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Chromatographic separation of reaction products from the choline acetyltransferase (ChAT) and carnitine acetyltransferase (CrAT) assay: Differential ChAT and CrAT activity in brain extracts from Alzheimer's disease vs. Controls

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Abstract

Choline acetyltransferase (ChAT) catalyzes the reaction between choline and acetyl coenzyme A to form acetylcholine (ACh) in nerve terminals. ACh metabolism has implications in numerous aspects of physiology and varied disease states, such as Alzheimer's disease, therefore a specific, sensitive and reliable method for detecting ChAT enzyme activity of great utility in a number of situations. Using an existing radionuclide-based enzyme activity assay, we have observed detectable signals from non-cholinergic cells, suggesting a contaminant in the assay producing an artifactual signal. Previous reports have suggested that L-acetylcarnitine (LAC) contaminates many assays of ChAT activity, due to difficulties in separating LAC from ACh by organic extraction. To determine the source of this hypothesized artifact and rectify the problem, we have developed a chromatography-based assay for the detection of acetylcholine and other contaminating reaction products of this assay, including acetylcarnitine. Our first goal was to develop a simple and economical method for resolving and verifying the identities of various reaction products or contaminants that could be performed in most laboratories without specialized equipment. Our second goal was to apply this separation method in postmortem human brain tissue samples. We successfully detected several contaminants, especially in assays using human brain tissue, and allowed the separation of the intended ACh product from these contaminants. We further demonstrate that this assay can be used to measure carnitine acetyltransferase (CrAT) activity in the same samples, and assays comparing ChAT and CrAT show that CrAT is highly active in neuronal tissues and in neuronal cell cultures. Thus, the simple chromatography-based assay we describe allows the measurement of specific reaction products separated from contaminants using commonly available and inexpensive materials. Further, we show that ChAT activity is significantly reduced in brain extracts from Alzheimer's disease compared to controls.

Keywords

Aging; Alzheimer; Dementia; Brain tissue; Carnitine; Choline; Chromatography; Cortex; Human brain; Neurochemical assay; Post-mortem brain; Synapse; Chloramphenicol Acetyltransferase

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Introduction

Cholinergic systems are involved in numerous physiological processes and disease states. These include cardiovascular control (Sartori et al., 2005), myasthenia gravis (Conti-Fine et al., 2006), memory (Brito et al., 1983), and Alzheimer's disease, (Lahiri et al., 2003; Terry and Buccafusco, 2003), among others. Thus, an assay that can determine the activity of the acetylcholine (ACh) generating enzyme choline acetyltransferase (ChAT), is of relevance in many situations. To this end, we have recently reported a refined technique for the detection of high affinity choline uptake and ChAT activity from the same population of cells (Ray et al., 2009). Of particular interest here, the ChAT assay depends on the reaction of choline with [^{14}C]-labeled acetyl coenzyme A (AcCoA) to produce [^{14}C]-ACh, followed by organic extraction of the [^{14}C]-labeled ACh product. In similar assays, it was found that carnitine indigenous to the tissue sample may react with the [^{14}C]-labeled acetyl coenzyme A in a reaction catalyzed by carnitine acetyltransferase (CrAT) to form L-acetylcarnitine (LAC) (Banns and Ekstrom, 1981). Using the existing technique, this radiolabeled LAC byproduct is extracted and detected with the ACh, introducing a source of non-specific activity into the assay.

Sensitive techniques have been developed that could potentially isolate the intended product of an ACh assay from potential contaminants, such as LAC. Most common among these is high performance liquid chromatography (HPLC) using several detection methods (Stadler and Nesselhut, 1986). However, these techniques require large investments in instrumentation, and the set-up and optimization for these techniques can be time consuming. Other techniques can also assess the levels of the enzyme itself within a tissue sample, such as Western blot or RT-PCR, however, such results may not adequately reflect enzyme activity because the activities of these enzymes are rapidly regulated post-translationally through multiple phosphorylation sites (Dobransky and Rylett, 2005). Therefore, we have devised a simple and economical paper chromatography-based method using inexpensive and widely-available materials for the isolation of the intended ACh product, detection of LAC contamination in ChAT assay products, and quantification of these reaction products.

Subsequently, we applied this novel separation method to *post-mortem* human brain tissue samples. The optimized assay described herein detected and resolved several contaminants from the intended ACh product. Thus, quantification of the specific product was possible in human brain tissue samples that produced substantial confounding non-specific products. We also show that with minor modification, this assay can be used to measure CrAT enzymatic activity in the same samples. In comparing ChAT and CrAT, we show that CrAT is highly active in neuronal tissues and in neuronal cell cultures. Furthermore, by discriminating the reaction products, we report that ChAT activity is significantly reduced in brain tissues affected by Alzheimer's disease compared to control tissues.

Materials and Methods

Primary neuronal cell culture sample preparation

Protein samples from primary cerebrocortical fetal rat neuron (PRCN) cultures and human frontal cortex were used in these assays. PRCN cells were cultured as described previously (Bailey and Lahiri, 2010; Bailey et al., 2011). Briefly, cells from the cerebral cortices embryonic day 16 pups (Harlan, Indianapolis, IN) were dissociated by trituration and seeded into poly-D-lysine (Sigma-Aldrich, Saint Louis, MO) coated tissue culture plates (Corning, Corning, NY) and maintained in Neurobasal medium supplemented with B27 serum replacement, an antibiotic cocktail, and 20ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA). We have observed previously that these cultures undergo time-dependent

neuronal differentiation, followed by synapse loss and neurodegeneration (Bailey et al., 2011). Cells were lysed in Mammalian Protein Extraction Reagent (M-PER; Millipore, Billerica, MA) containing a protease inhibitor cocktail (Roche, Indianapolis, IN) per manufacturer's instructions.

Human brain tissue sample preparation

Human *post-mortem* brain tissue from the frontal cortices was provided by Dr. Bernardino Ghetti. This tissue was lysed by sonication in M-PER buffer with protease inhibitors. Lysates were cleared by centrifugation at $10,000 \times g$ for 10 minutes, and protein concentrations of the supernatants were determined using the Bradford technique (Bio-Rad, Hercules, CA). Volumes of lysis buffer were adjusted to equalize the protein concentrations of all samples to 5 $\mu\text{g}/\mu\text{l}$.

Enzyme activity assay of ChAT and CrAT

In the initial assay, activity of the choline acetyltransferase enzyme (ChAT) was measured in the lysates of primary cerebrocortical cells that were maintained in culture for varied periods of time. These cells were lysed in M-PER buffer (Pierce, Rockford, IL) with protease inhibitor cocktail (Roche, Indianapolis, IN) using an established protocol (Ray et al., 2009). Four microliters of lysate was incubated with 10 μl of an assay buffer that consists of phosphate buffered saline (pH 7.4) plus 0.14 μM EDTA and 0.11 mM [^{14}C]-acetylcoenzyme A (60.0 mCi/mmol or approximately 200,000 CPM per reaction; Perkin-Elmer), and 0.086 μM eserine sulfate (Sigma-Aldrich, St. Louis, MO) to prevent breakdown of the [^{14}C]-ACh product by cholinesterases that may be in the tissue samples. The reaction is then stopped by the addition of 25 μl 0.5 M HCl on ice (Sigma-Aldrich). The [^{14}C]-ACh product was then extracted from the reaction mix by the addition of 100 μl of 3-heptanone (MP Biomedicals, Solon, OH) containing 30 mg/ml tetraphenyl boron (Strem Chemicals, Newburyport, MA). Samples were vortexed for 10 seconds, followed by centrifugation at $12,000 \times g$. Seventy five microliters of the organic phase containing the reaction product was then transferred to a scintillation vial with counting cocktail (EconoSafe, Research Products International, Mount Prospect, IL) and read in a scintillation counter. Under the conditions described here, ChAT enzyme activities in the samples were found to be proportional to the scintillation counts. In the samples we have used, primary neuronal cultures lysates have produced 5 μg to 10 μg per assay. These data were normalized to the Cell Titer-Glo (CTG; Promega, Madison, WI) cell viability assay, performed per manufacturer's instructions. This CTG assay produces a luminescent signal that corresponds to cellular ATP content, and thus should reflect the relative number of viable cells in each cell culture well. For a more detailed description of this portion of the assay, please see our recent publication (Ray et al., CPNS in press).

The ChAT assay was also performed using human cerebral cortex extracts. An equal amount of protein from each sample was used in the assay (4 μl or 20 μg of protein). Different processing of cell culture and brain tissue samples was required because Western blotting of cell culture lysate samples revealed media protein (e.g., albumin) contamination, invalidating the equal protein normalization. Conversely, cell viability assays appropriate for cell culture samples would not be valid for brain tissue samples because the results of this assay would be expected to vary due to multiple factors, such as *post mortem* interval. This assay has also been used successfully to measure ChAT activity of multiple cell lines, rat and transgenic mouse brain samples using the methods described herein.

Chromatography of reaction products

To identify reaction products and possible contaminants in the assay, the above procedure was performed, omitting the organic extraction step. The reaction mixture was lyophilized in

a SpeedVac (Savant, Hyannis, MA) then resuspended in 5 μ l methanol and spotted onto Whatman DE81 ion exchange chromatography paper (Sigma-Aldrich). This was placed in a chromatography tank containing the solvent mix: 1-butanol:ethanol:acetic acid:water (8:2:1:4) at room temperature. Several modifications of the solvent ratios were tested in an effort to optimize separation, but unless otherwise indicated, this 8:2:1:4 ratio was used in all experiments shown here. The chromatography tank was sealed and allowed to develop until the solvent front approached the top of the chromatography paper, approximately 1.5 hours. The paper was then air dried in a fume hood and wrapped in cellophane and exposed to ECL film overnight or up to 3 days if necessary to visualize the radioactive product(s) signal(s). To develop the non-radioactive Rf standards (choline, acetylcholine, carnitine, and LAC) spotted at the same time as the experimental samples, the chromatography paper was sealed in an iodine (I₂) chamber for 10 minutes to overnight until all four spots were visible. Typically, carnitine and LAC require longer exposure times. The iodine-stained paper was placed in a plastic sleeve and the paper and film were then scanned. The resulting images were overlaid using ImageJ software (Abramoff et al., 2004), allowing identification of the reaction product(s).

Quantification of signal and data analysis

Quantification of the radioactive product separated by the chromatographic technique was performed using ImageJ. Scanned images of the ECL films were subjected to densitometric analysis of the specific spots visualized on the ECL film corresponding to the correct Rf value, as compared to the standards. Experiments were carried out with n=3 for cell culture samples and n=6 to 8 for human brain tissue samples. Between-group comparisons were made using a 2-tailed t-test using SPSS software, and p<0.05 considered to be significant.

Results

ChAT and CrAT activity in the initial primary cerebrocortical culture experiments

Primary rat cerebrocortical neurons were cultured as described previously (Bailey et al., 2011). These cultures are known to exhibit time-dependent neurodegeneration with profound synaptic pruning between day 12 and day 16 *in vitro* (Bailey and Lahiri, 2010). Therefore, it would be expected that any observed ChAT activity would follow a similar pattern, with any ChAT signal detectable at day 12 would be absent at day 16. While complete loss of the synaptic marker synaptophysin was observed in these samples, there was no significant change in ChAT activity (figure 1). These results suggested that the signal obtained in these assays was not the result of ChAT activity, but rather, some contaminant of the assay. CrAT enzyme activity using carnitine endogenous to the cultured cells was hypothesized to be the most likely source of this contamination, as has been previously suggested (Banns and Ekstrom, 1981).

Separation and visualization of Rf standards

Standards of choline, ACh, carnitine, and LAC (all from Sigma-Aldrich) were dissolved in methanol and 100 μ g of each spotted onto the chromatography paper, singly or in combination, as indicated (figure 2) to determine resolution and any possible interference between the compounds. The compounds were resolved and visualized by overnight exposure to I₂ vapor. Choline and carnitine were well resolved from each other. The acetylated forms of these compounds appear very close, or even slightly overlapping with, their unacetylated forms, but these were also sufficiently resolved to determine which compounds were spotted onto the chromatography paper in every combination (figure 2). Furthermore, none of the compounds appeared to alter the resolution or Rf values of any of the other compounds when spotted together. Thus, the chromatography paper used can accommodate a large amount of these substrates while maintaining adequate resolution. It

should also be noted that the best results were observed with freshly dissolved standards. Chromatograms with carnitine and LAC performed with solutions prepared more than approximately 48 hours in advance showed additional spots of slightly higher R_f (data not shown), which suggests spontaneous modification or degradation of these molecules in the methanol solution.

Determination of the detection limit of I_2 visualization for ACh and LAC

The detection limit of this assay was tested using non-radioactive standard solutions of ACh and LAC. A chromatography paper was loaded with 1 μ l each of 1:1 serial dilutions of both compounds starting with 100 μ g/ μ l, including 50 μ g/ μ l, 25 μ g/ μ l, 12.5 μ g/ μ l, 6.25 μ g/ μ l, 3.13 μ g/ μ l, and 1.56 μ g/ μ l. The chromatogram was developed and exposed to I_2 overnight to maximize detection. Based on thresholding of the scanned image using ImageJ software (Abramoff et al., 2004), both compounds were just detectable at 3.13 μ g/ μ l, and reliably visualized at 6.25 μ g/ μ l (figure 3).

Optimization of the solvent mixture

Various modifications to the solvent mixture were tested in an effort to increase the resolution of this technique. Adjusting the 1-butanol:ethanol ratio of the previously reported solvent produced higher R_f values and improved resolution somewhat. Two examples are shown in which the 1-butanol:ethanol ratio was changed to 6:4 or 5:5 (keeping acetic acid and H_2O constant at 1:3; figure 4). The 6:4 ratio increased the average R_f values of acetylcholine from about 0.4 using the previously reported solvent ratio (McLean and Jewers, 1972) to around .6, while higher ethanol concentrations increased the R_f values further, but reduced separation between the compounds (figure 4, "Solvent B"). A solvent mixture of 6:4:1:3 proportions of 1-butanol:ethanol:acetic acid:water (figure 4, "Solvent A") was used for all other experiments described here (figures 2, 3, 5, and 6).

Identification of reaction products in a tissue culture model

PRCN cultures were previously found to be positive for ChAT activity using the scintillation counting version of this assay (figure 1). This was unexpected as there are no intrinsic cholinergic neurons in the cerebral cortex. Therefore, we tested these samples along with homogenate of adult rat cerebrocortical tissue, which is known to receive cholinergic projections, as a positive control, and the lysis buffer (M-PER) as a negative control. The assay was performed in duplicate, substituting an equimolar concentration of carnitine for choline as the substrate to determine the relative activities of these enzymes in these cell cultures. The reaction products were lyophilized and dissolved in 5 μ l of methanol and spotted on chromatography paper. The chromatogram was then exposed to x-ray film for 48 hours. The autoradiogram showed strong ChAT activity in the cortical homogenate positive control only, and strong CrAT activity in both the rat cortex and cerebrocortical cultures (figure 5). The chromatogram was then developed in I_2 vapor to visualize the standards (figure 5), and the developed chromatogram and film were scanned to produce a composite image, which confirms the identity of the reaction products (figure 5). Based on the results of this visualization method, we determined that the ChAT activity observed in these cell cultures is due to high levels of CrAT enzyme activity reacting the [^{14}C]-acetyl CoA with endogenous carnitine, and not genuine ChAT activity.

Quantification of specific ChAT assay product in human brain

To further validate this technique in a relevant sample set, a the ChAT activity assay was then performed using *post mortem* human brain tissue obtained from individuals with Alzheimer's disease and age-matched controls and the reaction products were separated using the chromatography technique described here (figure 6). Loss of cholinergic terminals

in the cerebral cortex is an established, consistent feature of Alzheimer's disease, thus, a valid technique should show a reduction in ChAT activity in these patient samples. The chromatogram was first visualized by autoradiography to visualize the radioactive enzymatic reaction products, then exposed to I_2 to develop the standards. We observed several reaction products in the autoradiogram of the brain tissue, which correspond to the major product, ACh, as well as LAC and an as-yet unidentified product (figure 6). Densitometric analysis of the film with ImageJ showed a significant decrease in ChAT activity, as expected.

Discussion

Our interest in this assay stems from our investigations of the cholinergic systems, which degenerate in Alzheimer's disease, and our work in the development of cholinesterase-based therapeutics aimed at treating this disease (Bailey and Lahiri, 2011; Greig et al; 2005; Lahiri et al., 1994; Lahiri et al., 2007). While studying cholinesterase inhibiting drugs, we observed apparent ChAT activity in cultured cerebrocortical neurons. While cholinesterases would be expected in these cultures, ChAT would not, given that the cerebral cortex has no intrinsic cholinergic neurons. This observation prompted us to further refine our existing ChAT assay (Ray et al., CPNS, in press) to ensure the specificity of the reaction product. Thus, we have developed a simple, rapid, and economical method to separate the ACh product of interest in this assay from potential contaminants, particularly LAC produced by CrAT enzyme and carnitine substrate endogenous to the tissue sample.

Neurons intrinsic to the cerebral cortex are not known to produce acetylcholine, rather, the cortex receives its cholinergic terminals via projections from subcortical structures. However, drugs such as rivastigmine that target cholinergic systems have been found to have neuroprotective and amyloid modulating effects in these cultures (Bailey et al., 2010; Bailey et al., 2011), therefore this highly sensitive radioisotope-based method of measuring ChAT activity (Ray et al., 2009, Ray et al., in press) was used to investigate the possibility of cholinergic neurons in these cultures. The primary cerebrocortical cultures as used here are known to degenerate in a time-dependent fashion, starting between day 12 and day 16 *in vitro* (Bailey and Lahiri, 2010; Bailey et al., 2011), and detection of ChAT enzyme activity at day 16 *in vitro* would suggest a non-synaptic, non-neuronal source. Thus, this initially determined activity was considered to be questionable. LAC was previously determined to be a contaminant of some assays of ChAT enzymatic activity (Banns and Ekstrom, 1981), and was therefore selected as the most probable source of this unexpected signal. Subsequent analysis, as detailed herein, determined that this activity was, in all probability, the result of the CrAT enzyme catalyzing a reaction between carnitine endogenous to the tissue sample and [^{14}C]-acetyl coenzyme-A included in the assay to form [^{14}C]-LAC. This hypothesis was further supported by replacing the choline substrate in the assay with carnitine. In this experiment, a substantially larger signal was observed with carnitine as the substrate than with choline. Thus, the CrAT enzyme was found to be much more active in these samples than ChAT, and is the probable source of the signal observed initially.

Using cell culture samples, these assays produced very strong signals when carnitine was provided as the substrate, which may represent saturation of the assay. Our goal was to identify possible contaminants in our ChAT assay, and conditions were therefore optimized for ChAT activity detection. It appears that in these tissues, CrAT is far more active than ChAT, and the assay would need to be re-optimized to measure CrAT in non-saturating conditions. This could be accomplished by simply reducing the incubation time in the assay. Given the potential importance of CrAT in normal physiology and some disease states, this simple technique for detecting CrAT activity and isolating the reaction products could be of great utility in a variety of situations. A limitation of this method is the number of samples

that can be spotted on a single sheet of chromatography paper. This is not a problem with smaller sample sets, such as the human brain tissues we used here, however, with larger sample sets, it may become impractical to process all samples using this technique. In this case, this technique may serve as a quality control measure applied to a subset of samples. Higher throughput might have been achievable with modifications to this process. In particular, this method could be used to process greater numbers of samples with greater convenience to the experimenter if thin layer chromatography (TLC) were to replace the paper chromatography used in the procedure reported here. We have previously applied TLC for use in chloramphenicol acetyltransferase (CAT) based reporter gene assays in order to compare promoter activities of genes such as APP and BACE1 (Lahiri and Robakis, 1991; Song and Lahiri, 1998). In this instance, TLC readily resolves the products of the CAT assay, including 1- and 3-acetylated chloramphenicol, in chloroform-methanol (19:1) (Lahiri and Nall, 1995). This method was unsuccessful for resolving products of ChAT and CrAT enzyme activities, possibly due to factors such as differences in partition coefficients of the samples. TLC was also attempted using several commercially available substrates, including silica and cellulose, but these did not provide adequate separation of the R_f standards and in some cases, apparent R_f changed based on the amount of the compound loaded on the TLC plate. Therefore, the paper method as described, though less convenient than TLC, provided superior resolution of the R_f standards and reaction products.

Several different detection methods have been developed for the measurement of ACh in biological samples. Detection of ACh in, for example, very low concentration microdialysates requires a very high degree of sensitivity. This has been achieved using high performance liquid chromatography in conjunction with several detection methods, including fluorescence and electrochemical methods (Tsai, 2000; Jin 2010). These have been reported to detect as little as 10 fmol of ACh. In comparison, the I₂ detection method used here was sensitive down to about 3 µg, which equates to 16.5 nmol of ACh. Thus, this method is not recommended for analysis of ACh levels in low-concentration biological samples. However, since the samples used here are from an assay of enzyme activity, this is largely irrelevant since the parameters of the assay, e.g., incubation time and substrate volume, can be easily modified to compensate for any sensitivity issues encountered. We have found this technique useful in several instances, allowing us to measure ChAT activities without the purchase of expensive instrumentation and with a reasonable amount of effort: we have found that an experienced technician can complete the enzyme assay and chromatography steps in three to four hours, followed by overnight film exposure followed by I₂ vapor exposure for 1 hour to overnight.

ChAT activity is known to be reduced in AD brain (Bao et al., 2012). To further validate, adapt, and demonstrate the utility of this method, we conducted an assay including samples from the brains of human subjects both confirmed AD and unaffected age-matched controls. ChAT activity was found to be reduced as expected. This method also demonstrated in these samples that multiple products are produced in this assay. This is the result of the [¹⁴C]-AcCoA in the assay buffer reacting with other substrates in the tissue sample, including carnitine. This method allowed the separation of the product of interest ([¹⁴C]-ACh) from other products resulting from the reaction of [¹⁴C]-AcCoA with endogenous substrates without the use of highly specialized or expensive instrumentation. When the assay was performed using choline and carnitine as substrates side-by-side, it was also demonstrated, similar to rat brain samples, that CrAT activity is much higher than ChAT activity. Thus it should be expected that any amount of carnitine present in the tissue sample will be acetylated and contribute to non-specific activity in the reaction product. Thus, a method to separate specific and non-specific products, such as the method described here, should be employed when measuring the products of ChAT enzymatic activity.

We have demonstrated that the reaction products and substrates, including those that contaminate the assay, can be effectively separated and identified by a simple and inexpensive paper chromatography technique, and visualized at low microgram quantities by I₂ vapor exposure, or using autoradiography with radiolabeled substrates if higher sensitivity is required. In experiments detailed here, these visualization techniques were combined, using I₂ visualization for Rf standards and autoradiography for detection of the reaction products that were present in much lower abundance. In brain tissue samples, it was clear that multiple reaction products were produced in the ChAT assay. Consistent with previous findings (Banns and Ekstrom, 1981), LAC appears to be one of the contaminating reaction products, although it was not the only contaminant of the ChAT assay detected. This clearly demonstrates the necessity of this kind of separation technique to monitor for such contaminants that could represent confounding variables, and exclude them from analysis. Failure to exclude these non-specific reaction products could potentially mask the effect of a disease state or experimental manipulation under investigation. Further, we have successfully applied our separation method in neuronal cells and in postmortem human brain tissue samples. Our assays comparing ChAT and CrAT show that CrAT is highly active in neuronal tissues and in neuronal cell cultures. Further, by discriminating the reaction products from the ChAT and CrAT assay, we report that ChAT activity is significantly reduced in brain extracts from Alzheimer's disease vs. Control subjects.

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Figure 1. Level of Synaptophysin and unexpected ChAT signal in cerebrocortical cultures

The culture techniques used here are known to produce time-dependent synapse pruning and neurodegeneration starting between day 12 and day 16 *in vitro*. This was confirmed in these samples by Western immunoblot. The synaptic marker synaptophysin was readily detectable in cell lysates at day 12, and below the detection limit at day 16. The ChAT activity assay in the same samples did not reflect the expected reduction in synaptic ChAT activity. The hypothesis was therefore proposed that the assay product was largely the result of contaminating enzyme(s) and/or substrate(s).

Figure 2. Resolving Rf standards

A. To investigate the resolving capacity of this paper chromatography method, 50 μ g each of choline, ACh, carnitine, and LAC were spotted onto the chromatography paper either singly or in the combinations indicated. B. A detailed view of the last four lanes is shown with the identities of the standards and blanks left by the omitted compounds indicated. Sufficient separation was observed such that four distinct spots could be identified. None of the standards were observed to react or interfere with any of the others.

Figure 3. Detection limits of I₂ vapor visualization for ACh and LAC

To determine the detection limits of the I₂ based detection technique, serial dilutions of ACh and LAC were spotted onto chromatography paper. These included 1 μl each of 100 μg/μl, 50 μg/μl, 25 μg/μl, 12.5 μg/μl, 6.25 μg/μl, 3.13 μg/μl, and 1.56 μg/μl solutions in methanol were spotted onto the paper and resolved in the chromatography tank. After overnight exposure to I₂ vapor, 3.13 μg spots were barely visible for both ACh and LAC, and the 6.25 μg spots were readily visible.

Figure 4. Optimization of the chromatography solvent

Several different combinations of solvents were evaluated for their ability to separate the four standards, and two representative combinations are shown here. The best combination tested was a slight modification of the previously reported solvent combination (McLean and Jewers, 1972), containing 1-butanol:ethanol:acetic acid:H₂O in ratios of 6:4:1:3. Adjusting the 1-butanol:ethanol ratio had the greatest influence on Rf values, with this combination ("Solvent A") producing values closest to 0.5 of all combinations tested. Solvent A was used in all other chromatography experiments reported here.

Figure 5. Combined autoradiography and I_2 vapor detection of reaction products and Rf standards

(A) To detect the radiolabeled reaction products of this ChAT assay, the chromatogram was dried and wrapped in cellophane and exposed to X-ray film for 48 hours. After the film was developed and adequate signal was observed, (B) the Rf standards were then visualized in I_2 vapor. The x-ray film and I_2 labeled chromatogram were scanned. (C) The images were overlaid using ImageJ software in order to compare the radiolabeled reaction products with the Rf standards. ChAT activity was only detectable in the rat cortex positive control, while CrAT activity was apparent in the rat cortex and both PRCN samples. We have previously observed in the PRCN model that synaptic protein markers are readily detectable at day 8, but are virtually absent at day 20. This suggests that the ChAT activity previously observed by scintillation counting was due to high CrAT activity acetylating endogenous carnitine. No signal was observed in the M-PER buffer negative control.

Figure 6. ChAT and CrAT activity in human frontal cortex

ChAT activities were measured in human *post-mortem* frontal cortex samples. Radiolabeled reaction products were detected by autoradiography and the Rf standards detected by I₂ vapor exposure and the images have been superimposed for comparison. As expected, significant decrease in ChAT activity was detected by this assay in Alzheimer's disease patients, compared to age-matched controls. Additionally, a detailed view of a single lane of the chromatogram shows multiple spots corresponding to different reaction products. The predominant product corresponds to ACh, however, other signals (e.g., LAC) appear to be strong enough to contribute significant non-specific signal to the assay.