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Quantitative ^{1}H-NMR analysis of amniotic fluid

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Abstract: Ten amniotic fluid samples (36–38 weeks gestation) are analysed by NMR spectroscopy. Of the species identified in the spectra, valine (mean 198 μM: SEM 57 μM), lactate (9.73 mM; 2.05 mM), alanine (689 μM: 115 μM), acetate (6.87 mM: 1.54 mM), citrate (363 μM: 59 μM), glucose (4.54 mM: 1.28 mM) indoxyl-sulphate (n = 4, 270 μM), histidine (n = 6, 125 μM: 31 μM) and formate (n = 4, 92 μM) are quantified using standard addition. The factors governing the detection limits and lowest quantifiable amounts are discussed as are the extension of the work into *in vivo* magnetic resonance spectroscopy (MRS) in the clinic.

Keywords: *NMR; quantitation; amniotic fluid.*

Introduction

Proton magnetic resonance spectroscopy (^{1}H-MRS) of whole bodies is becoming more common with the advent of wide-bore, high-field spectrometers and efficient water suppression sequences. The major application of this technique is in the *in situ* analysis of malignant tissue [1] and the brain [2]. However, it is, in principle, applicable to any large homogeneous volume in the body such as amniotic fluid in the womb. In turn MRS analysis offers the ability for repeated analysis in a non-invasive manner, and could be of future importance in monitoring pregnancy assuming ethical approval were to become routinely available.

The approach outlined above will have an impact on clinical MRS only if spectroscopy can be used to both speciate and quantitate a number of key compounds in amniotic fluid which can act as predictive markers for certain disease conditions, for example, diabetes, pre-eclampsia and foetal kidney malfunction [3]. As such, we have made a preliminary study of the ^{1}H-NMR spectroscopy of isolated amniotic fluid.

Experimental

Amniotic fluid was obtained at Caesarean section (36–38 weeks) from 10 patients with healthy pregnancies. The fluid was centrifuged (1500 rpm, 5 min) to remove any cells or heavy debris. A 5.0 ml volume of fluid was then removed and freeze-dried. Samples were stored at −20°C until analysis commenced. Lyophilized samples were then resuspended in 0.5 ml of deuterium oxide and placed in a 5 mm diameter NMR tube. This procedure allowed us to pre-concentrate the sample (×10) and introduce a deuterium lock for the spectrometer.

^{1}H-Spectra were obtained using a Bruker AMX400 operating at 400.14 MHz. Samples were maintained at 20°C during spectral acquisition. The water resonance was suppressed by pre-saturation (58 dB) at the water frequency for 2 s. This also allowed sufficient time for complete (T_1) relaxation which is necessary for accurate quantitation. The free induction decay was generated by a 12.5 μs pulse width (90°). Each data set consisted of 800 scans collected in 32 k of memory zero filled to 64 k. Total data accumulation time inclusive of presaturation was 35 min. A 1 Hz line broadening function was applied before Fourier transformation.

Quantitation of the NMR spectra was achieved by three standard additions of known concentration (10 μl, 0.354 mM) of acetate or alanine directly into the analytical solutions. The key resonances in the spectrum were integrated before and after each addition of the

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internal standard. The calibration curve provides the concentration of the primary compounds, acetate or alanine. By compensating for the number of equivalent hydrogens associated with the functional groups it is possible to quantitate the other important species in the spectrum by direct comparison of the integrals of the other major resonances.

Results and Discussion

The ^{1}H-NMR spectrum of 10-fold concentrated amniotic fluid re-dissolved in ^2H_2O is shown in Fig. 1. The assignment of the resonances was made by the addition of the relevant reference materials directly into the samples and follow a previous study on amniotic fluid [3]. The low field resonances (6–8 ppm) are also observed in NMR spectrum of human urine [4]. Thus these resonances can be used as markers for foetal renal output. Initially the resolution of MRS will not be as good as the NMR experiments presented here and as such we should expect to observe only a small subset of the species, i.e. those with a relatively high concentration, those with a large number of protons in identical magnetic environments and those that occur in regions of the spectrum which are not crowded.

It is possible to quantify a number of species in the spectrum using standard addition. The detection limit (3 × signal to baseline noise) and the lowest quantifiable concentration are estimated to be 16 and 40 μM, respectively. There are a number of important caveats to these values. In this study, each is based on the

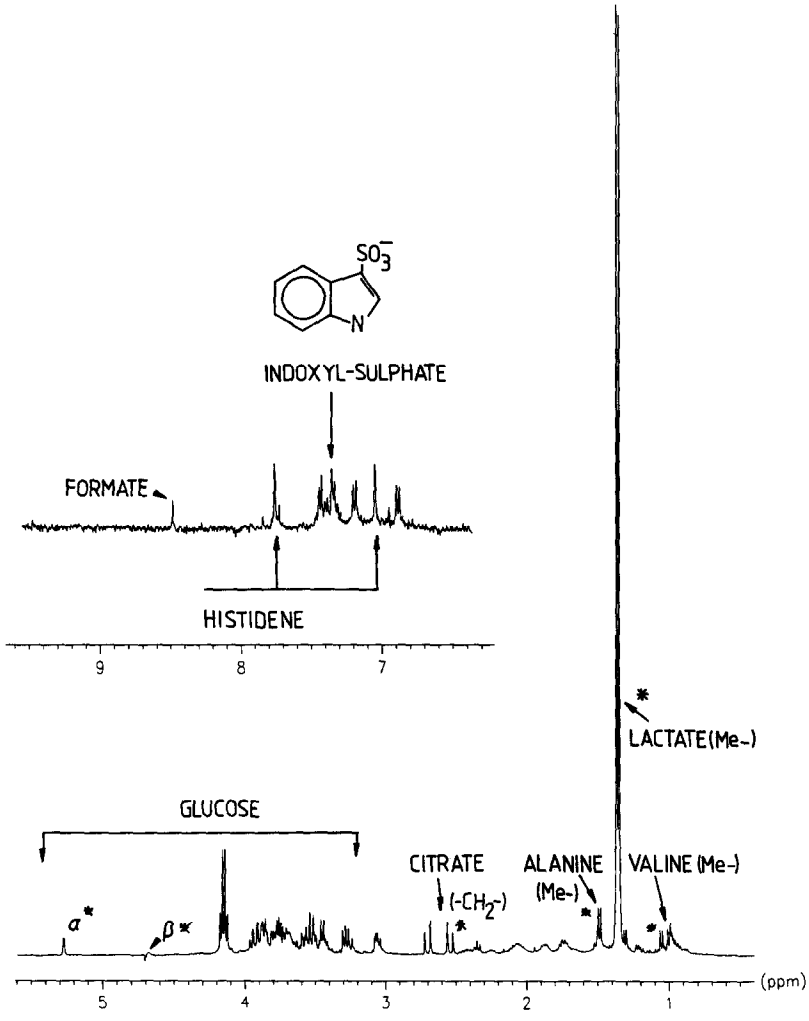

Figure 1
The 400 MHz ^{1}H-NMR spectrum of human amniotic fluid collected at Caesarean section (weeks 36–38). The acquisiton parameters are described in the Experimental section. The assignments of the resonance follow previous studies on biological fluids [3, 4]. The resonances identified (*) were those used for the respective quantitations.

resonance being a singlet which is equivalent to a single resonance corresponding to an individual proton (e.g. formate $HCOO^-$). If the resonance is split into a doublet or higher multiplet the detection limit will rise. Alternatively, if there is more than a single proton associated with the resonance (e.g. methyl-) the detection limit will fall. As such the speciation of the resonances play a significant role in the potential for quantitation.

The detection limit and lowest quantifiable amount are determined not just by the analyte concentration but by how many scans are accumulated to form the data set. Any improvement in signal-to-noise should be expected to be roughly proportional to the square route of the number of scans. It is also possible to increase the signal to noise at the expense of resolution by using a line broadening function during Fourier transformation.

The amniotic fluids used in this study are all expected to have similar ionic strength, viscosity and protein content. However, great variation is expected between other biological samples (e.g. erythrocytes, urine, plasma) and the detection limit and lowest quantifiable amounts will vary between samples.

Figure 2 shows the quantitative analysis (mean and standard error of the mean, SEM) of valine, lactate, alanine, citrate, glucose and acetate in 10 patient samples. Where available published values (mean and SEM) derived from other studies are also shown [5]. Except for the two amino acids alanine and valine, the NMR approach is in good agreement with these values. The valine and alanine resonance occur in a region of the spectrum which is coincident with broad resonances from lipoproteins (HDL, VDL and LDL) [6] which are present in low concentrations. The presence of the broad lipo-proteins resonances will contribute to an increase in the integrals of the alanine and valine methyl-resonances leading to a higher than expected value of their concentration in the fluid. The standard deviation in the measurements is small (1.5%) compared to the standard error in the data and as such, quantitation by NMR is a viable prospect for biological solutions such as amniotic fluid.

Renal function is normally measured by the plasma concentration of urea, or creatinine. Urea is not visible in Fig. 1 due to exchange with the deuterium in the solvent, although this problem does not exist for *in vivo* MRS, spin

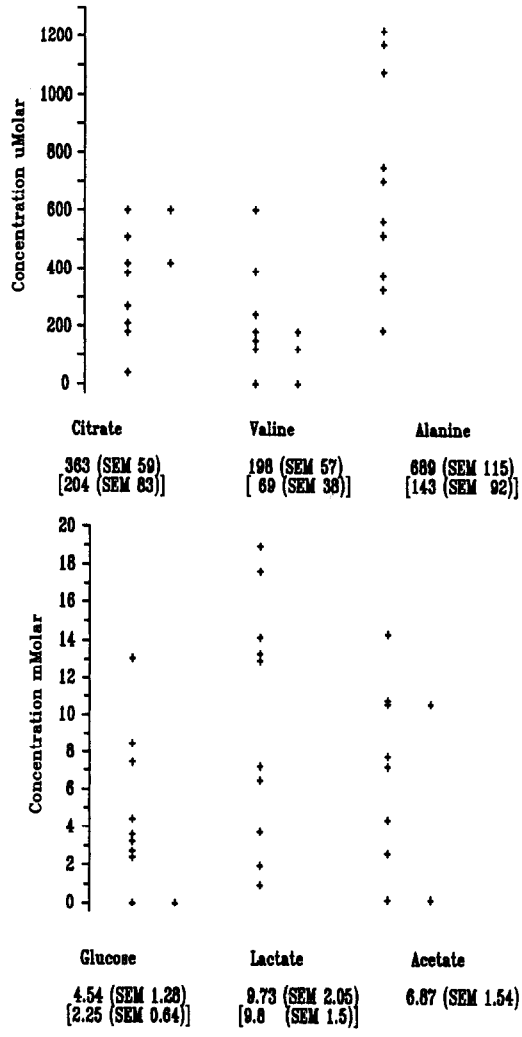

Figure 2
Quantitative data derived from 10 amniotic fluid samples. The data presented show the spread in the values. The mean and standard deviations are quoted as are the expected literature values (square brackets) for lactate, valine, alanine, citrate and glucose [5].

transfer during the water suppression would be expected to broaden the lines and reduce NMR visibility. Creatinine occurs in a region of the spectrum (3.1, 4.1 ppm) occupied by the plethora of glucose resonances. Using NMR, renal function may be best assessed through a combination of indoxyl-sulphate, histidine or formate (Fig. 1), species which are routinely observed in the ^{1}H-NMR spectra of urine [4]. Formate was observed in nine of the spectra. However, only in four was it present in sufficient amounts to quantify (mean 92 μM). Similarly, histidine is assessed through two resonances which can be observed in the aromatic region (Fig. 1) being detected in seven samples and quantifiable in six (mean

162 μM: SEM 31 μM). The lowest quantifiable amount of indoxyl-sulphate is complicated by the fact that it is assessed via singlet resonance in a crowded region of the spectrum. Its detection limit is estimated to be 30 μM (n = 5) with its lowest quantifiable amount being 60 μM (n = 4, mean 270 μM).

MRS should be able to detect, but probably not quantify indoxyl-sulphate, histidine or formate, and should therefore, be expected to assess kidney function *in vivo* through these species. MRS is also capable of monitoring glucose quantitatively. Although *in vitro*, NMR can distinguish between the alpha (4.4 ppm) and beta (5.2 ppm) anomers of glucose (Fig. 1), these will anomerise quickly, after collection, to an equilibrium position. Using *in vivo* MRS, however, total glucose and the relative concentration of the alpha and beta anomers can be measured as a function of metabolism and subsequently may have some importance in diabetic pregnancy.

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