ON THE USE OF 13-C LABELLED ANHYDRIDES AS CHEMICAL PRECURSORS OF SHORT CHAIN FATTY ACIDS FOR DNP-MRS.

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INTRODUCTION *In vivo* Magnetic Resonance Spectroscopy (MRS) combined to Dynamic Nuclear Polarization (DNP) has been shown to be a powerful tool for assessing metabolic alterations associated to the onset and progression of cancers [1] and cardiovascular diseases [2]. Being short chain fatty acids, *e.g.* acetate and butyrate, preferred substrates for the heart, particularly when oxidative metabolism is increased, these compounds have a great potential as metabolic reporters in cardiac applications. Unfortunately their typical low-temperature physical state have required, until now, the use of glass forming additives for obtaining preparations of butyric and acetic acids suitable for DNP procedures [3], with clear drawbacks in terms of maximum achievable concentration and metabolic interference. Here we demonstrate that the need of introducing a vitrifying agent can be overcome by exploiting symmetric anhydrides as DNP chemical precursors of said acids and we propose the use of anhydride-specific lipophilic radicals as an additional mean for achieving hyperpolarized preparations containing only the metabolic substrate of interest.

METHODS *Materials*: The following substrates have been analyzed and compared: [1,1'-$^{13}C_2$]-butyric anhydride; [1,1'-$^{13}C_2$]-acetic anhydride; [1-^{13}C]-butyric acid / dimethyl sulfoxide (DMSO) solutions at various relative concentrations; [1-^{13}C]-acetic acid / DMSO solutions at various relative concentrations. Finland trityl radical, tris(8-carboxy-2,2,6,6-tetramethylbenzo[1,2-d:4,5-d′]-bis(1,3)dithiol-4-yl) methyl (acid form) has been used as polarizing agent (PA) for the acid solutions; Finland trityl methyl ester, tris(8-methoxycarbonyl-2,2,6,6-tetramethylbenzo[1,2-d:4,5-d′]-bis(1,3)dithiol-4-yl) (ester form) was used as PA for anhydrides. *Glass forming test*: Glass versus crystal formation was checked by visual inspection after flash freezing of small drops into liquid nitrogen. *Polarization build-up curves:* The build-up curve of the hyperpolarized signal for the 4 compounds doped with trityl radicals was monitored by acquiring a ^{13}C solid state MR spectrum generated by a radiofrequency pulse with a flip angle of 3° every 300 s. The polarization time (T_{pol}) was evaluated by fitting the build up curve with a pseudo-exponential law that takes into account the artificial reduction introduced by the RF pulses themselves. Solid state polarization levels were evaluated by comparison with a reference sample. *Dissolution procedure*: Dissolution of samples based on acetic (or butyric) acid was performed with a phosphate buffer added with calibrated amounts of ethylenediaminetetraacetic acid (EDTA) and NaOH to obtain a neutral solution. Anhydrides were first dissolved with a strong basic buffer to allow a fast hydrolysis and then transferred into a syringe containing a neutralization solution made by phosphate buffer and HCl. *Liquid state relaxation curves*: The decay of the hyperpolarized signal after dissolution was monitored by acquiring a series of ^{13}C MR spectra with flip angle = 5° separated by a delay of 3 s. The longitudinal relaxation time T_1 was estimated by fitting the data to an exponentially decay law. Liquid state NMR signal enhancement was then determined as ratio between the integrated area of the hyperpolarized signal over the thermal equilibrium signal.

RESULTS The addition of 20 – 50 % (33 – 66 %) in volume of DMSO was found to be mandatory in order for butyric (acetic) acid to form a glass upon sudden freezing at 77 K whether, remarkably, butyric and acetic anhydride do vitrify as neat compounds. The maximum achievable polarization at solid state (P_{max}) and built-up time (T_{pol}) of butyric acid formulations are not substantially affected by the DMSO / butyric acid ratio as long as the DMSO content is between 20 and 33 % of the whole formulation volume. Conversely, the DNP behaviour of acetic acid is more markedly affected by DMSO and reaches optimal levels at a ratio 1 / 1 (acid / DMSO). The PA concentration (c) dependence of P_{max} and T_{pol} is reported in Figure 1 for butyric anhydride and for a butyric acid formulation containing 20 % of DMSO. For both butyric anhydride and acid formulations, P_{max} goes through a smooth maximum at c ≈ 10 mM, whereas T_{pol} decreases monotonically as a function of c. In the light of the moderate loss of ^{13}C polarization and of the substantial reduction in polarization time observed on varying c between 10 and 12.5 mM, this latter PA concentration can be likely considered as an optimal trade-off between efficacy and efficiency of the DNP procedure. Analogous results in terms of optimal radical concentration were found for acetic acid/anhydride. A summary of DNP parameters characterizing the two anhydrides and the two acids / DMSO formulations, all at c = 12.5 mM, is provided in Table 1. In order to obtain an injectable solution of the desired short-chain fatty acids, the solid state preparations have been rapidly dissolved with a basic dissolution medium, which assured a complete hydrolysis of both anhydrides in less than 10 s. Moreover, being the esterified PA poorly soluble in such aqueous solution, it precipitates upon dissolution and can be filtered out to increase tolerability of the final formulation. The obtained liquid state initial polarizations (P_{max-LS}) measured after 30 s of handling time are shown in Table 1 and are regarded as being promising for future clinical applications. In most compounds, the loss of polarization observed upon sample transfer from solid to the liquid state can be basically accounted for by low field longitudinal relaxation during transfer time.

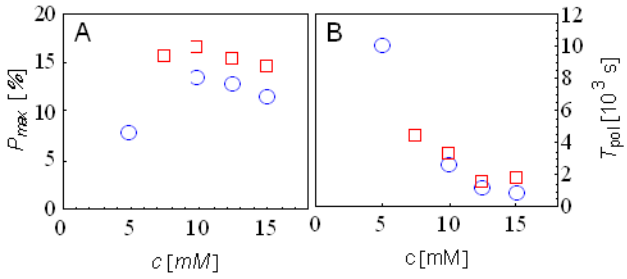

Figure 1. End-polarization (A) and polarization build-up time (B) of butyric anhydride (circles) and butyric acid added with 20 % of DMSO (squares) versus PA concentration.

Compound	DMSO %	T_{pol} (s)	P_{max} (%)	P_{max-LS} (%)
Butyric anhydride	0	1100	13	7
Butyric acid	20	1600	16	10
Acetic anhydride	0	675	7	4
Acetic acid	50	1100	7	3

Table 1. Build-up time, polarization at solid and liquid state of optimized formulations at PA concentration = 12.5 mM.

CONCLUSIONS: Pure hyperpolarized solutions (free of radicals and glass-forming agents) of butyric and acetic acid for metabolic imaging purposes can be obtained starting from the relevant symmetric anhydrides added with proper lipophilic radicals. The concept of using chemical precursors to overcome critical steps in the process of hyperpolarization, such as the achievement of a solid - highly concentrated - glassy solution upon flash freezing, is easily broadened and promise to solve several challenges in the formulation of metabolic probes.

REFERENCES: **1.** Golman, et al. Cancer Res 2006:66(22) **2.** Golman, et al. MRM 2008:59 **3.** Jensen et al. J Biol Chem 2009:284(52)