High Resolution ^{1}H-NMR Spectroscopy of Brain Extracts: Chloroform/Methanol Extraction is Superior to Perchloric Acid

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Introduction

Cell and tissue extraction is an important and widely-used technique in NMR spectroscopy studies of many disease states. A number of different extraction techniques exist for this purpose but the most ubiquitous is the perchloric acid (PCA) method which extracts the water-soluble metabolites.

A less widely-used technique is the methanol-chloroform-water extraction (M/C) which facilitates the simultaneous extraction of both the water-soluble metabolites and the organic-soluble lipid components from the same tissue sample. *In vivo* proton NMR spectroscopy has shown significant changes in lipids as well as the amino acids in many different diseases such as multiple sclerosis and high-grade brain tumours (1,2). Therefore, it may be very useful to extract and investigate both pools of metabolites *in vitro* as well.

The important requirements of an extraction technique are that it is capable of extracting a reasonably high amount of the total tissue metabolites (efficiency) and that it is highly reproducible (low variability). It is well established that PCA extraction fulfills these criteria for the water-soluble metabolites (3) and the M/C extraction fulfills these criteria for lipids (4). However, the M/C extraction has not yet been established as a reliable technique for proton NMR spectroscopy of the water-soluble metabolites. In this study we have compared the quantitative aqueous metabolite yields and protein pellet content for both the PCA and M/C extracts on rat brain tissue.

Methods

Funnel freezing: Adult male Sprague-Dawley rats (n=21) were anaesthetized with halothane/N_2O/O_2 mixture (4% halothane during surgery and 1% during funnel freezing). Brain tissue was frozen *in situ* by pouring liquid nitrogen through a funnel directly onto the exposed skull of the rat (5). Each frozen brain was divided equally in half for extraction by the PCA and M/C techniques.

Extraction: The frozen brain tissue was kept under liquid nitrogen and ground to a fine powder with a mortar and pestle.

PCA: Cold 12% perchloric acid (3ml/g tissue) was added to the powder under liquid nitrogen. The samples were then centrifuged at 13,000 RPM for 20 minutes. The supernatant was removed and neutralized with 1M KOH and the protein pellet kept for further analysis. The precipitated salt was removed by centrifugation and the supernatant was freeze-dried overnight.

M/C: Cold methanol and cholorform in a ratio of 2:1 (3ml/g tissue total) was added to the ground tissue and allowed to thaw. After approximately 15 minutes, chloroform and distilled water in a ratio of 1:1 (1ml/ g tissue each) was added to the mixture to form an emulsion. The sample was then centrifuged at 13,000 RPM for 20 minutes. The upper phase (methanol and water) was then separated from the lower (organic) phase and the protein pellet was retained for further analysis. The two phases were dried at room temperature under a stream of nitrogen gas.

Protein analysis: Protein pellets were dissolved in 2ml/g tissue of 1M NaOH and analyzed for protein content against a set of protein standards (Bio-Rad, UK) with an LKB Ultrospec II spectrophotometer.

^{1}H-NMR spectroscopy: All dried samples were redissolved in D_2O and adjusted to pH 7.0. A known amount of an internal standard (TSP) was added to each sample. Spectroscopy was performed at 25°C with a Varian Unity plus spectrometer operating at 500 MHz. Fully relaxed spectra were acquired. Resonance assignments were made based on published chemical shifts and coupling patterns of known compounds (6). Peak areas were integrated using standard Varian software.

Statistical Analysis: A multivariate analysis (Hotellings T-squared test) was carried out comparing the metabolite yields for 7 different metabolites between the two different extraction techniques. These metabolites represent those most important to ^{1}H NMR, and include non-polar (Ala), polar (Gln), and acidic (Glu) amino acids, other anions (Lac, NAA), cations (Choline, Glycerophosphocholine), and zwitterions (Creatine). Post hoc tests with correction factors for type 1 errors (Bonferroni and Roy-Bose) were used to discriminate individual metabolite differences between extraction methods. Protein content between extraction methods was analyzed with a paired T-test.

Results/Discussion

Metabolite yields in μmol/g wet weight of tissue

	MC Mean	SEM	CEV	PCA Mean	SEM	CEV
Total Cr	8.75	0.15	8	7.74	0.24	14
Cho	1.36	0.03	9	1.28	0.04	14
Gln	5.37	0.07	6	5.18	0.20	18
Glu	9.40	0.11	5	8.64	0.32	17
NAA	7.09	0.13	8	6.51	0.22	16
Ala	0.65	0.01	9	0.57	0.02	19
Lac	1.98	0.07	15	1.46	0.05	17
Protein	9.73	1.30		15.10	1.30	

The following abbreviations have been made: CEV= *Co-efficient of variation (SD x 100/mean);* Total Cr= *Creatine+Phosphocreatine;* Cho=*Choline-containing compounds;* Gln= *Glutamine;* Glu= *Glutamate;* NAA= *N-acetylaspartate;* Ala= *Alanine;* Lac= *Lactate.*

The metabolite yields were all greater with the M/C than the PCA extraction, with a highly significant overall effect ($P=0.002$). Total Cr, Ala, and Lac were significantly different ($P<0.05$) between the two groups on post hoc tests. In addition, the M/C metabolite values consistently displayed a lower coefficient of variation (5 - 9% as compared to 14 - 19% in PCA, with the exception of lactate). The variation in protein is the same for both techniques which is an important consideration for studies of cell extracts in which metabolite concentrations are expressed relative to protein.

Conclusion

M/C extraction is a superior technique for ^{1}H-NMR spectroscopy. Not only is M/C more efficient and more reproducible, but it allows the simultaneous extraction of the aqueous and lipid metabolite pools from the same cell or tissue sample.

References

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