HBsAg is found not only on the outer surface of Dane particles but also in small serum particles (20-nm spheres and tubular particles) and in the cytoplasm of hepatocytes. HBcAg has been demonstrated in the core of Dane particles and in the nuclei of liver cells. The third antigen related to hepatitis B virus is hepatitis B e antigen (HBeAg), a soluble protein seen only in HBsAg-positive sera.

Studies of these three antigenic systems of the hepatitis B virus (HBsAg, HBcAg, and HBeAg) have added considerably to our knowledge of the pathogenesis of hepatitis (1). HBsAg and HBeAg can now be radioimmunoassayed routinely in the sera of patients. Because of the advantage of commercial kits, but determinations of HBcAg are still difficult, requiring the preparation and standardization of radiolabeled antisera.

HBcAg is a reliable marker of circulating hepatitis B virus, and its determination is important for clinical and epidemiological studies (2, 3). HBcAg in the sera of patients with chronic inactive hepatitis has been recently correlated with an unfavorable course of the disease (4).

As with HBeAg, which has recently been identified in the core of Dane particles (5, 6), HBcAg may also indicate infectivity. Heretofore, it has been detected mainly by solid-phase RIA, developed originally by Purcell et al. (7, 8). We describe here a simple and sensitive RIA for detecting HBcAg in the serum. Utilizing the sera of patients with chronic hepatitis, we compared the results of this method with those of the standard solid-phase RIA and correlated the results of both methods with the presence of HBeAg.

Materials and Methods

We used sera from 99 patients with biopsy-proven chronic hepatitis; 49 of these sera were HBsAg-positive. We also used sera from 30 healthy controls.

Procedures

Preparation of HBcAg-rich serum pellets. One milliliter of serum, diluted twofold with Ca2+ and Mg2+-free phosphate-buffered saline (Dulbecco’s; Gibco, Grand Island, NY 14072), was placed in 10-mL polycarbonate tubes (Nalge Co., Rochester, NY 14620). Dane particles were precipitated by centrifugation with a fixed-angle rotor (4 h, 39,000 × g, 4 °C). The supernate was aspirated with a Pasteur pipette, and the internal surface of the centrifuge tubes was promptly dried with sterile cotton swabs. Each pellet was then suspended in 8 mL of phosphate-buffered saline and centrifuged again, as before. The centrifuge tubes were then rinsed again with cotton swabs and the pellets resuspended in 50 µL of phosphate-buffered saline. This washing and thorough drying were intended to remove trace amounts of antiHBc that may have been present in the pellets obtained after the first centrifugation. Duplicate samples of pellets were prepared for analysis by RIA.

Solid-phase RIA. The solid-phase RIA for HBcAg has been extensively described in previous papers (4, 7). Briefly, a

---

1. Departments of Pathology1 and Medicine2 (Division of Liver Diseases), Mount Sinai School of Medicine of the City University of New York, and Immunopathology Laboratory, Veterans Administration Medical Center, Bronx, NY.
2. Address reprint requests to Dr. Paronetto at Laboratory Service, Veterans Administration Medical Center, 130 West Kingsbridge Rd., Bronx, NY 10468.
3. Nonstandard abbreviations: HBsAg, hepatitis B surface antigen; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; antiHBc, antibodies to hepatitis B core antigen; antiHBs, antibodies to hepatitis B surface antigen; RIA, radioimmunoassay.
4. Received May 21, 1981; accepted Nov. 24, 1981.