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Concentration Measurement by Proton NMR Using the ERETIC Method

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The ERETIC method (Electronic REference To access In vivo Concentrations) provides a reference signal, synthesized by an electronic device, which can be used for the determination of absolute concentrations. The results presented here demonstrate the accuracy and precision of the method in the case of ^{1}H high resolution NMR. Five tubes were filled with D_2O solutions of trimethylamine hydrochloride (TMA) 3.84 mM and sodium lactate at concentrations ranging from 5.25 to 54.11 mM. Results obtained with the ERETIC method were compared to those obtained by using TMA as an internal reference. The standard deviations were the same for the two methods and always lower than 1% of the mean. The accuracy (difference between true value and measured value) was slightly better for the ERETIC method than for the internal reference. No significant variation was observed when the experiments were performed over 56 h. Measurements were repeated once a month during three months. As the values obtained showed a standard deviation of only 3%, we can conclude that the ERETIC method has a good stability and only requires monthly calibration. Furthermore, it must be noted that nothing is added to the sample and that the reference signal frequency can be freely chosen to fall within a transparent region of the spectrum.

The determination of the quantity of material present in a given sample is of fundamental interest in many aspects of chemistry and biology. NMR is a powerful tool for this purpose because of the direct proportionality of signal intensity to the number of resonating nuclei. The quality of quantitative NMR is dependent on experimental conditions,[1] and many aspects of signal acquisition or data processing must be optimized to get good accuracy and precision.[2] Critically, a calibrated reference signal needs to be acquired in the spectrum simultaneously with the signal from the analytes. In high resolution NMR, this reference is provided by either a part of the sample spectrum or a spectral line from a compound added to the sample, the so-called internal reference. In the latter case, the more frequently used, the reference compound has to satisfy several constraints: it must be soluble in the sample, chemical interaction with the sample and overlap between sample and reference lines must be avoided, and the longitudinal relaxation time (T1) must be close to or smaller than that of the sample because the recovery time, thus the experimental duration, is determined by the longer T1. Hence, there are many situations in which it is very difficult to find the ideal reference compound.

We propose an alternative to the internal chemical reference based on a calibrated reference signal which is not a real NMR line but an NMR-like, electronically produced signal. This approach applies the ERETIC method (Electronic REference To access In vivo Concentrations), which has already been proposed for assessing metabolite concentrations in vivo.[3,4] In ERETIC, nothing is added to the sample and the reference line can be freely positioned in a transparent region of the spectrum. Thus, all of the drawbacks of the internal reference method cited above are avoided and the recovery of the uncontaminated sample for further analysis is easy.

EXPERIMENTAL SECTION

The ERETIC Method. The ERETIC method provides a reference signal, synthesized by an electronic device, which can be calibrated against absolute concentrations. The reference signal provides a pseudo-FID that has all of the characteristics of a real NMR signal and whose parameters (frequency, magnitude, phase, T2) are controlled from the spectrometer console.[3,4] The pseudo-FID was produced by multiplication of an exponentially decreasing signal (low frequency component) and a sinusoidal signal at the observed frequency (high frequency component) (Figure 1). The high frequency component was provided by the second channel of the spectrometer. It was derived after the frequency modulation and before the amplifier. The frequency, the magnitude, and the phase of this signal are therefore freely chosen by the operator as decoupler parameters. The low frequency component was produced by a capacitive network operating with a square wave input. The time constant of the network was modified in order to match the ERETIC line width to the desired value. The square wave signal used was a trigger present on our spectrometer and reflecting the transmitter status (high level when the second channel was transmitting). It was derived from the frequency

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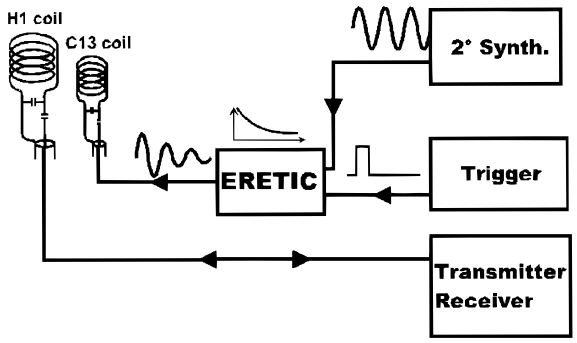

Figure 1. Material configuration used for the ERETIC method. The electronic device (labeled ERETIC on the figure) is connected to a trigger signal and the second radio frequency channel of the spectrometer. The pseudo-FID is transmitted by the coil tuned to the carbon frequency.

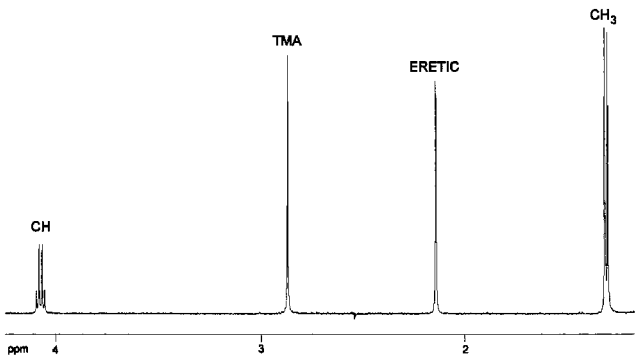

Figure 2. Typical spectrum of a solution of sodium lactate 25.55 mM and trimethylamine hydrochloride (TMA) 3.84 mM with the ERETIC reference peak.

synthesis card, and its onset and duration are determined by the pulse program.

After mixing of the two components, the pseudo-FID was transmitted through the carbon coil which was not tuned at the operating frequency (here the proton frequency) and acted therefore as a broad band antenna for the ERETIC signal.

After calibration of the ERETIC peak, the concentration of any compound can be simply measured by

$$[\text{Comp}] = k[\text{ERETIC}]\, A_{\text{Comp}}/A_{\text{ERETIC}} \qquad (1)$$

where k takes into account the number of protons per chemical group, A_{comp} is the area of the peak to be quantified, A_{ERETIC} is the area of the ERETIC peak, [Comp] is the concentration of analyte, and [ERETIC] is the equivalent concentration of the ERETIC line determined after a calibration acquisition by the expression

$$[\text{ERETIC}] = [\text{REF}]\, A_{\text{ERETIC}}/A_{\text{REF}} \qquad (2)$$

where [REF] is the concentration of a calibration solution and A_{REF} is the area of the calibration peak.

Sample Preparation. Five tubes 5 mm in diameter were filled with 500 μL of D_2O solutions of trimethylamine hydrochloride (TMA) 3.84 mM and sodium lactate at concentrations ranging from 5.25 to 54.11 mM. A solution of TMA was prepared by dissolving 10.90 mg of TMA in 29.70 mL of D_2O. Final solutions were prepared by dissolving the appropriate amount of sodium lactate (5.77, 5.80, 8.71, 13.74, 18.11 mg, respectively) in 9.80, 4.74, 4.82, 4.80, and 2.99 mL, respectively, of the TMA solution. To obtain a better accuracy, D_2O was weighed and volumes were calculated from D_2O density (1.105 kg^{-1}) assuming that dissolution did not induce significant variations in volume.

Trimethylamine hydrochloride and DL-lactic acid sodium salt were purchased from Fluka Chemika, Buchs, Swizerland. D_2O was purchased from Euriso-top, Saclay, France.

NMR Spectra. All experiments were performed on a DRX 500 Bruker spectrometer. A dual probe ($^1H/^{13}C$) was used. The proton channel was used for transmitting rf pulses and receiving. Fully relaxed spectra were acquired with the following parameters: flip angle 90°, repetition time 20 s, SW = 3000 Hz, SI = 16K, NS = 40, RG = 1. An exponential multiplication was applied to the FID inducing a line broadening of 1 Hz. No baseline correction was used.

Spectra were processed in the frequency domain using the Interliss software[5] (Eurofins Scientific, Nantes, France).

The T1 values of lactate and TMA were determined by using an inversion recovery sequence[6] with nine inversion time values ranging from 5 ms to 10 s and by using the T1 calculation software of the spectrometer.

Determination of the Concentrations. Calibration of the ERETIC peak was performed using eq 2 and from acquisitions with the tube (E) containing the highest lactate concentration (54.11 mM). The ERETIC peak area was compared with the area of the methyl line of lactate.

Determination of the lactate concentrations was done for each tube using eq 1 and the methyl resonance of the lactate. For the tube E, the spectra used were different from those used for calibration of the ERETIC peak.

For each spectrum (Figure 2), the lactate concentration was determined by the ERETIC method and by using TMA as an internal reference.

Precision and Accuracy. For each measurement, five spectra were acquired in order to calculate the precision (δ) with the standard deviation. Accuracy was evaluated by the parameter Δ defined as follows:

$$\Delta = \frac{C_{\text{meas}} - C_{\text{th}}}{C_{\text{th}}} \times 100 \qquad (3)$$

where C_{meas} is the measured lactate concentration and C_{th} is the true concentration.

In order to evaluate the ERETIC signal stability, 14 acquisitions were performed over a period of 56 h. Furthermore, measurements on the five tubes were repeated once a month during three months.

RESULTS AND DISCUSSION

T1 Measurements. The value found for TMA was 2.9 s and did not depend significantly on the lactate concentration. The T1 of lactate ranged from 2.2 to 4.4 s for the CH group and from 1.8

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Table 1. Accuracy Δ and Precision δ of the Lactate Concentration (mM) Determination by the ERETIC Method and by the Use of TMA as Internal Reference

tube	true concn (mM)	[lactate] ± δ (mM)		Δ (mM)	
		ERETIC	TMA	ERETIC	TMA
A	5.25	5.19 ± 0.05	5.46 ± 0.05	−0.06	0.21
B	10.92	11.01 ± 0.07	10.88 ± 0.05	0.04	−0.04
C	16.13	16.27 ± 0.06	16.56 ± 0.15	0.14	0.43
D	25.55	25.74 ± 0.14	25.51 ± 0.06	0.09	−0.14
E	54.11	54.55 ± 0.20	53.84 ± 0.22	0.23	−0.27

to 2.2 s for the CH_3 group. The T1 values of the lactate groups decreased with increasing concentration.

These results show that the proton longitudinal relaxation time of TMA is of the same order as that of the lactate groups. The line from the CH_3 group was used as this has the smaller T1 and gives the higher signal-to-noise ratio. With the values found for T1 and taking into account a repetition time of 20 s, we can be sure that proton spectra were fully relaxed for TMA and the lactate methyl group for all concentrations measured.

Measuring Conditions. Flip angle (90°) and repetition time are not the best measuring conditions in term of signal-to-noise ratio. However, Cookson and Smith[7] have shown that, for high accuracy, optimal signal-to-noise ratio is always achieved by using large pulse angles of 70−90° rather than small pulse angles. The spectral width (3000 Hz) and the number of sampling points (16K) induced a sampling period of 2.73 s. Taking into account the line broadening, these parameters were sufficient to avoid any truncation of the FID. Measuring conditions were therefore matched to quantitative analysis.

Determination of the Concentrations. Lactate concentrations determined by the ERETIC method and with TMA as an internal reference method are summarized in Table 1. Δ was calculated using eq 3 and δ is the standard deviation.

The precision (δ) was not significantly different for the two methods and was always lower than 1% of the mean. The accuracy (Δ) was slightly better for the ERETIC method than for the internal reference.

Calibration of the ERETIC peak was achieved using acquisitions performed with the tube containing the highest lactate concentration (54.11 mM); however, when another tube was used, the accuracy was not significantly different (results not shown).

Figure 3 shows the evolution with the time of the peak area for the ERETIC signal and the signal from the methyl group of lactate and the TMA line. It should be noted that the biggest variation was obtained for the TMA signal. During this period of time the standard deviation of the ratios I_{CH3}/I_{ERETIC} and I_{CH3}/I_{TMA} were calculated. The former was 0.76% of the average value while the latter was 1.68% of the average value.

These results clearly demonstrate that the ERETIC method gives at a minimum the same precision and accuracy as the use of an internal reference such as TMA. Experiments performed over 56 h show a greater stability for the ERETIC signal than for the TMA line. This could be explained by a lower sensitivity of the ERETIC signal to variation in coil tuning. The probe we used contains two coils. The first was tuned at the proton frequency and was used for receiving both NMR and ERETIC signals and for transmitting rf excitation. The second was the coil regularly assigned to the carbon observation. Its tuning was not modified, and it was used for transmitting the ERETIC signal. The ERETIC is therefore transmitted by a coil (the carbon coil for experiments described here) which is not tuned at its operating frequency (here the proton frequency). Thus, during emission, ERETIC is not affected by the probe tuning, and during reception both ERETIC and NMR signals are reduced by the same factor by variation in coil loading. It must be noted that, because of the latter point, calibration and measurements have to be performed with the same coil loading and so with the same solvent and the same tube size.

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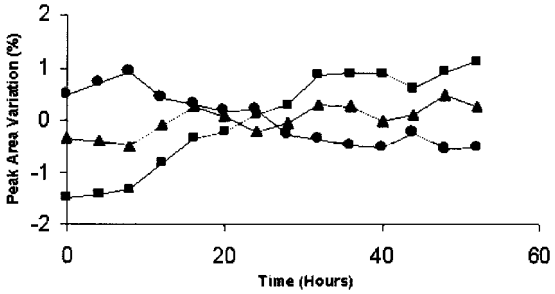

Figure 3. Evolution with time (in hours) of the peak area as a percentage of the average value. ● ERETIC, ▲ TMA, and ■ CH_3 of lactate.

Table 2. Stability in Time of the Determination of the Lactate Concentrations (As Defined in the Experimental Section) Determined by the ERETIC Method for Two Sets of Experiments (M and M+1) Separated by One Month[a]

tube	true concn (mM)	M (mM)	M+1 (mM)	
		Cal/M	Cal/M	Cal/(M + 1)
A	5.25	−1.15	−2.84	0.90
B	10.92	0.79	−3.09	0.63
C	16.13	0.86	−3.01	0.72
D	25.55	0.72	−3.97	−0.28
E	54.11	0.81	−4.46	−0.79

[a] The calibration of the ERETIC signal was achieved using the signal of tube E in the set M (Cal/M) or in the set M+1 (Cal/M+1). Values listed are accuracies calculated using eq 3.

A small variation of the area of the ERETIC signal with time was observed. This variation probably originates from instability of the spectrometer over period of acquisition. Spurious signals produced by the electronic device generating ERETIC could also be a contributing factor. This latter point is currently under investigation. However, the maximum variability in the ERETIC signal area is significantly inferior to the overall precision of the method (Figure 3).

To evaluate long-term stability in time, measurements were repeated once a month during three months. The standard deviation of measured concentrations was 3% over this period.

Typical values of the accuracy are given in Table 2 for two sets of measurements (M and M + 1) separated in time by one month. Concentrations were obtained by the ERETIC method as described in the Experimental Section. In order to evaluate the stability in time of the ERETIC signal, concentrations were calculated in two ways for the set M + 1: first, by using the

equivalent concentration of the ERETIC signal determined one month sooner (Cal/M column) and second, by recalibrating the ERETIC signal using eq 2 and an acquisition performed on the tube E in the set M + 1 (Cal/(M + 1) column). Table 2 shows that Δ was enhanced approximately three-fold when the calibration of the ERETIC signal was not repeated. It can be concluded that, when an accuracy of only a few percent is needed, adjustment of the ERETIC signal can be done with a periodicity of several months. However, for an accuracy of one percent or better, ERETIC must be calibrated at least monthly.

CONCLUSION

The results presented here demonstrate that the ERETIC method, in the concentration range explored, has an efficiency (precision and accuracy) which is the same or better than that obtained in the same conditions with an internal reference, provided that the ERETIC signal is calibrated regularly. This efficiency is achieved without addition of any substance to the sample. This major advantage simplifies sample preparation, allows the recovery of the uncontaminated sample for further analysis, and avoids chemical interaction with the sample. No overlap between sample and reference lines can occur because all of the parameters of the ERETIC line can be freely chosen. Furthermore, in the case of the internal reference method, it is essential to choose a reference compound with a relaxation time near to or smaller than that of the sample, otherwise the repetition time, and thus the experimental duration, must be increased. This drawback is also eliminated by the use of the ERETIC method.

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