Published in final edited form as:

Nucl Instrum Methods Phys Res B. 2010 April 1; 268(7-8): 1313–1316. doi:10.1016/j.nimb.2009.10.161.

Method for evaluating the potential of ^{14}C labeled plant polyphenols to cross the blood-brain barrier using accelerator mass spectrometry

Elsa M Janle^{1,*}, Mary Ann Lila², Michael Grannan¹, Lauren Wood¹, Aine Higgins¹, Gad G Yousef², Randy B Rogers², Helen Kim³, George S Jackson⁴, and Connie M Weaver¹

¹ Purdue University, Department of Foods and Nutrition, West Lafayette, IN, USA

² University of Illinois, Department of Natural Resources & Environmental Sciences Urbana IL, USA

³ University of Alabama at Birmingham, Department of Pharmacology, Birmingham AB, USA

⁴ Purdue University, Department of Physics, West Lafayette, IN, USA

Abstract

Bioactive compounds in botanicals may be beneficial in preventing age-related neurodegenerative diseases, but for many compounds conventional methods may be inadequate to detect if these compounds cross the blood brain barrier or to track the pharmacokinetics in the brain. By combining a number of unique technologies it has been possible to utilize the power of AMS to study the pharmacokinetics of bioactive compounds in the brain at very low concentrations. ^{14}C -labeled compounds can be biosynthesized by plant cell suspension cultures co-incubated with radioisotopically-labeled sucrose and isolated and separated into a series of bioactive fractions.

To study the pharmacokinetics and tissue distribution of ^{14}C labeled plant polyphenols, rats were implanted with jugular catheters, subcutaneous ultrafiltration probes and brain microdialysis probes. Labeled fractions were dosed orally. Interstitial fluid (ISF) and brain microdialysate samples were taken in tandem with blood samples. It was often possible to determine ^{14}C in blood and ISF with a β -counter. However, brain microdialysate samples ^{14}C levels on the order of 10^7 atoms/sample required AMS technology. The Brain Microdialysate_{AUC}/Serum_{AUC} ranged from .021- to .029, with the higher values for the glycoside fractions. By using AMS in combination with traditional methods, it is possible to study uptake by blood, distribution to ISF and determine the amount of a dose which can reach the brain and follow the pharmacokinetics in the brain.

Keywords

Blood Brain Barrier; Grape polyphenols; Pharmacokinetics; Accelerator Mass Spectrometry

© 2009 Elsevier B.V. All rights reserved.

*Corresponding author: Elsa M. Janle 700 West State Street West Lafayette IN 47907-2059 Phone: 765-494-1566 FAX: 765-494-0906 janle@purdue.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

With the aging population, neurodegenerative diseases such as Alzheimer's disease (AD) have become significant medical problems[1]. Currently there are no cures for these diseases and medications have only limited efficacy[2]. Supplements of bioactive plant polyphenols may be beneficial in protecting against the oxidative stress that is thought to be a major contributing factor in the development of neurodegenerative diseases[3].

One of the major issues in the efficacy of polyphenol rich supplements is bioavailability. Bioavailability can be extremely complicated to determine. Many plant polyphenols are destroyed in the digestive process. It is important to understand the pharmacokinetics and tissue distribution of the various polyphenol constituents of plant based supplements. It is not sufficient just to determine concentrations in blood because the blood brain barrier (BBB) blocks many compounds from entering the brain, and the biological half life of compounds in the brain may be different from blood. Therefore to understand the mechanisms of potentially beneficial compounds, one must understand their kinetics in brain. Previous studies have used ^{14}C -labeled compounds to study their ability to cross the blood brain barrier by harvesting brain, and determining the ^{14}C by AMS [4]. In these studies we used microdialysis (MD) to measure changes in ^{14}C -labeled chemicals over time in the brain extracellular fluid of awake, freely moving animals.

It has been shown in this study that ^{14}C from labeled plant polyphenols, obtained by growing plant tissue in the presence of ^{14}C -labeled starting materials and chemical fractionation, can be tracked into tissues even in extremely small amounts because of the sensitivity of AMS. Fractionation of plant extract, bioavailability and tissue distribution testing of the plant polyphenols can provide insights into which compounds actually reach the target tissues and may be the effective ingredients. In this study total ^{14}C label from both dosed compounds and metabolites was tracked into blood, ISF and brain.

2. Materials and Methods

2.1 Preparation of ^{14}C labeled plant polyphenols

^{14}C labeled polyphenols from different plant sources can be produced as described previously [5;6]. Cell suspension cultures of plant material were co-cultured with ^{14}C labeled sucrose in a specially designed chamber (Figure 1). Cells were harvested, lysed and polyphenols were extracted with 70% aqueous acetone. Extracts were lyophilized and fractionated by vacuum chromatography on a Toyopearl resin polymer (TOSOH Bioseparation Specialists LLC, Montgomeryville, PA). Polyphenols were prepared from grape (*Vitis vinifera*).

2.2 Membrane Probes

Two types of membrane probes can be use for sampling interstitial fluids: MD and ultrafiltration (UF) [7;8] (Figure 2). The membranes on both the MD and UF probes are polyacrylonitrile hollow fibers with a 220 μm I.D. and a 320 μm O.D. The molecular weight cut off of the probe membranes is 30 kD making it suitable for sampling low molecular weight polyphenols and metabolites. The driving force with UF probes is a negative pressure; with MD it is a concentration gradient. UF probes consist of one to three looped fibers attached to a microbore tube. The microbore tubing is attached to a negative pressure source which can be either a peristaltic pump or a Vacutainer™. The fibers can vary in length depending on the size of the tissue to be sampled. In MD a fluid is pumped through the membrane and molecules diffuse across the membrane in response to a concentration gradient.

For both types of probe it is necessary to perform *in vitro* recovery studies to determine the efficacy of the transfer of the measured compound across the membrane. UF recovery is the

same regardless of the applied negative pressure or probe dimensions. With MD both the probe size and flow rate influence the recovery. Therefore it is imperative to perform the recovery studies under the same conditions that will be used for sampling.

Details for probe recovery studies have been described previously[9]. Briefly, glycerine coating the fibers must first be removed by pumping saline through the probe. The probe is then placed in a standard solution of the compound to be tested and the first sample is discarded. For MD probes the flow rate should be the same as will be used in the in vivo tests.

2.3 Animal models

All animal procedures were approved by the Purdue Animal Care and Use Committee. Ten male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing about 250 g were used for pharmacokinetic and tissue distribution studies. Rats were placed on an AIN-96M polyphenol-free diet (Dyets, INC, Bethlehem, PA) for 5 days to prevent confounding effects of polyphenols present in standard rodent chow. Rats were prepared for study by implantation of jugular or femoral catheters for blood sampling, subcutaneous ultrafiltration probes for sampling of ISF and brain MD probes to determine which compounds cross the BBB. Rats were anesthetized by intraperitoneal injection of 10:1 mixture of ketamine (100mg/ml, Fort Dodge, IA) and xylazine (100 mg/ml Lloyd Laboratories, Shenandoah, IA) dosed at 0.1 ml/100g body weight. Probes were sterilized using Serad hydrogen peroxide sterilization. Catheter, ultrafiltration probe and brain probe guide were implanted under sterile conditions as described previously [10]. After surgery rats were placed in the Culex™ automated blood sampling system (Bioanalytical Systems, INC, West Lafayette, IN) . The TEND function of the Culex™ kept the catheter patent. Rats were allowed to recover for 2 days.

2.4 Pharmacokinetics and tissue distribution

Rats were fasted 8 hours. One hour before dosing, the stylet was removed from the probe guide and a 4 mm MD brain probe (Bioanalytical Systems, West Lafayette IN) was inserted. The probe inlet tube was attached to a syringe pump and the probe was perfused with artificial cerebrospinal fluid at the rate of 1 μ L/min. Brain probes are available with membrane lengths of 1-4mm. The choice of probe size is determined by the size of the target and the sensitivity of the assay: smaller probes provide better spatial resolution but lower recovery. Consideration of assay sensitivity is also necessary when optimizing flow rate: slower flow provides higher recovery but less temporal resolution.

Baseline, pre-dose samples were taken of blood ISF and brain MD. Rats were gavaged with 0.5 ml doses of one of 5 fractions of grape polyphenols. Because of the difference in efficiency of 14 C incorporation into the different compounds, doses were in the range of 4-10 μ Ci. Sample collection was programmed into the Culex and blood samples were taken at 5, 15 and 30 minutes, 1, 2, 4, 6, 8, 10, 12 and 24 hours post dose. Hourly ISF and brain MD samples were collected. Samples were collected in capped vials in a refrigerated fraction collector. At the conclusion of the pharmacokinetics study the rats were sacrificed by CO_2 overdose.

2.5 Tissue Distribution

AMS can also be used to determine if there is uniform distribution of compounds in the brain or if they concentrate in specific locations. Compounds that accumulate in areas where there are changes taking place in neurodegenerative diseases might be good prospects to investigate for therapeutic qualities. When tissue distribution studies were done the animal was sacrificed and the vascular system was immediately flushed with cold saline to remove blood and any 14 C compounds that might be in blood. To look for differences in distribution within the brain, the brains were frozen at -80°C , placed in a brain matrix (AL-1130, Roboz Surgical, Gaithersburg, MD) and sliced into 1 mm sections. Half of each slice was analyzed by AMS.

2.6 Sample analysis

100 μL of serum or ISF were mixed with 15 ml of EcoLite™ scintillation fluid (MP, Irvine, CA) and counted in a β -scintillation counter. Brain samples were sent to the PRIME Lab AMS facility at Purdue University for analysis.

Samples to be measured by AMS were dried by vacuum centrifugation, combusted and converted to graphite using the high throughput method developed at CAMS [11]. The graphite was placed in the ion source [12] which generates ^{13}C -currents of 500-750 nA with the corresponding $^{14}\text{C}^{4+}$ ion detection rates of about 200 Hz for a sample ^{14}C -enrichment of about 2×10^{-12} of total carbon. The ^{14}C : ^{12}C ratios were analyzed with no $\delta^{13}\text{C}$ correction. A $\delta^{13}\text{C}$ was not deemed necessary since the average uncertainty associated with the AMS measurements was 3.9%. Since the ^{14}C enrichment of each of the fractions varied, the dose of each fraction contained slightly different levels of ^{14}C , therefore concentrations were expressed as a percent of dose per ml sample. Carrier was added to the unknown in the form of methanol/tributyrin (Sigma chemical, St. Louis, MO) cocktail. This mixture contained 92 ml of methanol and 8 ml of tributyrin. The tributyrin had a background of 0.24 Fm (fraction modern) $\pm$ 0.008 Fm. It should be noted that we have switched to the tributyrin produced by MP Biomedicals (Solon, OH) since it has a much lower background of about 0.1 Fm [13]. 48.66 mg of carbon from the tributyrin/methanol carrier (990 ml of solution) was added to 10 μL of the unknown brain MD, which contained less than 100 μg of carbon. Then 50 μL of this sample mixture (containing 2.43 mg of carbon from the carrier) was vacuum centrifuged, combusted, and graphitized. We knew the amount of ^{14}C in the sample since we knew the measured ratio and the amount of stable carbon which was all contributed by the carrier. We were then able to subtract off the small amount of ^{14}C from the tributyrin, which was usually a negligible amount since our average measured ratio was 18.8 Fm. We were then able to calculate the activity in units of CPM/ μL of brain MD. Since the amount of brain MD collected was known and the activity of the dose was known it was then trivial to convert the data into percent dose.

To evaluate bioavailability, areas under the %dose vs. time curve were calculated for plasma, ISF and brain. Amounts remaining in brain and ISF were negligible by 24 ours. Plasma was extrapolated by using the slope of the 12 to 24 hour data. To evaluate the efficiency with which fractions were able to enter the brain, the total percent of dose accumulated in the brain slices within 24h was divided by the plasma area under the curve.

3.0 Results and Discussion

Grape polyphenol fractions I-III which contained mostly proanthocyanidin monomers, dimers and trimers were poorly absorbed compared to fractions IV and V which contained the anthocyanin glycosides. Figure 3 shows the relationship between the plasma, ISF and brain MD for two of the fractions. Probe recoveries were 93% for UF probes and 11.2% and 6.3% for fractions IV and V for MD probes. Concentration in target tissues is not necessarily proportional to plasma concentrations. The maximum plasma concentration of fraction V was about 10% greater than fraction IV. However, ISF concentrations were about 77% greater for fraction IV. Areas under the time x concentration curve (AUC) were calculated for serum and brain MD. When comparing AUC for plasma to brain the plasma values represent all labeled material in the serum, both free and bound. The $\text{Brain}_{\text{AUC}}/\text{Serum}_{\text{AUC}}$ ranged from .021- to .029, with the higher values for the glycoside fractions. Investigators who have measured the $\text{Brain}_{\text{AUC}}/\text{Plasma}_{\text{AUC}}$ utilizing non-protein bound concentrations of drugs in plasma have greatly differing ratios ranging from 0.002-1.6[14;15;16;17;18]. The ratios of the grape polyphenol fractions are less than the majority of drugs, but this is partially due to our use of total concentrations vs. free concentration used by other authors.

The ^{14}C values of the brain slices represented the total amount of ^{14}C remaining in the brain after 24 hours. We did not measure any unlabelled brain slices. However, since the average ratio was 1000 F_m for the AMS measurements any small differences in the background ratio for each rat brain are negligible. The fact that material remains after 24 hours may be relevant to long-term use of supplements for prevention of disease because bioavailability in the brain may be greater than indicated by plasma pharmacokinetics. The total ^{14}C remaining in the brain for each fraction can be seen (Table 1). There were lesser amounts for fractions I to III (.007 to .016%) and greater amount for fractions IV and V (0.125% and 0.119%). Fraction II with the lowest serum AUC had greater residual % of dose than fractions I and III with higher serum bioavailability. Fraction IV had the greatest % residual dose in the brain even though it had lower serum concentration than fraction V. There were no significant differences in ^{14}C content of slices going from front to back indicating the distribution was diffuse not localized in one particular area. It is still possible that there was localization in specific smaller structures and future studies will examine individual structures.

Brain microdialysate samples contained from 10^7 to 10^{10} ^{14}C atoms per sample. Assuming a contemporary ratio in unlabeled rat brains and a 2% precision, doses of 0.02 μCi could be used and still be within the range of sensitivity of the AMS.

4.0 Conclusion

Determination of the potential of bioactive compounds to reach the brain is difficult because the BBB restricts access of many chemicals making concentrations which reach the brain low. Use of ^{14}C labeled plant polyphenols for in vivo studies and AMS to analyze the samples make it possible to track even the small amounts of material which crosses the BBB and enters the brain. Use of AMS tracking of plant polyphenols into the brain may be a useful technique of identifying components of plant extracts which may have potentially beneficial in preventing neurodegenerative disorders.

Acknowledgments

The authors gratefully acknowledge technical assistance of Pamela Lacheik, Jane Einstein and Tom Kubley.

This project was supported by the NIH Office of Dietary Supplements and NCCAM Grant P50 AT 00477.

References

1. Brookmeyer R, Johnson E, Ziegler-Graham K, Arrighi H. Alzheimer's and Dementia 2007;3:186.
2. Khachaturian Z, Khachaturian A. Alzheimer's and Dementia 2009;5:81.
3. Rossi L, Mazzitelli S, Arciello M, Capo CR, Rotilio G. Neurochem Res 2008;33:2390. [PubMed: 18415677]
4. Palmblad M, Buchholz BA, Hillegonds DJ, Vogel JS. J. Mass Spectrom 2005;40:154. [PubMed: 15706618]
5. Grusak MA, Rogers RB, Yousef GG, Erdman JWJ, Lila MA. Plant 2004;40:80.
6. Yousef GG, Seigler DS, Grusak MA, Rogers RB, Knight CT, Kraft TF, Erdman JW Jr. Lila MA. J Agric Food Chem 2004;52:1138. [PubMed: 14995111]
7. Janle, EM.; Kissinger, PT. Microdialysis and Ultrafiltration. In: Coburn, T., editor. Advances in Food and Nutrition Research. Academic Press; San Diego: 1996. p. 183
8. Leegsma-Vogt G, Janle E, Ash SR, Venema K, Korf J. Life Sci 2003;73:2005. [PubMed: 12899925]
9. Janle EM, Cregor M. Curr Sep 1996;15:31. [PubMed: 11540471]
10. Janle EM, Portocarrero C, Zhu Y, Zhou Q. Journal of Herbal Pharmacotherapy 2005;5:55. [PubMed: 16520298]

11. Ognibene TJ, Bench G, Vogel JS, Peaslee GF, Murov S. *Anal Chem* 2003;75:2192. [PubMed: 12720362]
12. Jackson GS, Elmore D, Caffee M, Mueller KA, De Bonte B, Muzikar P, Alexander B. *Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms* 2004;223-224:155.
13. Gilman SD, Gee SJ, Hammock BD, Vogel JS, Haack K, Buchholz BA, Freeman SP, Wester RC, Hui X, Maibach HI. *Anal Chem* 1998;70:3463. [PubMed: 9726169]
14. Doran A, et al. *Drug Metab Dispos* 2005;33:165. [PubMed: 15502009]
15. Wu YT, Lin LC, Tsai TH. *J Chromatogr A* 2009;1216:3501. [PubMed: 19022451]
16. Tong X, Ratnaraj N, Patsalos PN. *Epilepsia* 2009;50:174. [PubMed: 19016829]
17. Kou Y, Liu Y, Xue M, Xu Y, Liu H, Ruan J, Liu K. *Int J Pharm* 2008;353:88. [PubMed: 18155373]
18. Lin LC, Chen YF, Tsai TR, Tsai TH. *Anal Chim Acta* 2007;590:173. [PubMed: 17448342]

Figure 1.

Cell suspension cultures of plant material were co-cultured with ^{14}C labeled sucrose in a specially designed plexiglass chamber. ^{14}C labeled polyphenols are isolated, fractionated and used for in vivo bioavailability studies.

Figure 2.

Membrane probes were used to sample interstitial fluid in target tissues. Brain MD probes have 1-4 mm membranes. UF have one to 3 fibers which are 1-6 cm in length.

Figure 3.

Pharmacokinetic profiles of two fractions of ^{14}C labeled grape polyphenols in serum, ISF and brain. ISF was sampled with UF probes and brain with MD probes. Serum and ISF refer to left axis and brain MD to right axis.

Table 1¹⁴C label in plasma and brain

Fraction	Serum AUC (% dose × h)	% dose in Brain	Brain ¹⁴ C/ Serum AUC
I	1.34	.007 %	0.0050
II	0.82	.016%	0.0200
III	1.11	.011%	0.0101
IV	2.07	.125%	0.0605
V	3.46	.119%	0.0343